Micropropagation of Orchids

Second Edition

VOLUME I

Joseph Arditti

Blackwell Publishing

Micropropagation of Orchids

Volume I

This greatly expanded and updated edition of this classic reference work provides the user with comprehensive details of all procedures for propagating orchids through the culture of tissues *in vitro* (micropropagation).

Presented in two volumes, the book opens with a detailed and illustrated historical section which covers the discoveries, developments, and people which made micropropagation possible. The next section discusses in detail the principles and components of culture media which are used in micropropagation. The main component of the two volumes, however, is the third section. This comprises the detailed procedures for the culture of over 100 orchid genera. Classical, established, and the very latest techniques of clonal propagation of organs, tissues, and cells are presented. Every one of the listed methods includes tables of complete recipes for a wide range of culture media.

This new edition will continue to be *the* key reference for all those interested in and involved in orchid micropropagation, growing, and production.

Dr Joseph Arditti majored in floriculture as an undergraduate and received his doctorate from the University of Southern California in 1965. After serving as a lecturer for one year at the University of Southern California, he accepted a faculty position at the University of California, Irvine in 1966 where he taught General Botany, Horticulture, and Plant Physiology while engaging in research on various aspects of orchid biology in the US, Indonesia, Singapore, and Malaysia. Dr Arditti retired in 2001 and is now Professor Emeritus. He continues to write and travel worldwide to lecture at scientific meetings and to orchid groups. He is acknowledged to be one of the world's leading experts on orchid biology and propagation.

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Second edition

Volume I

Joseph Arditti
Professor Emeritus
Developmental and Cell Biology
University of California, Irvine

Blackwell
Publishing

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For
my 22-year-old son Jonathan
. . . yet again

My brother Mordi . . . again
and his Virginia Quintana

And the memory of my parents
Salomon (1902–1993) and Rebecca (1905–1997) Arditti

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Preface

Micropropagation of Orchids was "born" in 1974 when I initiated my *Orchid Biology, Reviews and Perspectives* (*OB*) series. Since a book containing only scientific literature reviews on orchid topics did not seem to have much of a financial or sales future I decided to include in the first volume a practical appendix entitled *Clonal Propagation of Orchids by Means of Tissue Culture – A Manual*. My hope was that the manual would attract buyers. I wrote the appendix while on sabbatical leave at one of my most favorite places on earth (the Bogor Botanical Gardens in Indonesia) using a portable manual typewriter which was a gift from my late father. Tissue culture propagation was relatively new then and the existing methods and literature citations were covered in 90 pages. The appendix accomplished its mission by attracting buyers and the series got its start in 1977.

By 1990 the first volume of *Orchid Biology, Reviews and Perspectives* was out of print and second hand copies were in considerable demand because of the appendix. The tail was wagging the dog. It was time to update and expand the manual and write a book on the subject which had acquired a name by then: micropropagation. I invited my colleague Dr. Robert Ernst to join me and we expanded the manual into *Micropropagation of Orchids* (*MO*1). It was published by John Wiley and Sons in 1993. *MO*1 included all of the procedures in the manual and almost all of the methods published between 1974 and 1990 in its 682 pages (nearly 7.6 times as many pages as the manual).

Like *OB*I, *MO*1 went out of print about 15 years after it was published. Demand for second hand copies rose quickly. Many people wrote me asking where to find a copy. By the year 2000 it was clear that the time had come to write a second edition of *MO*. I retired on July 1, 2001 and started to write a few months after that. My plan was to include in *MO*2 all the procedures in *MO*1 because they are still useful, and as many of the methods that were published after 1990 as possible (and hopefully all). What I found was that more new methods were published between 1990 and the year 2000 than from 1949 to 1990 (the period covered by the manual and *MO*1). Suggestions that orchid micropropagation was a mature field with a decreased number of publications seem to have been grossly exaggerated (to paraphrase Mark Twain) and completely unrealistic. This meant that writing would take a long time and it did. I finished the first draft in early 2004, edited it after that and stopped adding procedures on May 1, 2004, my 72nd birthday. There is no question

that many new methods will be published in the years after that, but *MO*3 will have to be written by someone else.

Chapter 1 in *MO*1 had presented a reasonably accurate history of orchid micropropagation, but I was made to remove parts of the story which placed the discovery and its discoverers in proper perspective because they questioned established dogma and the claims of the presumed discoverer. Professor (now emeritus) Abraham D. Krikorian and I used the excised parts as the basis for an extensive and precise history of orchid micropropagation, which was published in the *Botanical Journal* of the Linnean Society of London. This article served as the foundation for the history chapter in the present edition of *MO* which pulls no punches, tells the story as it happened, and places all historical figures in proper perspective. In retrospect I regret buckling down under intense pressure and allowing the history chapter in *MO*1 to be emasculated. I apologize for my lapse in good sense and momentary weakness. History must be reported as it really happened even if the actual facts may offend some people (even friends) because (to quote E. Mach, 1838–1916) "It is hardly possible to state any truth strongly without apparent injustice to some other." I thank Professor Krikorian for allowing me to quote liberally from our joint publication.

Chapters 2 and 4 were rewritten and revised not due to any shortcomings but because I decided to emphasize a few points more strongly, add information, and reword or restate several subjects. Both chapters are longer as a result of these changes.

With one exception, reviews of *MO*1 were very positive. The sole exception was critical of the absence of: (1) information about the use of colchicine to increase chromosome numbers in orchids, and (2) advice on how to combat internal contamination. This current edition also does not have information on the use of colchicine because this topic is not part of micropropagation. It belongs in a book on cytology, cytogenetics, or bioengineering. In view of the large amount of information that had to be included in *MO*2 my criteria for inclusion were very strict, narrow, and based entirely on the last half of the term micro*propagation*. Bioengineering, cytology, cytogenetics, physiology, molecular biology, and seed germination were excluded. As is obvious, even with this strict policy, the book grew to be very large.

Only published information was included in the manual and *MO*1 and is part of *MO*2. No published information on internal contamination in orchid cultures and how to handle it was available when the manual and *MO*1 were written. And, I could find none when writing this edition. Thus, when faced with the choice of presenting or not presenting non-existent (i.e., not available in the literature) information I chose the latter. However, I did include information on a variety of antibiotics and anticontaminants because they can be used to combat any contamination.

Most published papers on orchid micropropagation and tissue culture techniques contain information on media, culture conditions, and procedures which is sufficient for a presentation in *MO*, but some do not. When information was missing I made a few logical assumptions. For example, if a paper did not describe culture conditions in detail I assumed that standard culture room temperature and illumination will be suitable. In the few cases of truly atrocious papers, I made more (perhaps too many) assumptions and also called attention to their low quality.

Many years ago a thoughtful reviewer of one of my early papers pointed out that I failed to evaluate the quality and content of several articles that were mentioned in a review of the literature. He/she indicated that a certain amount of expertise is

implied in the writing of a paper or a review (and by implication a book) and that readers have a right to expect evaluations, criticisms, praise, opinion, and advice from an expert. *MO*1 was largely devoid of such comments, but I added several to this edition when they were called for. Some of these comments are negative. They may cause unhappiness in some quarters and/or generate criticism, but I think that the reviewer of long ago was right in suggesting that readers have a right to expect guidance and the opinions of an author who presumes to write a review or a book.

The author of a second edition has the advantage of hindsight since comments by readers, discussions with friends, opinions by users of the book, and statements by reviewers point to strengths and weaknesses. What I learned from comments about *MO*1 is that users of that book appreciated having the following:

- redundancy ["move the tissue to the first medium (Table XYZ-1) and then to the second solution (Table XYZ-2) before returning it to the first substrate (Table XYZ-1) and then taking it to the third (Table XYZ-3)" was liked by users who when asked and given a choice preferred this type of writing to "move the tissue to the first medium and then to the second solution before returning it to the first substrate and then taking it to the third"],
- repetitions ("tell me how to prepare a sterilant every time I need to use it, don't refer me to 100 pages back"),
- details ["dissolve it in 70% ethanol (73 ml 95% ethanol diluted to 100 ml with distilled water)" was preferable to "dissolve it in 70% ethanol"],
- clarity (short declarative sentences rather than long and involved ones),
- unambiguous instructions ("don't give me a choice between two sterilants, tell me which one to use"),
- simple language ("don't use a long chemical name if the compound has a trade-name or list both"), and
- self-standing procedures ("list all media and solutions with every procedure, don't tell me to use the medium in Table JOA-1 on p. xxx first, then the solution in Table MA-3 on p. yyy, the substrate in Table VQ-9 on p. zzz after that and finish with Table SUN-8 on p. aaa. This will make me leaf through the book endlessly in search of media and I will not like it").

I wrote *MO*2 in the same manner. What several users did not like was the fact that in many cases tables that pertained to one procedure were mixed with pages that contained text about another method. I reorganized this edition so that tables and text which relate to a procedure are together. Tables and sometimes text do not always fill a page. To not have empty spaces throughout the book and avoid mixing procedures in such cases I added illustrations, chemical formulae, and miscellaneous information or historical vignettes in boxes. These items are not numbered because some may have to be removed due to space limitations which could arise during typesetting. I hope that readers and users of the book will find these items to be interesting and even illuminating. They can be ignored by those who will find them uninteresting and distracting.

Ideally every procedure in this edition and in the previous versions would have been tested before being included in the book. However, given the number of procedures this is clearly impossible due to limitations of time, laboratory facilities, funds, and availability of orchids for experimentation. In fact, it would be illegal to import

some species due to CITES. Therefore procedures are presented without having been tested in the hope that they do work. However, it is reasonable to assume that procedures which are affected by the previous history of the donor plant may not work with plants that were grown under different conditions.

I could not have written this book without help from many individuals and sources. My thanks go to:

- Professor P. N. "Dhanny" Avdhani, National University of Singapore, my friend since 1969 for good ideas and stimulating discussions,
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- Suan Wong (Mrs. C. S. Hew) for an extensive retrospective literature search which produced a very detailed and inclusive printout of relevant publications. I could not obtain such a printout from the library of my own institution, the University of California, Irvine,
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- Kathryn Kjaer, University of California, Irvine library for tending to my current awareness profile and literature searches efficiently, willingly, and well (she is not in charge of retrospective searches),
- Hideka Kobayashi for finding and sending me many papers from journals which are not available at the University of California, Irvine library (the selection of plant science journals in the UCI library is meager and reflects the negative attitude toward plant sciences in the School of Biological Sciences),
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- Dr. John Yong, Nanyang Technological University in Singapore for allowing me to use illustrations, and
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My special thanks go to my son Jonathan Omar for just being around and to my brother for maintaining my computers when they needed it and making it easier for me to write.

I also thank Ward Cooper, my editor; Jane Andrew, easily the best copy editor I have ever worked with and her assistant Pat Croucher for ferreting out errors, finding omissions, correcting mistakes, and generally making the book much better; as well as Rosie Hayden, Caroline Milton, Rachel Moore, Delia Sandford, Nancy Whilton, and the rest of the staff at Blackwell Publishing for their high level of professionalism.

Most of all I thank my Jonathan for being my son and making my life better by just being around.

Joseph Arditti
Irvine, California

Preface to the First Edition

Photocopy machines, pocket calculators, and micropropagation of orchids through tissue culture appeared on the scene almost simultaneously, and the world has not been the same since. Now it is hard to imagine how it was ever possible to get along without these advances. Those who are in their thirties and forties have never known a world without them. The large, slow, and primitive copiers of the 1960s gave rise to the small, fast, and versatile photocopiers of today. Pocket calculators became smaller (some even moved to the wrist in combination with watches) and more sophisticated. It is possible to suggest that one evolutionary branch led from them to the personal and "notebook" computers of today.

Mass rapid clonal propagation of orchids led to the development of similar procedures for other plants and eventually to the isolation and culture of cells and protoplasts. The combination of molecular biology and tissue, cell, and protoplast culture is the basis of plant biotechnology that holds the promise of improved crops, safer chemicals, and perhaps a better environment. Tissue culture was and is used for the mass rapid clonal propagation of outstanding hobby crosses and commercial cut-flower cultivars of orchids. In the former case it resulted in reduced prices of desirable plants to levels within the reach of most growers, whereas in the latter instance it is responsible for the tremendous growth of the orchid cut-flower industry in Thailand, Singapore, Malaysia, Indonesia, and other countries.

Research on orchid tissue culture as a means of micropropagation is being carried out in many laboratories all over the world. Papers based on this research are published in numerous journals and in several languages (but fortunately for English-speaking people mostly in English). Even with computerized literature searches it is not always possible to trace all existing papers because some publications are not recorded in the relevant databases. Some papers (in both popular and obscure journals) do not contain enough details to be useful for the average grower or even the experienced scientist. Moreover, most orchid growers and propagators are familiar with only one or at most two languages (usually their own and English). These limitations may deny some growers access to certain methods. The tissue culture propagation appendix in *Orchid Biology, Reviews and Perspectives*, Vol. 1, written by one of us (J. A.), to provide access to most available methods in the mid-1970s, is now outdated. This book is intended to update the appendix by including all the information it contains as well as procedures that have developed since

it was written. However, despite all our efforts we may not have included all existing methods.

Procedures must be presented in a clear easy-to-follow format to be useful. Comments by users and reviewers suggest that the format used in *Orchid Biology*, Vols. 1 (tissue culture) and 2 (seed germination), is appropriate, and we have adopted it for this book. Procedures for which complete details are not available are described briefly.

Despite the bewildering number of formulations in this book, only a relatively small core of basic media are used for orchid tissue culture. These media are usually modified to meet the needs of individual orchids or the preferences of researchers. In writing this book we were faced with the need to choose between two formats: (1) listing only basic media and indicating modifications in each procedure, or (2) providing complete recipes in every case. We selected the latter despite the enormous amount of additional work it entailed because it is more convenient and (in computer jargon) more "user-friendly."

It is easier to follow a table that includes all components of a medium than to try to make sense of instructions in the following form: "for buds use Doe's medium, but replace 2 mg of hormone *X* with 1.5 mg of growth regulator *Y* and 0.5 mg of hormone *Z*. Also add 6 mg of hormone *W* in place of substance *V*. Replace vitamin A with an equal amount of vitamin B, and leave out vitamin C. Finally, add concoction RX7 instead of extract 300ZX. For stem explants use 1 mg hormone *A*, 0.5 mg growth regulator *B*, and 0.25 mg substance *T*. Do not alter the vitamin mixture, but use less agar and replace extract 300ZX with filtrate D1600, except for older stems when juice TR6 must be employed at 100 ml l^{-1}."

To provide a wide selection we have included in this book most, perhaps all, available methods for clonal propagation of orchids through tissue culture. Testing all of these procedures clearly would have been an impossible task, and for this reason we do not have firsthand experience with many of them. The outlines we present are based on the literature and as a consequence are limited by the amount of detail and degree of accuracy of each original communication.

Orchid nomenclature is in a constant flux and subject to disagreements among taxonomists. Rather than determine the "correctness" of names, we have chosen to use the ones employed by original authors.

Another point to keep in mind is that procedures are sometimes suitable only for certain cultivars, hybrids, species, and genera grown under specific conditions. This fact is not always evident. For these and other reasons we cannot guarantee success for any of the procedures and cannot assume responsibility for failures. Those who wish to propagate expensive and/or rare plants would be well advised to experiment first with less valuable ones.

Orchid tissue culture research is an active field, and new procedures were published while we were writing the book. This means that we had to add new methods to earlier sections while writing later ones (e.g., add a procedure to *Cymbidium* while writing about *Vanda*). If all figures and tables were to be numbered consecutively this would have meant constant renumbering. To avoid this onerous task we used prefixes to number the tables and illustrations in each section. These prefixes are the abbreviations of generic (natural and hybrid) names adopted by the Sander's List of Orchid Hybrids. In cases where abbreviations do not exist in the List we devised provisional ones that follow its format.

Full appreciation of present procedures requires a knowledge of their history, which is the reason for the first chapter. We thank Dr. Abraham (Abe) D. Krikorian, Department of Biochemistry, State University of New York, Stony Brook, for providing some of the information in this chapter through his excellent reprints and several informative discussions. However, the opinions in the chapter are our own.

Conversations with those who have used the appendices in *Orchid Biology, Reviews and Perspectives*, Vols. 1 and 2, indicated that a more general discussion of methods and procedures would be of benefit, especially to those who may not be completely familiar with the methodology. Chapter 2 was written to meet this need. Not all orchid laboratories have access to reference books that contain conversion factors, lists of abbreviations, definitions of units, information about reagents, and similar data. Chapter 2, which contains some information of such nature, is intended to make this book as much as possible a self-standing reference.

Detailed indices are indispensable tools in a book like this. Preparing such indices manually is an extremely unpleasant task. The indexing capabilities of word-processing programs cannot be used to prepare book indices since they must be based on page proofs, which are not stored in files. A computer program written especially for this purpose by Kevin J. Hackett in 1983–1986 for *Orchid Biology, Reviews and Perspectives*, Vol. 4, and modified in 1989 by Handajany Suryadharma and Ling Shao (computer science students from Indonesia and Hong Kong, respectively, at the University of California, Irvine) made indexing this book much easier.

We thank those who provided us with illustrations; they are acknowledged in the captions. We are grateful to the following for reading and commenting on parts of the manuscript and/or engaging us in helpful discussions: P. N. Avadhani, C. S. Hew, and A. N. Rao (Botany Department, National University of Singapore), Djunaidi Gandawijaja (Bogor Botanical Gardens, Indonesia), Abdul Karim B. Abdul Ghani (Botany Department, University Kebangsaan Malaysia, Bangi, Selangor, Malaysia), Franz Hoffmann (University of California, Irvine), Syoichi Ichihashi (Department of Biology, Aichi University of Education, Aichi, Japan), Helen Nair (Botany Department, University of Malaya, Kuala Lumpur), Leslie Paul Nyman (California State Polytechnic University, Pomona, California), Tim Wing Yam (formerly of the University of Hong Kong, then a postdoctoral fellow in our laboratory and now at the Singapore Botanic Gardens), and Gu Zhuping (Biology Department, Lanzhou University, Lanzhou, China).

Mordi Arditti provided hard disks for J.A.'s computers. Mak Chin On and Jill Lim Kok Eng (owners of Maryland Orchids, Singapore) and Mr. and Mrs. (Noes) Soediono (proprietors of Flora Sari Orchids, Jakarta, Indonesia) argued eloquently for Chapter 2, and the book is better as a result. G. C. Stephens as department chairman and dean provided funds for the necessary computers, peripherals, and software, and Emma Webb, J.A.'s former technician, gave us instructions on how to prepare better illustrations; we are thankful to all.

A book of this type requires special editorial and production skills, and the staff at Wiley were more than equal to the task. We thank our editor Philip C. Manor and the staff, including Jennifer Dowling, Ruth Ellowitz, Melanie Field, Joanne Kelman, Maggie Kennedy, and the copyeditor Susan Middleton for being so competent, helpful, and efficient.

Finally, two personal comments: First, both of us came to the United States as young adults without resources (J.A. did not even have a high school education or diploma and still does not). We feel that adopting this great and free country as our own was the wisest decision we ever made. The United States accepted us, gave us the same opportunities it affords its native sons, and thereby allowed us to become all we could be. Even more importantly, it gave our children (J.A.'s seven year-old Jonathan and R.E.'s Nina and Olivia and their children) the opportunity to be born as American citizens. No one could ask for more. We are deeply grateful and very appreciative. Second, we met in 1966 and have worked together ever since. Publication of this book will mark 25 years of fruitful collaboration. We are happy for that.

Joseph Arditti and Robert Ernst
Irvine, California
September 1990

CHAPTER ONE

History

A reasonable case can be made that new orchid propagation methods were always in the forefront of the biotechnology (or at least propagation methods) of their time. The first method for orchid seed germination (Moore, 1849; for reviews see Arditti, 1984; Yam et al., 2002*a*) was a radical departure from the manner in which other seeds were germinated 155 years ago. David Moore's (1807–1879) approach was innovative and a major horticultural and biological advance.

Half a century after Moore's discovery, Noël Bernard (1874–1911) made another quantum jump when he formulated a method for symbiotic germination of orchid seeds in vitro (Bernard, 1899, 1909; F. Bernard, 1990; for reviews see Boullard, 1985; Arditti, 1990; Rasmussen, 1995; Yam et al., 2002*a*). His is probably the first method for in vitro propagation of any plant. It utilizes what were at the time modern and advanced microbiological procedures. Bernard also predicted that a day would come when orchid growers would have laboratories as part of their establishments. This is the case at present not only for orchids, but also for other plants.

Lewis Knudson's (1884–1958) method for the asymbiotic germination of orchid seeds (Knudson, 1921, 1922; for reviews see Arditti, 1984, 1990; Yam et al., 2002*a*) was the first procedure for in vitro propagation of any plant in pure (i.e., axenic) culture. His method was a significant conceptual and technological innovation which foreshadowed modern biotechnology.

David Moore may have based his work (Moore, 1849) on reports that orchid seeds can germinate if scattered at the base of a mature plant. However Bernard's discovery and method were not based on any previous procedures and/or research by others. They were solely a result of his brilliance (Bernard, 1899, 1909; Boullard, 1985; Arditti, 1990; F. Bernard, 1990; Yam et al., 2002*a*). Knudson developed the asymbiotic method as a result of a sharp mind, incisive reasoning and on the basis of his own pioneering research with other plants (Knudson 1921, 1922; for a review see Arditti, 1990). The micropropagation of orchids by means of tissue culture has a more complex history, which is not free of controversy and includes unusual episodes (Arditti, 1977*b*, 1985, 2001; Arditti and Arditti, 1985; Torrey, 1985*b*; Arditti and Krikorian, 1996; Easton, 2001).

Terminology

As is very often the case, popular usage brought about some confusion regarding several terms associated with micropropagation. There is also some misuse. Given these facts, it is appropriate to describe and define a number of relevant terms at the outset (others are in the glossary, which please see). A number of the definitions presented here are taken from three scholarly and thoughtful reviews (Krikorian and Berquam, 1969; Krikorian, 1975, 1982).

Cell culture is the culture of isolated cells in vitro.

Clone as a term was introduced in 1903 and is based on the Greek word *clon*, which means twig, spray, or slip of the type used for vegetative propagation (Weber, 1903; Krikorian, 1982). Originally referred to plants produced through vegetative propagation methods like cuttings, layering, budding, and grafting, clone signified that "plants grown from . . . vegetative parts are not individuals in the ordinary sense, but are simply transplanted parts of the same individual, and in heredity and all biological and physiological senses such plants are the same individual" (Weber, 1903, cited by Krikorian, 1982). Because this definition can be applied to plants produced in vitro from a variety of explants, the term clone is now also used to describe individuals propagated in this manner. However, it is necessary to keep in mind that the tissue culture process, especially if the tissues are proliferated extensively, can be mutagenic and therefore some clones produced in vitro may not be completely (1) genetically uniform, or (2) similar to other clones or ramets of the same hybrid or cross.

Explant is a portion of a plant (tissue, organ, a few cells, or part of a callus mass) taken for culture in vitro. In practice the term is sometimes assumed to imply a relatively small amount of tissue, but this usage is neither universal nor a requirement.

Medium is a liquid or solidified solution used for the culture of explants, callus, organs, cells, protoplasts, etc. *Medium* is the singular form of the word whereas the plural is *media*. Using "media" as singular is incorrect. Therefore, it is proper to speak of "one medium" and "many media." "One media," "many medias," and "several mediums" are incorrect and simply bad English.

Mericlone was proposed by Mr. (at the time Lieutenant) Gene Crocker (Fig. 1-1) who originated it by condensing the words "meristem" and "clone." It was popularized by the late Gordon W. Dillon, long-time editor of the *American Orchid Society Bulletin* and executive secretary of the American Orchid Society (Dillon, 1964). This term is a very clever merchandizing tool, but, as has already been pointed out (Krikorian, 1982; Arditti and Krikorian, 1996), "mericlone" is unfortunate for several reasons:

1. It is a linguistic abomination as for example: "to mericlone," "mericloning," "to make a mericlone," "this plant has been mericloned," "to mericlone a mericlone" (meaning that a plant produced in vitro will be/is/was propagated a second time in the same manner), or "meristemmer" (Rutkowski, 1967). Fortunately no one seems to have bestowed upon him/herself the title of "mericloner" in the title of an article.
2. It is inaccurate since in most cases the explant is a shoot tip, not a meristem.

FIG. 1-1–1-6. **Early plant physiology and tissue culture researchers. 1. Lieutenant and later orchid grower Gene Crocker (source: Joseph Arditti (JA)). 2. Professor Gottlieb Haberlandt (White, 1943). 3. Professor Hans Fitting (photograph by Brigitta H. Flick, signature from a letter to JA). 4. Professor Kenneth V. Thimann (photograph from University of California Santa Cruz website, signature from autographed book owned by JA). 5. Professor Frits W. Went (photograph and signature from Went, 1990). 6. Professor Johannes van Overbeek (photograph from Skoog, 1951, signature from a letter to JA). Flowers below Fig. 1-6 (one facing left and the other right) are of *Schoenorchis funcifolia*, a Javanese orchid, whose carbon dioxide fixation was studied by Professor Went at the Bogor Botanical Gardens between 1927 and 1933 (orchid drawings from Smith, J. J. 1914. *Die Orchideen von Java*. E. J. Brill, Leiden, the Netherlands).**

3 The term is not really necessary because in principle there is no difference between cloning in vitro or through standard vegetative propagation methods.

4 It is misleading since it implies that all plants produced by this method are identical, which is not the case. Separate rules govern the naming of orchid crosses, clones, and mutants (Batchelor, 1982). These rules and the terminology they employ belong to the realms of plant and orchid taxonomy and nomenclature and are beyond the scope of this book.

Meristem is a well-defined term that describes the apex of a shoot tip. In common usage, especially among orchid growers, this term is erroneously used to describe the shoot-tip apex, which includes the apical meristem and some leaf primordia.

Micropropagation was first proposed in 1968 and defined as an aseptic procedure for the asexual production of plantlets from organs, tissues, and cells bypassing the sexual process or other means of asexual propagation (Krikorian, 1982; Hartman and Kester, 1983). This term should never be used to describe in vitro seed germination as is being done in the literature (see Perner, 1999, for one example).

Organ culture pertains to the culture of isolated juvenile or mature organs (leaves, roots, buds, shoot tips, flowers).

Ortet, from the Latin *ortus* (origin), was coined in 1929 to designate the "original plant of seedling origin from which members of a clone or ramets have originated" (Stout, 1929; Krikorian, 1982).

Protocorm, a term coined by Melchior Treub in 1890 (not by Noël Bernard as stated by Dr. Phillip Cribb in the first volume of *Genera Orchidacearum*, which was published in 1999), was applied to orchids by Bernard between 1899 and 1910. It refers to the small spherical, tuber-like bodies, formed by germinating orchid seeds (spherules is an incorrect name for these structures and must not be used). The term must not be used to describe similar bodies formed from explants or tissues in vitro (see below).

Protocorm-like body (PLB) is the proper term for structures that resemble protocorms and are formed by tissue explants and/or callus in vitro. This term was coined by Georges Morel in his first English language article on shoot-tip culture (Morel, 1960). It is the only first that can be attributed to him in connection with orchid micropropagation.

Protoplast culture should be applied only to cultures of isolated protoplasts (cells whose walls have been removed).

Ramet, based on the Latin *ramus* (branch), is an independent member of a clone (Stout, 1929; Krikorian, 1982).

Seedling is a young plant obtained from seed. This term may not be used to describe young plants obtained through tissue culture. The proper term for these is "plantlets."

Shoot tip is a meristem with several subjacent leaf primordia. This is the orchid explant generally cultured under the name "meristem."

Tissue culture is often used inappropriately to describe the culture of organs, tissues, cells, and protoplasts in vitro. This term should be applied only to the culture of tissues or tissue explants (meristems, callus sections, parenchyma pieces, tuber portions, and the like), not protoplasts, cells, or organs.

Origins of Orchid Micropropagation

Orchid micropropagation did not originate suddenly and de novo in the mind of one person despite a self-serving effort to create such an impression (Morel, 1960). The roots of orchid micropropagation are intertwined with the history of tissue

culture but they also have other origins (this chapter was revised extensively with new information, some of it taken verbatim or nearly so, from Arditti and Krikorian, 1996; I thank Professor Emeritus Abraham D. Krikorian for allowing me to use both text and photographs). Its origins lie in several lines of research and came from the work of many scientists, some of them well known and others not as appreciated as they should be (Arditti and Krikorian, 1996). The different lines of research will be discussed separately and brought to where they converged and gave rise to orchid micropropagation as it is known and practiced at present. A short outline of the history of plant hormones will also be presented because these substances are of critical importance to the culture in vitro of plant cells, tissue, and organs as well as to the differentiation of cultured plantlets (see Krikorian, 1995, for a more extensive history).

Plant Hormones and Propagation Additives of Plant Origin

Inclusion of plant hormones in culture media used for tissue culture, to control development and differentiation in vitro, and micropropagation is taken for granted at present. Yet, a century ago the existence of plant hormones was only being suggested.

Auxins

Gottlieb Haberlandt (1854–1945; Fig. 1-2), Professor of Plant Physiology in Berlin, was the first to propose the existence of plant hormones by stating that pollen tubes affect ovary growth through the release of substances he called *Wuchsenzyme* ("growth enzymes") and suggesting that if vegetative cells were cultured together with pollen tubes "perhaps the latter would induce the former to divide" (Haberlandt, 1902, English translation by Krikorian and Berquam, 1969; Arditti and Krikorian, 1996; Laimer and Rücker, 2003).

Pollen tubes do indeed release a substance which brings about post-pollination phenomena and ovule development in orchids. This was first shown by Hans Fitting (1877–1970; Fig. 1-3), before he became Professor of Botany in Bonn in his work with *Phalaenopsis* pollinia and pollination at the Bogor (then Buitenzorg) Botanical Gardens (Kebun Raya) in Indonesia (at the time the Netherlands Indies) in 1909 (Fitting, 1909*a*, 1990*b*, 1910, 1911, 1921 and a number of letters to JA in 1968 and 1969; for reviews see Arditti, 1971*a*, 1979, 1984, 1992; Avadhani et al., 1994). Fitting, who was "The first investigator to work with hormones and active extracts in plants" (Went and Thimann, 1937), went on to become one of the most prominent plant physiologists of his time and chancellor of the University of Bonn immediately after World War II.

He named the substance *Pollenhormon* and thus became the first plant scientist to use the word hormone in connection with plants and to suggest that they produce hormones.

From the time he named it (Fitting, 1909*a*, 1990*b*) and until his death (in letters to JA) Fitting maintained that *Pollenhormon* was a specific substance or hormone different from auxin. Present evidence suggests that Fitting's extracts in Bogor probably contained several substances including auxin (see Avadhani et al., 1994, for a

review). Fitting did not pursue the matter further, probably because he became interested in other phenomena including the sensory physiology of plants (Frits Went in conversation with JA; Arditti and Krikorian, 1996). Had he continued his work on *Pollenhormon*, Fitting might have discovered auxin.

The first intimation that *Pollenhormon* is or contains auxin was by Friedrich Laibach (1885–1967; he became prominent for his work with *Arabidopsis*), who reported that the active substance can be extracted with diethyl ether (Laibach, 1932). Several years after that, Kenneth V. Thimann (1904–1998; Fig. 1-4) demonstrated that the ether extract contained auxin (for reviews see Thimann, 1980; Avadhani et al., 1994; Arditti and Krikorian, 1996).

Auxin was discovered in Holland by Frits W. Went (Went, 1926, 1990; Went et al., 1928; Went and Thimann, 1937; Fig. 1-5) before Laibach extracted it from *Pollenhormon*. It was identified as indole-3-acetic acid (IAA) in 1934 (Went and Thimann, 1937; Haagen-Smit, 1951) and made successful tissue culture possible (Gautheret, 1935, 1937, 1983, 1985; Loo 1945*a*, 1945*b*). At present IAA and a number of synthetic auxins are used in orchid micropropagation.

Coconut Water and Cytokinins

In his classic paper Haberlandt suggested that "one might also consider the utilization of embryo sac fluids" (Haberlandt, 1902; Krikorian and Berquam, 1969; Laimer and Rücker, 2003). Two years later, E. Hannig followed this advice and screened the effects of such fluids from *Raphanus* and *Cochlearia* on the growth of embryos from the same species (Hannig, 1904; Krikorian and Berquam, 1969; Laimer and Rücker, 2003). European botanists of that period may not have been acquainted with the liquid endosperm of coconuts; however anyone who has spent time in the tropics will be familiar with the colorless liquid endosperm in green coconuts, which is a very refreshing drink by itself on a hot day or with a meal any time. This is coconut water. Coconut milk is a white liquid obtained by extracting, grating, or squeezing the solid white endosperm in mature nuts (in green nuts the endosperm is jelly-like and clear), which is dried to make copra. Like many Dutch botanists Johannes van Overbeek (1908–1988; Fig. 1-6) spent time at the Bogor (Buitenzorg) Botanical Gardens where he must have become acquainted with coconut water.

Later, when Albert Blakeslee (1874–1954; Fig. 1-7), a well-known geneticist at the time, wanted to culture recalcitrant immature embryos of *Datura stramonium* in vitro his associates Johannes van Overbeek and M. E. Conklin suggested the use of liquid endosperm of coconuts (i.e., coconut water) as a medium additive. Their suggestion was good; the *Datura* embryos grew well in its presence (van Overbeek et al., 1941, 1942) and an effective complex additive became available for plant tissue culture (van Overbeek et al., 1944; Steward and Shantz, 1955; Pollard et al., 1961; Tulecke et al., 1961; Raghavan, 1966). It can induce cell division in quiescent cells of carrot root phloem explants when added singly (Caplin and Steward, 1948; Krikorian, 1975; Gautheret, 1985).

Five years later Ernest A. Ball (Fig. 1-8) used coconut water to culture apical meristems (Ball, 1946; Krikorian, 1975, 1982). Frederick C. Steward (Fig. 1-9) and S. M. Caplin first reported on the use of coconut water for carrot root explants in 1948. After that, F. C. Steward made extensive use of coconut water for the culture

FIG. 1-7–1-12. **Students of in vitro culture of plants. 7. Professor Albert F. Blakeslee (Skoog, 1951). 8. Professor Ernest A. Ball (from a Kodachrome transparency by Joseph Arditti, signature from Ph. D. Dissertation by Michael S. Strauss). 9. Professor Frederick C. Steward alone (a) and with Mr. Russell C. Mott (b) and a flowering *Cymbidium* plant derived from cell suspension culture (courtesy Professor Emeritus Abraham D. Krikorian). 10. Dr. Georges Morel (Orchid Orlando, no date, signature from a letter to Hans Thomale). 11. Professor Folke Skoog (Janick, 1989). 12. Professor John T. Curtis (Skoog, 1951). The line drawing under Fig. 1-10 is of a *Phalaenopsis* flower. Professor E. A. Ball co-authored a paper on the micropropagation of this orchid. The line drawing under Fig. 1-12 is of a *Cymbidium* flower. Professor F. C. Steward produced flowering size plants (Fig. 1-9b) from cell suspension cultures of this genus.**

of carrot cells and the regeneration of plants (Krikorian, 1975, 1982; Steward and Krikorian, 1975). In 1950 L. Duhamet used coconut water to culture crown gall tissues (Duhamet, 1950). Also in 1950, Georges Morel (Fig. 1-10) cultured *Amorphophallus rivieri*, *Sauromatum guttatum*, *Gladiolus*, *Iris*, and lily in media containing coconut water (Morel, 1950). At present coconut water is used widely in tissue culture and micropropagation of many plants.

When used in combination with 2,4-dichlorophenoxyacetic acid (2,4-D) coconut water induced cell division in potato tuber explants even if neither of the two had any effect if added alone (Steward and Caplin, 1951). When the cytokinin zeatin was isolated from coconut water (Leetham, 1968) some (Galston, 1969; Skoog, 1994)

suggested that this explained the reasons for its activity, but there is an alternate view (Steward and Krikorian, 1971).

F. Mariat may have been the first to publish on the use of coconut water (erroneously referring to it as milk) and copra extract as an additive to media employed for orchid seed germination. When added at a concentration of 2% it did not inhibit germination and development but the seedlings were yellowish green. At higher levels coconut water was inhibitory to germination and development and the seedlings died (Mariat, 1951; for reviews see Arditti, 1967, 1979; Arditti and Ernst, 1984). Experiments with *Phalaenopsis* seedlings showed that coconut water can induce proliferation of protocorms (Ernst, 1967*b*). Coconut water is added at present to some orchid culture media (for some examples see Murashige, 1962; Jasper, 1966; Hahn, 1970; Pages, 1971; for reviews see Arditti, 1977*a*, 1977*b*; Holdgate, 1977; Rao, 1977; Zimmer, 1978; Fast, 1979; Sagawa and Kunisaki, 1984; Chen, 1985; Bouriquet, 1986; Czerevczenko and Kushnir, 1986; Griesebach, 1986; and procedures in Chapter 3). There is no consensus of opinions regarding the reasons for the effects of coconut water on orchids.

In the 1940s and 1950s research on plant tissue culture expanded, gained momentum, encountered new problems, and came up against recalcitrant tissues that required new approaches. One of these tissues was tobacco pith (Gautheret, 1985; Skoog, 1994). Folke Skoog (1908–2001; Fig. 1-11) and his students and associates at the University of Wisconsin formulated a number of media and evaluated the growth-enhancing properties of several substances in an effort to culture this tissue (Skoog, 1944, 1951; Skoog and Tsui, 1948; Skoog and Miller, 1957). One of the substances they tested was herring sperm DNA which had been stored for a very long time. In fact the time frame was long enough to raise the possibility that this DNA may have been left over from orchid seed germination experiments by Professor John T. Curtis (1913–1961; Fig. 1-12). However, Professor Carlos O. Miller (b. 1923; Fig. 1-13), one of the co-discoverers of cytokinins, thinks that the relationship between Curtis and Skoog was such that they would not have shared a reagent, not even one that had been languishing on a shelf for a long time. In any case, the research in Skoog's laboratory resulted in the discovery of the first cytokinin – kinetin (Strong, 1958; Miller, 1961, 1977; Leopold, 1964; Skoog et al., 1965; Gautheret, 1985; Skoog, 1994).

The discovery of cytokinins closes the circle as it were because by then the need for auxin and some vitamins (Gautheret, 1945) for explant cultures had already been established. The availability of kinetin enabled Toshio Murashige (b. 1930; Fig. 1-14) to formulate the widely used Murashige–Skoog (MS) culture medium for plant tissue culture (Murashige and Skoog, 1962; Smith and Gould, 1989; Skoog, 1994). Since an appropriate medium is a major factor in the establishment of cultures (Krikorian, 1982, 1995), many tissues and explants which were difficult or impossible to culture until then could now be cultured. The MS medium is used in many orchid micropropagation procedures.

Banana

Powdered banana was first incorporated in a medium for orchid seed germination in Brazil (Graeflinger, 1950 as cited by Withner, 1959*a*). The addition of banana to culture media for orchid seedlings became popular after that with a number of orchid

FIG. 1-13–1-18. **Plant scientists. 13. Professor Carlos O. Miller (photograph courtesy C. O. Miller, signature from a note to JA). 14. Professor Toshio Murashige (Janick, 1989). 15. Professor Roger J. Gautheret. 16. Seigneur du Monceau et de Vrigny, Henri-Louis Duhamel du Monceau. 17. Professor Herman Vöchting (photograph and signature from Fitting, 1919). 18. Professor Karl von Goebel (Wittrock, 1897–1903). The line drawings are of *Paphiopedilum* flowers, an orchid for which Professor T. Murashige developed a shoot-tip culture procedure.**

propagators claiming to have been the first to use it. Some growers homogenize banana fruit pulp with their media whereas others stir puree into their solutions, and there are those who simply submerge a few banana slices per flask. All of these enhance seedling growth. Banana-containing media are always easy to recognize due to their darker color (even when claims are made for a "secret non-banana" additive).

Opinion varied for a period as to whether green bananas (Hey and Hey, 1966) enhanced growth better than ripe ones. Preferences still exist among growers. However, there is no question that pulp from ripe bananas does stimulate the growth

of seedling and immature embryos of *Vanilla* (Withner, 1955) and *Phalaenopsis* seedlings (Ernst, 1967*b*).

According to one report "green and not ripe [bananas] . . . grated, cooked gently for 40 minutes, [and] strained . . ." (Hey and Hey, 1966) enhanced seedling growth. That the extra work and tedium associated with this procedure are not necessary was demonstrated in experiments with banana pulp (Ernst, 1967*b*). Subsequent experiments (Pages, 1971) confirmed these findings (for a review see Withner, 1974*a*, 1974*b*). Banana pulp can also enhance the growth of plantlets obtained from explants in vitro (see procedures in Chapter 3). The reasons for the effects of banana are not clear. Attempts to fractionate banana pulp through serial extractions with several solvent produced inconclusive results (Arditti, 1968).

The effects of a number of other plant homogenates on seed germination and seedling growth have been evaluated (Arditti, 1967, 1979; Ernst, 1967*b*; Arditti and Ernst, 1984), but few if any of them are used with explants. Some are added to cultures of protocorm-like bodies or developing plantlets with varying result (see specific procedures).

Culture of Tissues and Organs

Roger J. Gautheret (1910–1997; Fig. 1-15), one of the earliest prominent figures in the history of plant tissue culture in France and later an historian of the field, wrote that "the progress of plant tissue culture was made possible by only a few genuine discoveries [which] . . . did not appear suddenly, but after a long and slow journey, unpretentiously covered by pioneers" (Gautheret, 1985). According to him, the earliest of these pioneers in the "prehistory" of plant tissue culture (Gautheret, 1985) was the Frenchman Henri-Louis Duhamel du Monceau (1700–1782; Fig. 1-16), who studied wound healing in trees while also writing about naval architecture (11 volumes) and science and art (18 volumes). In his book *La Physique des Arbres* (1756) he described swelling and the appearance of buds following the removal of bark and cortex from an elm tree (Gautheret, 1985). Gautheret's view is that this was the discovery of callus formation and "a foreword for the discovery of plant tissue culture. But in 1756 the bacteriological technique was not invented, asepsis was unknown, the concept of tissue culture had not been yet expressed, and finally nobody was able to appreciate Duhamel's discovery" (Gautheret, 1985). Perhaps so, but callus formation on mature trees after wounding bears little, if any, resemblance to tissue culture. Also, the development of grafting and budding techniques can be described as being equally relevant. But it may well be that in this account Gautheret was more interested in endowing one of his countrymen with a first rather than writing an objective historical account.

In an earlier historical presentation, Gautheret was more objective and made a convincing suggestion that "the history of plant tissue culture begins in 1838–1839 when [M. J.] Schleiden (1838) and [T.] Schwann (1839) . . . stated the . . . cellular theory and implicitly postulated that the cell [is] totipotent" (Gautheret, 1983). Schwann even suggested that "plants may consist of cells whose capacity for independent life can be clearly demonstrated . . ." (translated from German by Gautheret, 1985). That this is so was demonstrated experimentally and considered theoretically by A. Trécul in 1853, H. Vöchting (Fig. 1-17) in 1878, K. Goebel (Fig. 1-18) in 1902, J. Sachs

(1832–1897) between 1880 and 1882, J. Wiesner in 1884, and C. Rechinger in 1893. The latter suggested that excised plant sections could develop in a solution (Gautheret, 1983). He clearly proposed that isolated plant parts could be cultured in vitro.

Early Tissue Culture Attempts

Gottlieb Friedrich Johann Haberlandt (1854–1945; see Fig. 1-2), considered by some to have originated the concept that structure and function are intertwined in plants (that is, physiological plant anatomy), made the first attempt to culture plant cells (Haberlandt, 1902; Krikorian, 1975, 1982; Gautheret, 1985; for an annotated English translation accompanied by a scholarly essay see Krikorian and Berquam, 1969; Laimer and Rücker, 2003). Haberlandt's first attempt was to culture isolated leaf palisade and mesophyll cells of *Lamium purpureum*, stinging hairs of nettle, *Urtica dioica*, glandular hairs of *Pulmonaria*, stomatal cells of *Fuchsia magellanica* Globosa, pith cells from petioles of *Eichhornia crassipes*, and three monocotyledonous species, *Tradescantia virginiana* (stamen filament hairs), *Ornithogallum umbellatum* (stomatal cells), and *Erythronium des-canis* (stomatal cells). He used Julius Sachs's version of Knop's solution (1 g potassium nitrate, 0.5 g calcium sulfate, 0.5 g magnesium sulfate, 5 g calcium phosphate, and a trace of ferrous sulfate per liter; a medium which is still useful at present) and added to it sucrose, glucose, glycerin, asparagine, and peptone (except for glycerin these additives are still being used). In addition, he used light (natural daylight and photoperiods, April–June and September–November in Germany) and dark culture conditions as well as appropriate temperatures (18–24°C).

Haberlandt was unsuccessful – "cell division was never observed" (Krikorian and Berquam, 1969; Laimer and Rücker, 2003). In retrospect there are several reasons for his failure (Krikorian and Berquam, 1969; Laimer and Rücker, 2003). One was his selection of cells, which were mature, specialized, non-meristematic, and highly differentiated. The second was his culture medium; it lacked substances now known to be required by tissue and cells in vitro (vitamins, hormones, *myo*-inositol, and other additives). Third, "Haberlandt could not have been less judicious in his selection . . ." (Krikorian and Berquam, 1969; Laimer and Rücker, 2003) of plants. He not only used three monocotyledonous species but also ones which are recalcitrant. One reason for this selection may have been Haberlandt's strong belief in the cell theory. This is ironic since it was this belief which led him to try cell cultures in the first place. However, chance must have also played a role since in those days Haberlandt had nothing to guide him in the selection of "proper" plants and explants. In all fairness it is necessary to keep in mind that subsequent discoveries of plants which are easy to culture were often a matter of luck. Finally, "Haberlandt did not think it necessary to achieve complete sterility" (Krikorian and Berquam, 1969; Laimer and Rücker, 2003) and stated in fact that "the cultured plant cells were impaired only slightly in their progress by the presence of numerous bacteria in the culture solutions" (translation by Krikorian and Berquam, 1969; Laimer and Rücker, 2003). Cell and tissue culture at present would be unthinkable without complete sterility, or at least the inhibition of contaminants.

Assertions that Haberlandt's failure was due to the fact that ". . . he neglected Duhamel's results as well as Vöchting's and Rechinger's experiments . . . and [his] ignorance of the past" (Gautheret, 1985) are unjustified, have no scientific basis, seem unnecessarily harsh, and may be based more on national pride than on solid science. He would have failed with most explants (Duhamel's species included) since the vast majority of tissues require a richer medium and do not grow in a contaminated solution. Haberlandt was probably not aware of the procedure used to culture *Phalaenopsis* flower stalks at the time (Anonymous, 1891). His medium may have supported their growth, but contamination would have destroyed them.

A more judicious selection of plant material and some luck may have led to perhaps partial success, but attention to Duhamel could not have been the key to Haberlandt's failure. He tried to culture potato tuber tissue and also failed, probably because his medium lacked the very hormones he envisioned (Krikorian, 1982). Perhaps he might have succeeded with carrot explants, but he made no attempts to culture them. In a foreshadowing of the use of coconut water in culture media, Haberlandt suggested the use of embryo sac fluids and used liquids from *Raphanus* and *Cochlearia* to culture embryos (Krikorian and Berquam, 1969; Laimer and Rücker, 2003). Given this fact "it is tempting to speculate that perhaps Haberlandt . . . might have conceived coconut as being a source of readily available 'embryo sac fluids' had coconuts been generally available in Berlin" (Krikorian and Berquam, 1969; Laimer and Rücker, 2003). Or, perhaps, if he had noticed them in Indonesia.

"Haberlandt followed the literature intently" (Krikorian and Berquam, 1969; Laimer and Rücker, 2003), which is obvious from the extensive citations in his papers, and the reference to Fitting's research with orchid pollinia in connection with his own observations. He also cited Vöchting in 1913 (Krikorian, 1982). This is hardly indicative of neglect or ignorance of previous literature. Haberlandt may have chosen to ignore Duhamel's observation perhaps because he did not believe it to be relevant, which is probably the case. Regardless of his failure, Haberlandt ". . . ushered in . . . a new era of inquiry" (Krikorian and Berquam, 1969; Laimer and Rücker, 2003). Others followed in his footsteps and had more success. H. Winkler attempted to cultivate string bean segments and reported cell divisions but no proliferation (Winkler, 1908; Gautheret, 1985). In the same year S. Simon reported the formation of callus, buds, and roots from poplar explants (Simon, 1908).

Culture of Stem Tips

As a concept, the utilization of buds or stem tips for mass rapid clonal propagation is more than a century old. As far back as the 1890s Carl Rechinger in Vienna tried to culture stem sections and excised buds of *Populus nigra* and *Fraxinus ornus* as well as portions of roots on sand moistened with tap water (Rechinger, 1893; Krikorian, 1982; Gautheret, 1983). Rechinger failed in his attempts, but concluded that for proper development sections must be thicker than 1.5 mm. His procedures cannot be called "tissue culture" as the term is understood at present, but they foreshadowed current methods because he used a medium (tap water), support (sand), and explants. Classic tissue culture procedures at present include: (1) a nutrient substrate that includes organic components like sucrose, which make it advisable to use aseptic techniques;

(2) an explant; and (3) in some cases agar or gellan gum (i.e., Gelrite or Phytagel) as a solidifier or support. However, except for the explant, several of these factors are not necessarily absolute requirements. Some are a matter of convenience or may sometimes be invoked for pedantic rather than functional reasons. Even sterility, which is clearly preferable, is not an absolute requirement if microbial contaminants can be kept under control, inhibited, and/or prevented from smothering or attacking the explants as is now possible through the use of PPM™ (see Chapter 2; Thurston et al., 1978, 1979; Spencer et al., 1979/1980; Brown et al., 1982, 1984; Johnson et al., 1982; Cvitanic and Arditti, 1984).

Nearly 20 years after Rechinger, the German experimental morphologist Karl [later von] Goebel (1855–1932; Fig. 1-18), who also spent time at the Bogor Botanical Gardens, attempted to grow excised buds of the water fern *Ceratopteris thalictroides* in peat moss, but obtained only abnormal plants (Goebel, 1902; Krikorian, 1982). Like Rechinger's method, Goebel's procedure was not "tissue culture" as the term has been used during the last 30–40 years. However Goebel did use explants and a medium.

Research on the effects of the presence or absence of cork, water, and polarity on root formation in *Salix* by the German botanist Herman Vöchting (1847–1917; Fig. 1-17) was somewhat tangential (Vöchting, 1906). However, it was an important contribution to the quest for tissue culture (Krikorian, 1982).

Despite accelerated research in the field, about 15 years passed before William J. Robbins (1890–1978; Fig. 1-19) attempted the first stem- and root-tip cultures at the University of Missouri (Krikorian, 1982; Gautheret, 1983). He germinated seeds of peas, corn, and cotton under aseptic conditions, excised root and stem tips, and tried to grow them in the dark on sterile Pfeffer's solution with and without glucose or fructose (Knop, 1884; Pfeffer, 1900; White, 1943; Krikorian, 1975, 1982; Arditti, 1977*b*, 1992; Murashige, 1978; Arditti and Krikorian, 1996; see Arditti et al., 1982 for composition of this medium). The corn and pea explants grew normally, but those taken from cotton did not (Robbins, 1922*a*, 1922*b*). Cotton explants produced roots but showed characteristics that were "typical of plants grown in the dark" and were chlorotic (Robbins, 1922*b*).

The results obtained by Robbins are easy to explain at present. He did not have plant hormones at his disposal because they were yet to be discovered. And, despite eventually making major contributions to the understanding of the role of vitamins in plant tissue culture, he did not even know initially that they may be required by some explants. He also did not realize that his cultures would benefit from illumination. Still, Robbins and his associates succeeded in maintaining their root-tip cultures for almost 4.5 months (Robbins and Maneval, 1923, 1924).

Walter Kotte (1893–1970; Fig. 1-20), one of Haberlandt's students in the Pflanzenphysiologische Institut in Berlin-Dahlem, cultured pea roots independently of Robbins, but at the same time, Kotte used Knop's salts (Knop, 1884) as his basic salt medium. He added to it alanine, asparagine, glucose, glycine, a meat extract, a digest of pea seeds, and peptone. Kotte's medium was more sophisticated than the one used by Robbins and may have contained vitamins, some plant hormones, and inositol as components of the complex additives. The roots grew in his medium, but could not be subcultured (Kotte, 1922*a*, 1922*b*; White, 1943).

Philip R. White (1901–1968; Fig. 1-21) of the Rockefeller Institute for Medical Research at Princeton, New Jersey reasoned that apical and intercalary meristems

FIG. 1-19–1-26. **Pioneers of plant cultures in vitro. 19. Professor William J. Robbins (Gautheret, 1985). 20. Dr. Walter Kotte (photograph and signature from White, 1943). 21. Dr. Philip R. White (photograph from Gautheret, 1985; signature from White, 1943). 22. Professor Pierre Noubécort (Gautheret, 1985). 23. Professor Loo Shih Wei (from a transparency by Professor Franz Hoffmann taken in Beijing ca. 1985, English and Chinese signatures from a letter to Joseph Arditti). 24. Some of the earliest asymbiotic orchid seedlings produced by Professor L. Knudson (Knudson, 1924). 25. Professor Lewis Knudson (photograph courtesy Professor Emeritus Charles H. Uhl, signature courtesy Cornell University Archivist Kathleen Jacklin). 26. Professor Wilhelm Pfeffer (Wittrock, 1897–1903). The line drawings are of Chinese cymbidiums, orchids which interested Professor Loo (see Arditti, 1999, for an obituary).**

"would be best to choose [as] materials for our first experiments" (White, 1931, 1933*b*). During a visit to the plant physiology institute at the University of Berlin which extended from the winter of 1930 to the spring and summer of 1931, he attempted to culture root tips (White, 1932*a*, 1933*a*) and "some 400 stem tips"of the "common weed" *Stellaria media* in hanging drops of a nutrient solution (U+U) formulated for pure cultures of *Volvox minor* and *V. globator* (Uspenski and Uspenkaja, 1925). White had used this medium previously for cultures of root tips, embryos, and other explants (White, 1933*b*). He managed to keep the tips alive "for periods up to three weeks . . . [and] during this time there . . . occurred active cell division . . . growth . . . differentiation into leaves, stems and floral organs" (White, 1933*b*). However his results were disappointing by present standards. The reasons given for the limited success were accumulation of "excretory products, and the exhaustion of nutrient materials" (White, 1933*b*). Medium composition is a more plausible explanation. U+U contained no vitamins or hormones because some of them were yet to be discovered or studied, and others were still new to science, or yet to be established as requirements. Also, it did not contain ammonium ions.

More specifically, *myo*-inositol, although not really a vitamin and a substance not present in the U+U medium, was isolated from muscles in 1850, and was first used in plant tissue culture media much later. One of the earliest inclusions of inositol in a plant tissue culture medium was half a century ago (Jacquiot, 1951), but it acquired importance as a possibly useful inclusion in plant tissue culture media only after the sugar alcohols sorbitol, *meso*- or *myo*-inositol and *scyllo*-inositol were isolated and identified as components of coconut water (Pollard et al., 1961). Despite being implicated in signal perception as part of the phosphoinositide system, it must still be shown that inositol plays a major and positive role in the growth of plant tissues in vitro. Interpretation of results from the utilization of inositol have generally been indecisive (Åberg, 1961). However its addition seems to do no harm, and adding it routinely to the MS medium provides a safety margin.

Thiamine (vitamin B_1), a common additive to culture media at present, was isolated from rice bran in 1910–1911, but its structure was elucidated only in 1926. Niacin (nicotinic acid) was first produced by oxidizing nicotine in 1925, but added to culture media only several decades after that. Ascorbic acid (vitamin C) was first isolated in 1928, studied more extensively in 1933, and is used in plant tissue culture media rarely even at present. The structure of riboflavin (vitamin B_2), a vitamin used in some culture media, originally isolated from eggs, was described in 1935. Biotin, discovered in egg yolks in 1936, is not in common use even now. Pyridoxine (vitamin B_6), which is also used in many culture media, was isolated from rice and yeast in 1938. Pantothenic acid was isolated from liver and its structure was first elucidated ca. 1940. Folic acid was identified in 1948 after being crystallized from liver in 1943 and yeast in 1947 (for a review of vitamins and orchids see Arditti and Harrison, 1977). Of the plant hormones used in tissue culture, auxins were discovered in 1928 (Went, 1928, 1990) and cytokinins in 1955 (Miller, 1961). Information that vitamins and hormones are required by explants in culture started to accumulate ca. 1936–1938 (for reviews see White, 1943; Schopfer, 1949; Åberg, 1961).

Even without additives known to be required at present, White's medium was one of the best available at the time. Corn shoot tips cultured on it produced plants (Segelitz, 1938). Tips shorter than 2 cm required illumination. Longer shoots (2–4 cm) grew

in the dark (Segelitz, 1938). This is one of the earliest successes with the culture of a monocotyledonous plant in vitro. It was reported more than a dozen years before what was claimed to have been the first success with this group (Morel and Wetmore, 1951*a*). However, it should be noted that Morel and Wetmore dealt with callus production in their cultures. The difference between shoots and callus can be viewed as hair splitting, but this success was considered to be significant because monocotyledonous plants do not normally produce wound tissue and therefore cultures grew only with difficulty until more suitable procedures were developed (for discussions of monocotyledonous plant recalcitrance see Swamy and Sivaramakrishna, 1975; Hunault, 1979).

Announcements that plant tissues can be cultured "for unlimited periods of time" were made independently and at about the same time during this period, but not "simultaneously" as stated incorrectly (for a review see Gautheret, 1985) by P. R. White (Fig. 1-21; ca. December 31, 1938), R. J. Gautheret (Fig. 1-15; on January 9, 1939) and Pierre Noubécourt (1895–1961, Fig. 1-22; on February 20, 1939). These findings on the potentially unlimited growth of callus cultures set the stage for the first successful culture of a stem tip not many years after that.

The second monocotyledonous plant to be propagated by what can retrospectively be described as a "prehistoric" or crude form of tissue or explant culture was taro (*Colocasia esculenta*), an ancient and still very important crop in the Pacific region and Hawaii. To accelerate taro propagation an attempt was made to culture normally dormant buds "borne in the axils of the leaves on the surface of the taro corm" (Kikuta and Parris, 1941). Tuber slices, 2–5 cm thick, and buds "together with approximately 1 cubic centimeter of corm tissue," planted in sterilized soil produced plants. In other words, corm explants and excised buds cultured in sterile soil as a culture medium produced plants. There is no real or valid reason why only a semisolid or liquid solution can or should be defined as a culture medium. This method of taro multiplication (Kikuta and Parris, 1941) is analogous to current tissue culture propagation even if the procedures are somewhat crude and the cultures are not in vitro. Unfortunately, this method and related ones are mentioned only in a few instances (Arditti and Strauss, 1979; Arditti and Ernst, 1993; Krikorian, 1994*a*) and is generally missing from historical reviews (Gautheret, 1980, 1982, 1983, 1985). Taro was cultured in vitro for the first time 30 years later (Mapes and Cable, 1972; also see Arditti and Strauss, 1979; Krikorian, 1994*a*).

Another monocotyledonous crop, rye, was also cultured early (de Ropp, 1945). Stem tips (actually the plumules) of excised embryos were cultured on White's medium containing 2% (w/v) sucrose. When "any isolated stem tip developed a root the entire growing point was stimulated to meristematic activity, and leaves normal in form and size developed" (de Ropp, 1945). These explants were embryonic and it is possible to suggest that they were not equivalent to shoot tips of mature plants. However, present evidence (at least that obtained from orchids) suggests that embryonic stem tips from mature plants and seedlings are similar or for the most part do not differ substantially with respect to their requirements in vitro.

From the mid-1930s to the 1950s the California Institute of Technology (Caltech) in Pasadena was arguably the world center for research in plant physiology. Its faculty [which included such major figures in plant physiology as Kenneth V. Thimann (1904–1997; Fig. 1-4), James Bonner (1910–1996), Frits W. Went (1903–1990; Fig. 1-5), Herman Dolk (d. 1932), Arie J. Haagen Smit (1900–1977), Johannes van

Overbeek (Fig. 1-6), and others] attracted excellent graduate and postdoctoral students from all continents (Thimann, 1980). One of these was Shih Wei Loo (Loo Shih Wei, Chinese style; 1907–1998; Fig. 1-23). He came to the USA. In 1943, he earned his Ph.D. at Caltech in 2 years and in 1945 became research associate at the Botany Department of Columbia University in New York. A year later he moved to the Chemistry Department and stayed there until 1947 when he returned to China. There he was appointed Professor of Botany at Beijing University. In 1953 Loo moved to the Plant Physiology Institute in Shanghai where he remained until the end of his life. Loo suffered more than most during the cultural revolution but returned to his laboratory after the upheaval, resumed research, and trained graduate students until his last days (Arditti, 1999).

For his doctoral dissertation Loo cultured excised stem tips of *Asparagus officinalis*, 5–10 mm long, on a medium utilized by James Bonner for the culture of tomato roots (Loo, 1945*a*). Some of Loo's explants formed buds, but none produced roots. His conclusion was that growth of the excised stem tips was "potentially unlimited" (Loo, 1945*b*). It is clear at present that he was right. Also, it seems reasonable to assume that the tips would have developed roots if an auxin had been added to the medium. Following his move to Columbia University Loo published yet another report on asparagus shoot tips (Loo, 1946*a*). He demonstrated that a solution rendered semisolid with agar was "as good, if not better, than liquid medium." While doing that, he devised a simple method for supporting stem tips (Loo, 1946*a*). Growth of the explants remained normal. They were still alive after 22 months and following 35 transfers (Loo, 1946*a*).

Loo also cultured stem tips of the parasitic flowering plant dodder (*Cuscuta campestris*). His cultures did not produce roots and leaves but fortuitously they did flower in vitro (Loo, 1946*b*). This is probably the first instance in which "floral organs . . . developed on excised stems tips in vitro" (Loo, 1946*b*). Again, it is reasonable to speculate that dodder explants would have formed leaves and roots if Loo had added appropriate hormones to his medium (Galston, 1948). Unfortunately he did not (cytokinins were discovered in 1955). However he did conclude that the explants required sugar for growth in vitro. This was a relatively new conclusion (but in some cases orchid explants develop in a more desirable fashion only on a sugar-free medium). Loo also cultured and obtained flowering in vitro of the composite *Baeria chrysostoma*, a small annual sometimes grown in gardens and which belongs to a California genus consisting of ca. 20 species (Loo, 1946*c*).

Clearly, "Professor Loo's papers suggest that tissue culture of angiosperms and micropropagation would have advanced more rapidly had he remained in the USA and/or if conditions in China had been different. His important contributions to stem-tip culture and ultimately to micropropagation have thus far received credit only passingly in a few reviews (Krikorian, 1982; Gautheret, 1983) and a few research papers (Steward and Mapes, 1971*b*; Koda and Okazawa, 1980). Loo's work is certainly not as well-known as it should be" (Arditti and Krikorian, 1996). It is worth emphasizing here that Segelitz, de Ropp, and Loo (independently of each other), and not subsequent workers (Morel and Wetmore, 1951*a*; Gautheret, 1983, 1985), were the first to have significant success in culturing monocotyledons in vitro.

Frits W. Went (Fig. 1-5), discoverer of auxin, was associated indirectly (through a gift of auxin) with the first successful culture of an axillary bud meristem by Carl D. LaRue (1888–1955). It was that of water cress on White's mineral nutrients

supplemented with 20 g (w/v) sucrose l^{-1} and "hetero-auxin 1 part to 20 millions" (LaRue, 1936).

Ernest A. Ball (1909–1997; Fig. 1-8) was interested in shoot tips and apical meristems (Ball and Boell, 1944; Ball, 1972), "the capacity for growth and development of vegetative plant cells," "polarity of the buds and subjacent cells," "the relation between respiration and development, independence of the tip from the rest of the plant, production of subjacent tissues by the apex," and the "totipotentiality of all living plant cells" (Ball, 1946). He excised shoot apices of nasturtium, *Tropaeolum majus* L. ("55 µ high and 140 µ thick"), and lupine, *Lupinus albus* L. ("81 µ high and 250 µ thick"); the sections were 400–430 μm^3 in volume (Ball, 1946).

Ball made "no provisions to achieve and maintain asepsis," and "inoculations were performed in the laboratory," but his cultures did not become contaminated. He placed explants on Robbins' modification of "Pfeffer's Solution" plus microelements and in some cases "unautoclaved coconut milk" (actually coconut water). The medium was made semisolid with agar which changed in color from brown to white after being washed with thirty 24-hour changes of distilled water. His explants grew well (Ball, 1946 and clearly stated by Ball in many conversations with Joseph Arditti while he was at UCI). Any insinuations to the contrary (Morel, 1974) are entirely without foundation, self-serving, and disrespectful of a pioneering plant scientist. Loo Shih Wei and Ernest A. Ball succeed in culturing shoot tips before Georges Morel did. And, nursery owner Hans Thomale and Dr. Lucie Mayer, not Georges Morel, were the first to culture an orchid shoot tip (Arditti and Krikorian, 1996).

First Micropropagation of Orchids

More than 100 years ago British orchid growers placed *Phalaenopsis* flower stalk nodes in peat and succeeded in producing plantlets from their buds (Anonymous, 1891, 1892; for a review see Arditti, 1984). This method of propagating *Phalaenopsis* can be viewed as a simple or crude form of tissue culture because an explant (a bud on a stalk section) was placed in/on a "medium" (moss, albeit non-sterile) and "cultured" until it produced a plantlet or died. In addition to being of practical use, this propagation procedure proved that isolated buds can "be separated from the plant and continue to grow" as suggested by Schwann in 1839 (in Gautheret's translation, 1985).

Unfortunately this method of propagating orchids escaped the attention of botanists at the time (and for many years after that) probably because: (1) it was superficially similar to the rooting of cuttings (but in fact very different from it since buds on *Phalaenopsis* flower stalk produced shoots which developed roots and became plantlets in a manner similar to that of a bud, a callus section, or a protocorm-like body in vitro); (2) it was published in a very early, highly specialized and obscure journal which even at present is hard to find; (3) "an increasing number of scientists read no modern languages other than English" (Krikorian and Berquam, 1969); and (4) not many scientists take the time to read the old literature regardless of language and prominence (or lack of it) of journals.

At least one person did notice the articles at the time because according to a short notice a grower named Perrenoud (no first name given) who saw reports in so-called

"journaux anglais" placed sections of *Phalaenopsis* roots in humid enclosures and obtained a plant (Anonymous, 1891). This is reminiscent of micropropagation. No details are available, except that *Phalaenopsis* roots can produce buds and plants (for a review see Churchill et al., 1972*b*). Nevertheless this attempt can be described as being part of the pre-history of orchid micropropagation (Arditti and Krikorian, 1996).

Had this method not escaped attention, it and its discoverer (an unknown British orchid grower) could have been important signposts on the road to plant tissue culture and micropropagation as they are known at present. It is certainly more (1) relevant to tissue culture, (2) important as a "foreword," and (3) similar to micropropagation than the observations by the Seigneur du Monceau et de Vrigny, Henri-Louis Duhamel du Monceau (Gautheret, 1985; Fig. 1-16).

The modern history of orchid micropropagation started when (1) "a new [tissue culture or in vitro], simple and practical method for vegetative [clonal] propagation of *Phalaenopsis* [orchids] was developed at Cornell [University]" 5 years (Rotor, 1949) before the first published report of orchid stem-tip cultures, and (2) a German nurseryman suggested that this method can be used for micropropagation (Thomale, 1956, 1957). The nutrient medium used to culture the *Phalaenopsis* nodes was Knudson C (KC) formulated for the asymbiotic germination of orchid seeds by Lewis Knudson (1884–1958; Figs 1-24 and 1-25), Professor of Plant Physiology at Cornell University (see Arditti, 1990 for a history, additional photographs, and a biography).

Knudson's first solution, known as the Knudson B medium (KB), was a modification of Pfeffer's Solution, a formulation devised by the German plant physiologist Wilhelm Pfeffer (1845–1920; Fig. 1-26). It was, and still is, a reasonably good medium for orchid seed germination, but Knudson improved it and published his solution C (Knudson C, KC) in 1946 (Knudson, 1946). This medium is used very widely for orchid seed germination (Arditti et al., 1982) and the micropropagation of some orchids.

Gavino Rotor Jr. (Fig. 1-27) was born in Manila on March 26, 1917 (the biographical information and photograph presented here were provided by Dr. Rotor) and died in the mid or late 1990s. His mother, an orchid enthusiast, introduced him to her plants before he was 10 years old. By the time Gavino entered high school he knew the scientific names of the major Philippine orchid species. His interest in orchids is probably what led him to major in agriculture at the University of the Philippines where he received his B.S. in Agriculture in 1937.

World War II broke out while Rotor was waiting to go abroad for further study. This delayed but did not alter his plans. He "chose Cornell University for several reasons, the most important ones being Dr. Knudson's presence there and its impressive reputation in the horticultural sciences." After receiving his M.S. degree in 1947 and "hearing Dr. Kenneth Post's lectures on the effects of day length and temperature on the growth and flowering of various florist crops [Rotor] decided to focus on the responses of orchids to temperature and day length" for his doctorate at Cornell University His major professor was the floriculture crop physiologist Kenneth Post (1904–1955). Knudson was a member of his doctoral thesis committee.

In one of his letters Dr. Rotor wrote that he conceived the idea of propagating orchids while attending a lecture by Knudson on the role of sugars in plant growth (Knudson's interest in sugar metabolism and utilization by plants lead him to orchid

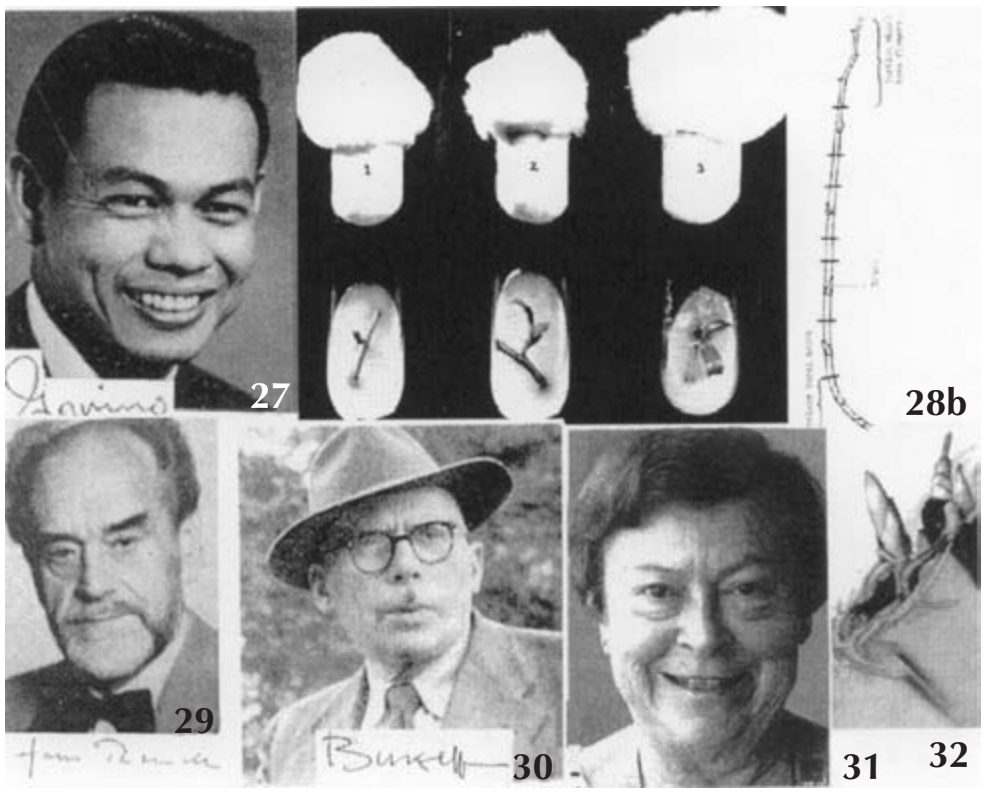

FIG. 1-27–1-32. **Pioneers in orchid propagation. 27. Dr. Gavino Rotor (courtesy the late Dr. Gavino Rotor). 28. The first ever attempt of orchid micropropagation – *Phalaenopsis* flower-stalk cultures: (a) explants in culture; (b) diagram showing how the flower stalk was sectioned (Rotor, 1949). 29. Hans Thomale, 1919–2002 (photograph courtesy Hans Thomale, signature from a letter by Hans Thomale to the author obtained with the help of E. Lucke and Dr. Norbert Haas-von Schmude). 30. Professor Hans Burgeff, 1883–1976 (photograph from Haber, 1963, signature from a letter to Professor Robert Ernst). 31. Dr. Lucie Mayer (courtesy Dr. Lucie Mayer obtained with the help of E. Lucke and Dr. Norbert Haas-von Schmude). 32. Shoot-tip explants of *Orchid maculata* (courtesy Hans Thomale obtained with the help of E. Lucke and Dr. Norbert Haas-von Schmude).**

seed germination). Rotor did not elaborate on how a lecture on sugars made him think of culturing the nodes of *Phalaenopsis* flower stalks. He cut inflorescences into segments and placed nodal sections, each with one bud, on KC in the hope that the buds would produce plants. The buds became swollen and leaves appeared after 14–60 days. Roots were produced after two or three leaves were formed (Fig. 1-28). Only seven of 65 buds failed to develop (Rotor, 1949). Rotor recalled in a letter that Knudson's "eyes brightened when [Rotor] showed him the first successful propagation . . . and told him how [he] got the idea from [Knudson's] lecture" (Arditti, 1990).

There can be absolutely no question that Dr. Gavino Rotor invented modern orchid micropropagation and was the first to publish a scientific report on clonal multiplication of a higher plant in vitro. His method involved a defined culture medium,

aseptic techniques, and explants. And, he called attention to the propagation potential. Some might argue that his procedure was not true micropropagation (Gautheret discounted its historical relevance when Joseph Arditti called his attention to it in response to a direct inquiry by him, perhaps because he was interested in glorifying his countrymen) because: (1) it produced only one shoot from each explant; (2) explants had pre-existing buds; and (3) Rotor's procedure did not involve callus formation or proliferation. However, multiple plantlet production, callus proliferation, and absence of pre-existing buds are not parts of the definition or requirements for micropropagation.

Rotor's micropropagation method was not widely noticed or appreciated at the time. One reason for this may have been its publication in a hobbyist publication, the *American Orchid Society Bulletin*. Orchid growers who read it probably found the procedure daunting and perhaps failed to grasp its importance. Scientists who would have appreciated Rotor's method and could use it probably did not read the *American Orchid Society Bulletin*. And so it was largely forgotten. When it was finally noticed, claims of priority by others had become accepted widely. However it is clear that in vitro clonal propagation (micropropagation, "mericloning," or any other term that may be used to describe the process for any higher plant in aseptic culture) was first carried out by Dr. Gavino Rotor Jr. in 1949 at Cornell University (those who cultured shoot tips of other plants before him do not seem to have appreciated the propagation potential of their procedures). The number of plants which can be produced by Rotor's method is not large, but it is of practical significance.

During the same time, Professor John T. Curtis (1913–1961; Fig. 1-12) and his associates in the Department of Botany at the University of Wisconsin published detailed descriptions of the formation of many growing points on proliferating callus of *Cymbidium* and *Vanda* (Curtis and Nichol, 1948). They used the word "calloid" to describe protuberances which developed from young asymbiotically germinated seedlings at the protocorm stage after treatment with barbiturates. These investigators noted that the tissue masses often had a capacity for continued growth into complete plants (Curtis and Nichol, 1948), appreciated the potential for clonal multiplication, and stated: "the practical ability to produce clonal lines of plants of potentially unlimited numbers would be of obvious value in many types of genetic and plant production work." However, there is a major difference between the drawing of attention to potential by Curtis and Nichol and the achievement of a well-conceived and clear goal by Rotor. With all that, it should also be noted that in their initial reports the (unfortunately) nearly forgotten Hans Thomale (Thomale, 1954, 1956, 1957) and the (unjustifiably) widely celebrated Georges Morel (Morel, 1960) also called attention to the potential of their findings (for historical accounts of the work by Thomale see Haas-von Schmude et al., 1995; Arditti, 2001; Easton, 2001). But, they did so after Rotor.

The Second Aseptic Culture of an Orchid Explant

The history of orchid micropropagation is elaborate, complex, and contains a few controversial episodes. In an effort not to offend a number of people, the history chapter in the previous edition withheld a number of facts and sugar coated others.

A subsequent review (Arditti and Krikorian, 1996), more concerned with historical accuracy than with offending individuals, is uncompromisingly accurate. As already mentioned this review (Arditti and Krikorian, 1996) served as a basis for a thorough revision of the present chapter. This is being done with permission (for which I am grateful).

Even before the availability of cytokinins (Skoog, 1944; Skoog and Tsui, 1948, 1951; White, 1951; Miller and Skoog, 1955) and the formulation of MS (Murashige and Skoog, 1962), several culture media were adapted for less demanding plants, especially with the addition of auxins, vitamins, and coconut water. Four such media were used to culture geranium, *Pelargonium zonale*, and cyclamen, *Cyclamen persicum* (Mayer, 1956). And this led a German horticulturist and nursery owner, Hans Thomale (Fig. 1-29), and a plant scientist, Dr. Lucie Mayer (Fig. 1-31), to the first reported culture of sections ("*Teilstücken*" or "*Pflanzenteile*") and tissues ("*Gewebe*") of orchids (pages 89–90 and figure 39 in Thomale, 1956; Figs 1-32–1-35).

Hans Thomale (Fig. 1-29) was born in Herne, Westphalia, Germany on October 16, 1919, raised in Cologne, and resided and grew orchids in Lemgo for many years. He started to study chemistry and medicine just before World War II broke out. When he "was half ready" Thomale was drafted and had to interrupt his studies. After World War II he "was forced to learn potato [cultivation] in a well-known nursery which had more orchids . . . than vegetables." The owner of the nursery, Mr. H. Kuhlman, also had a "daughter [Lieselotte] who [earned] the title 'Doctor of Botany' [while] I was forced to be a soldier." She became Mrs. Thomale and they parented two sons and a daughter.

Thomale became interested in orchid seed germination and asked Professor [Hans] Burgeff (1883–1976; Fig. 1-30) for his book *Samenkeimung der Orchideen* and used it to teach himself both symbiotic and asymbiotic seed germination. In 1946 he established a laboratory and utilized it to produce hybrids between the "many fine orchids [Mr. Kuhlman] bought [in] England and Belgium before the war . . . after that I tried to raise . . . orchids [via] clonal propagation" (quotations are from a letter by Mr. Thomale). The laboratory work brought him offers from Dorset Orchids Ltd., Plush, Dorset, UK in 1949 and Sanders Orchids, St Albans, Herts., UK in 1950 (neither business exists now) to establish laboratories for them. Thomale wanted to propagate both tropical orchids and those which were native to Germany. It is clear from his writings that Thomale read widely and was familiar with the work of Gautheret, Mayer, Rotor, Skoog, Tsui, and others.

Thomale based his own work with orchids on a paper by Dr. Lucie Mayer (Mayer, 1956; Fig. 1-31), who worked with him. On September 23, 1956 he was able to report to a meeting of the Deutsche Orchideen Gesellschaft (German Orchid Society) that explants of *Dactylorhiza (Orchis) maculata* (Fig. 1-33) and some tropical orchids in vitro produced shoots (Figs 1-32 and 1-34) and subsequently plants. Thomale recollected, albeit with some uncertainty, that Mr. Lecoufle of the French orchid firm Vacherot and Lecoufle (see below) was present at that meeting. A photograph of the *Orchid maculata* culture (Fig. 1-32) was published in the second edition of *Die Orchideen* (Thomale, 1957). The caption reads: "Section of *Orchis maculata* on agar medium (Mayer's method), which was induced to form roots and shoots" (Figs 1-32 and 1-34). Thomale appreciated immediately the potential of his discovery. He wrote (Fig. 1-35; Arditti and Ernst, 1993; Haas-von Schmude et al., 1995):

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Nur der Vollständigkeit wegen sei darauf aufmerksam gemacht, daß der Wunsch nach einer Vermehrungsart europäischer Erdorchideen dazu führte, dies auf Grund einer Arbeit von Dr. L. Mayer*) durch Kultur von steril gewonnenen Teilstücken auf Agar-Nährböden zu versuchen, was auch gelingt.

Bekannt war bisher, daß vegetative Teile von Orchideen, z. B. sterilisierte Stücke von *Phalaenopsis*-Blütenstielen, die über mindestens eine Adventivknospe verfügen, auf Agar-Nährböden zum Austreiben gebracht werden können. Neu ist hierbei, daß auch vollkommen indifferentes Gewebe einer Pflanze unter gewissen Nährbodenverhältnissen dazu gebracht werden kann, neue Wurzel- und Triebsprosse zu bilden.

Da zur Zeit der Drucklegung des Buches noch keine Veröffentlichung der Arbeitsweise vorliegt, muß es bei der Erwähnung der Tatsache bleiben, daß es bereits möglich ist, aus kubikzentimetergroßen Teilstücken irgendeines Pflanzengewebes neue Pflanzen zu erziehen. Eine Art der vegetativen Vermehrung von kaum zu übersehenden Möglichkeiten!

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FIG. 1-33–1-35. ***Orchis maculata*** **and the work by Hans Thomale, which lead him to suggest that shoot-tip cultures can be used for micropropagation. 33. Inflorescences during three stages of development: emerging, at the start of anthesis, and with open flowers (Landwehr, 1977). 34. Plants produced from in vitro explants like the one in Fig. 1-32 (courtesy Hans Thomale with help from Dr. Norbert Haas-von Schmude and Mr. E. Lucke). 35. The first description of shoot-tip cultures and the suggestion that such cultures have potential as a propagation method (Thomale, 1957:89–90).**

It should be noted that efforts to find a propagation method for European terrestrial orchids, based on the work by Dr. L. Mayer [Mayer, 1956], through the culture of sterile explants on an agar medium were successful. It is well known that vegetative parts of orchids, for example, sterile sections of *Phalaenopsis* flower stalks [Rotor, 1949], which bear at least one adventitious bud [Note in Arditti and Krikorian, 1996:

> these buds are lateral on the flower stalk and not necessarily adventitious, at least not in the strict sense of the word], can produce shoots when cultured on an agar medium. Recently it has become possible to culture undifferentiated tissues on certain nutrient media to produce roots and shoots from them. Since sufficient details were not available by the time this book went to press [i.e., the second edition which appeared in 1957; the first edition was published in 1954], it is only possible to mention that whole plants can be produced from tissue explants one cubic centimeter in size. *This is a form of vegetative multiplication whose potential cannot be overlooked*" [emphasis added]!

Thomale's work and his prediction about the use of explant culture as a means of mass rapid propagation was published (Thomale, 1957) before the first reports of *Cymbidium* "meristem" cultures (Morel, 1960; Wimber, 1963), but it was overlooked. Another important point is that Thomale behaved professionally by calling attention to Rotor's work, first by mentioning his name (Thomale, 1956) and later by referring to *Phalaenopsis* (Thomale, 1957). Had Thomale neglected to mention Rotor and *Phalaenopsis* he could have created the impression that he originated the entire idea of clonal propagation in vitro. Thomale did not describe his techniques in detail, but referred to Mayer's published procedure on which they were based. In fact, Dr. Mayer participated in Thomale's initial attempts (Haas-von Schmude et al., 1995; E. Lucke and N. Haas-von Schmude, Wettenberg, Germany, 1995, pers. comm.). Dr. Mayer (who retired to Madeira, Portugal) recalls that they also excised and cultured *Cymbidium* stem tips. They never published that part of their work and therefore cannot be credited with it.

Several reasons may be responsible for the fact that Thomale's work did not become well known: (1) his findings were first published in German in an orchid hobbyist publication, which at the time was not well known outside Germany (Thomale, 1956); (2) the second publication, also in German, was in a relatively obscure book (Thomale, 1957, the second edition of Thomale, 1954) aimed primarily at hobbyists and commercial orchid growers. As a result, few scientists read about Thomale's discovery. Practical growers who read it probably did not appreciate the technique and/or were bewildered by it (there is a parallel between Rotor's and Thomale's discoveries and publications and their fates).

Georges Morel (1916–1973; Fig. 1-10) is generally given exclusive, but completely undeserved (Arditti and Arditti, 1985; Haas-von Schmude et al., 1995; Arditti and Krikorian, 1996; Arditti, 2001; Easton, 2001), credit for being the first to culture an orchid explant in vitro. In fact it has been stated that: "few scientists or knowledgeable orchid growers subscribe to the widely publicized view that either Georges Morel or Michel Vacherot in France were the first to meristem orchids, yet these views are rarely challenged in print" (Easton, 2001). One reason for rare challenges is editorial interference (see below). Another is a strongly entrenched urban legend.

There is no question that Morel was familiar with Thomale's work at least as early as 1965 (Fig. 1-36). However, he cited it for the first time nearly 10 years later in a chapter written for Carl. L. Withner's, *The Orchids – Scientific Studies*. This was 14 years after Morel's fame in the orchid world had been firmly established (Morel, 1974; Haas-von Schmude et al., 1995), whereas Thomale was only known to *Paphiopedilum* growers for having formulated a seed germination medium for this popular genus.

CENTRE NATIONAL DE RECHERCHES AGRONOMIQUES

STATION CENTRALE DE PHYSIOLOGIE VÉGÉTALE

Etoile de Choisy - Route de St-Cyr
VERSAILLES (S.-&-O.)
Tél. : 950 75-22

Références à rappeler :
N/Réf. : GM/JL
VERSA PHYSIO VE 52.211

V/Réf. :

Objet :

RÉPUBLIQUE FRANÇAISE
MINISTÈRE DE L'AGRICULTURE

INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE

G. MOREL , Directeur de Recherches ,
à
Monsieur Hans THOMALE
Kastanienwald 19
LEMGO
Lippe
(Allemagne)

Versailles, le 15 Décembre 1965

Dear Sir ,

I have been asked by Dr. C. WITHNER to write a chapter on clonal propagation of Orchids for a new edition of his book .

I would like to know if you did other experiments on propagation of Terrestrial Orchids since the one you mentionned in your book, p. 89 .

May I use the picture Ab 39 , of Orchis maculata, for this paper ? In that case, could you be kind enough to send me a print of it ?

Yours sincerely ,

G. MOREL

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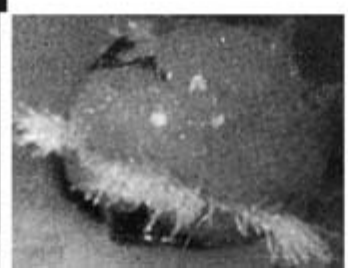


37

36

FIG. 1-36–1-38. **Correspondence and in vitro cultures by Dr. Georges Morel. 36. Letter from Dr. G. Morel to Mr. Hans Thomale requesting a copy of Fig. 1-32. This letter proves that Dr. Morel knew of Mr. Thomale's work long before he cited it (courtesy Hans Thomale with help from Dr. Norbert Haas-von Schmude and Mr. E. Lucke). 37. First photograph of a protocorm-like body published by Dr. Georges Morel (Morel, 1960). 38. *Cymbidium* plantlet produced from a protocorm-like body like the one in Fig. 1-37 (Morel, 1974).**

Even then Morel only cited Thomale's 1957 book and although he accurately reported that "pieces from the bulb of *Orchis maculata*, aseptically cultivated on nutrient medium, soon regenerated stems and roots . . ." he also added the qualifying statement "that [cases like this] are very exceptional." Morel included a copy of a photograph provided by Thomale (Fig. 1-32) in his chapter with the caption "Regeneration of roots and shoots occuring on a piece of tuber of *Orchis maculata*. (After Thomale.)" The wording ("stems and roots") minimizes Thomale's achievement by implying that what was produced was not plants, and the context (a section entitled "Regeneration from Inner Parenchyma") would seem to suggest that the new plants were produced from inner parenchyma rather than from buds, through bud formation, or via some other process commonly associated with tissue culture propagation (see Morel, 1974). Moreover, the photograph was not "after Thomale," it was provided by Thomale because Morel asked for it (Fig. 1-36).

By the time Thomale was given any recognition (Arditti and Ernst, 1993; Haas-von Schmude et al., 1995; Arditti and Krikorian, 1996; Arditti, 2001), essentially total credit for priority of discovery had been established for and by Morel. It is possible to argue that this occurred not only because Morel was already a well-known and established senior scientist in the world of plant physiology and plant pathology and had many friends, but also due to his extensive travels and lectures. Orchid scientists unfamiliar with the historical details presented here, admiring hobbyists, and grateful commercial growers have played a major role in elevating Morel to the position of being virtually the sole participant in "the invention." There was/is also resistance to new knowledge (Gaffron, 1969).

A note marking Thomale's 75th birthday (Lucke, 1994) does not even mention his discovery because a statement to that effect was edited out by the editors of *Die Orchidee* (Dr. Norbert Haas-von Schmude, Wettenberg, Germany, pers. comm.). An article marking the 25th anniversary of "mericloning" (Arditti and Arditti, 1985) was similarly "shortened at the advice of a reviewer." However Thomale's important contribution and priority over Morel were recognized eventually (Haas-von Schmude et al., 1995; Arditti and Krikorian, 1996; Arditti, 2001; Easton, 2001).

Now, after attention has been called to Thomale and his work and to his amazingly accurate prediction, it is no longer correct to state that ". . . Georg[e] Morel has realized for the first time the multiplication of Orchids [sic] by stem tips in vitro culture. Dr. [sic] Thomale seems to be unaware of the tissue culture history" (R. J. Gautheret, Paris, pers. comm.). Cauvinism, national pride, and not even loyalty to a ". . . late collaborator . . ." (R. J. Gautheret, pers. comm.), friend, and fellow countryman can justify the setting aside of historical facts and the rewriting of history. The ones unaware of history as it relates to orchids are Gautheret and those who blindly credit Morel with a discovery he did not make.

Plant Diseases and Meristems

The idea that healthy clones of horticultural plants can be obtained from stem tips, root cuttings, and even leaves is more than half a century old (see North, 1953; Krikorian, 1982; for literature citations). A method for establishing *Verticillium*-free clones of chrysanthemums by making tip cuttings from 10–15 cm (4–6 inch) long

shoots which were shown to be disease-free was reported by Arthur W. Dimock (1908–1972) during World War II (Dimock, 1943*a*, 1943*b*) and subsequently refined and extended to other diseases (Brierly, 1952; Dimock, 1956). Similar methods were used for carnations (Dimock, 1943*a*, 1943*b*, 1951; McFarland, 1948; Forsberg, 1950; Andreasen, 1951; Guba, 1952; Hellmers, 1955; Thammen et al., 1956).

That tips of virus-infected roots could be free of infection was reported 60 years ago (White, 1934*a*, 1934*b*, 1943). Before that, virus or "abnormalities" could not be seen in stem tips of tobacco, tomato, and *Solanum nodiflorum* (Clinch, 1932; Sheffield, 1933, 1942). Aucuba (*Aucuba*, Cornaceae, is a genus of ornamental shrubs known as Japanese, greenleaf, or sulfur leaf aucuba) and tobacco mosaic infections were obtained from isolated shoot and root tips (Sheffield, 1942), but this could have been due to the manner in which the tissues were excised, or to a low virus content (Samuel, 1934). By 1948 stem-tip cuttings were used to eliminate the spotted wilt virus from *Dahlia* (Holmes, 1948, 1955). This method was extended to leaf spots associated with the internal-cork disease in sweet potato, *Ipomea batatas* (Holmes, 1956*a*), as well as aspermy virus (Holmes, 1956*b*) and other viruses (Brierley and Olson, 1956) in *Chrysanthemum*.

The use of stem-tip cuttings to eliminate spotted wilt of *Dahlia* (Holmes, 1948) very clearly suggested that apical meristems might be virus-free. This was confirmed a year later in studies with tobacco mosaic infection of *Nicotiana tabacum* var. Samsun (Limasset and Cornuet, 1949). These findings were fortuitous. At present it is well known that apical meristems are not necessarily free of virus infection and this has led to considerable difficulties in freeing many clones and cultivars of viruses (Kassanis, 1967).

A problem facing French horticulture ca. 1950 was viral infection of certain potato and *Dahlia* cultivars which would have caused them to be abandoned (Lecoufle, 1974*a*, 1974*b*). Given the previous findings with *Dahlia* (Holmes, 1948) and tobacco (Limasset and Cornuet, 1949) the culture of stem tips provided a means of freeing these plants of viruses. And, indeed, Pierre Limasset (1911–1988) and Pierre Cornuet (b. 1925) "suggested to their colleagues Georges Morel and Claude Martin to cultivate shoot meristems of infected plants" (Gautheret, 1983, 1985). The suggestion was excellent, the attempts were successful, and virus-free *Dahlia* (Morel and Martin, 1952) and potato (Morel and Martin, 1955*a*, 1955*b*; Morel and Muller, 1964; Gautheret, 1983, 1985) plants were obtained from infected ones.

Dahlia and potato shoots obtained from stem tips in vitro by Georges Morel and his co-workers did not produce roots. Shoots produced by previous workers also failed to form roots in vitro. Therefore, following established laboratory practice the shoots produced by Morel and his associates were grafted onto healthy seedlings (Gautheret, 1983). Later, other investigators were able to get rooting (Quak, 1961; Hollings and Stone, 1983). Attempts to free potatoes of virus through the culture of shoot tips were also undertaken by a number of others (Kassanis, 1957; Pirie, 1973; see Hirst and Harrison, 1988, for historical perspectives).

The success with *Dahlia*, potatoes, and other plants (Morel and Martin, 1955*b*; Morel, 1964*a*) led Morel "an amateur orchid grower [who] had in his greenhouse a plant of *Cymbidium* Alexaderi 'Westonbirt' . . . the most famous *Cymbidium* of all time, which was, sadly, totally infected by *Cymbidium* mosaic virus [to apply] the same technique as he was using on his potatoes to the *Cymbidium* [and] produced

FIG. 1-39–1-40. **Advertisements for clonally propagated orchids. 39. Part of the cover of the Orchids Orlando catalog which offered orchids that were propagated clonally by the French firm Vacherot and Lecoufle (Orchids Orlando, no date). 40. A page from the Orchids Orlando catalog which tells how Georges Morel came to shoot-tip cultures of orchids (Orchids Orlando, no date).**

a protocorm [sic]" (Morel, 1960; Vacherot, 2000; Figs 1-37 and 1-38). As already mentioned, this achievement has been heralded in a wide array of publications. A particularly adoring pseudo-historical account in an advertisement-catalog makes the claim on its cover that "a funny thing happened to the orchid when they operated on a sick potato" (Fig. 1-39) and the text states that "a beautiful thing happened to the orchids when they operated on a sick potato [because] Dr. Georges Morel, distinguished French botanist, discovered the orchid meristem process while he was trying to figure out a way to prevent virus in potatoes" (Orchids Orlando, no date; Fig. 1-40). Less poetical but equally historically imprecise statements abound in the scientific and horticultural literature as well (for examples see Bertsch, 1966, 1967; Vacherot, 1966, 1977; Marston and Voraurai, 1967; Borriss and Hübel, 1968; Vanseveren and Freson, 1969; Hahn, 1970; Kukułczanka and Sarosiek, 1971; Lecoufle, 1971; Lucke, 1974; Allenberg, 1976; Champagnat, 1977; Rao, 1977; Loo, 1978; Murashige, 1978; Goh, 1983*b*; Bouriquet, 1986; Griesbach, 1986; Hetherington,

1992; Zimmerman, 1996). Much less frequently does one encounter attempts to be more precise about history (Arditti, 1977*b*, 2001; Stewart, 1989; Haas-von Schmude et al., 1995; Arditti and Krikorian, 1996; Easton, 2001). In some instances accuracy was treated gingerly due to editorial pressure (Arditti and Arditti, 1985; Lucke, 1994; N. Haas-von Schmude, Wettenberg, Germany, pers. comm.) or in attempts not to offend established interests (Arditti and Ernst, 1993). The present chapter, like the review (Arditti and Krikorian, 1996) on which parts of it are based, dispenses with such niceties in favor of historical accuracy despite a very real possibility of offending or antagonizing some readers.

Horticulture and plant agriculture are the major beneficiaries of stem-tip culture in terms of massive and rapid clonal propagation as well as generation of pathogen-free plants. The fact that both objectives can sometimes be accomplished simultaneously with one and the same explant has created "an apparent conception among horticulturists that tissue culturing and diseases-freedom [sic!] are synonymous. A similar misconception was true of the so-called meristem-cultured plants. A classic example of this misconception can be seen in the orchid industry. Before "mericloning" orchid viruses were a minor problem. However [they] are now common, widespread and costly" (Langhans et al., 1977) because careless culturing spread rather than contained or eliminated viruses (Toussaint et al., 1984).

The Third Aseptic Culture of an Orchid Explant

Most accounts and reviews of orchid micropropagation seem to start with a citation or at least a mention of Morel's first paper on *Cymbidium* shoot-tip culture (Morel, 1960). A few examples are: "the potential of propagating orchids through tissue culture was observed first by Morel" (Murashige, 1974). Similarly, ". . . credit for the initiation of meristem culture technique goes to the late Dr. G. Morel of INRA [Institut National de la Recherche Agronomique], Versailles, France" (Rao, 1977). Assertions that "the first application [of micropropagation] concerned the clonal propagation of orchids (Morel, 1960)" can be found in historical accounts by a "founding father"of plant tissue culture (Gautheret, 1983, 1985). And, since such reviews are often re-stated or quoted in other papers [e.g., "the potentials of tissue culturing for plant propagation . . . have been . . . reviewed by Murashige . . ." (Langhans et al., 1977)], an historical "factoid" has been elevated to truth and dogma. Once such a transformation happens, the forces which usually resist knowledge tend to maintain the status quo and thus strive to support dogma (Gaffron, 1969).

These factors seem to have come to bear on the history of orchid micropropagation. Attempts to question the accepted views have led to sharp exchanges in the literature (Arditti, 1985; Torrey, 1985*a*, 1985*b*). Editorial demands for changes in manuscripts (Arditti and Arditti, 1985; Lucke, 1994) had to be agreed to. The accepted history was examined carefully (Arditti and Krikorian, 1996) for the sole purpose of placing historical facts in the most accurate perspective possible. That careful re-examination (Arditti and Krikorian, 1996) served (in certain instances word for word) as a basis for what is presented here. Unfortunately, it may not be possible to provide an accurate presentation without creating an impression of an intent to diminish some reputations. Or, to quote the famed physicist Ernst Mach (1838–

1916) as he was quoted by the (well-known in his day) plant physiologist Hans Gaffron in 1969: "It is hardly possible to state any truth strongly without apparent injustice to some other." Indeed, the historical outline in this chapter may appear, to some at least, to be "unjust" only because many previous accounts have been imprecise enough to have done considerable disservice to the truth. And, unfortunately, in an effort not to offend, the first edition of this book (Arditti and Ernst, 1993) also failed to present full historical details.

Georges Morel (1916–1973; Fig. 1-10) was born on April 16, 1916 in Béthune, France and died suddenly around 6 p.m. on December 1, 1973 while going up the steps to his laboratory (Gautheret, 1977). His father, an architect with an interest in horticulture, died in 1928, also apparently of a heart attack. Young Georges attended l'Institution Saint Vaas de Béthune where he showed an interest in physics and chemistry. In 1934 Morel entered l'Institut de Chemie in Paris where his interests led him to agriculture, plant pathology, and the INRA, the French Institute of Agricultural Research (Gautheret, 1977), where he "was one of the most influential members" (Vacherot, 2000).

Drafted into military service in 1939, Morel served with an artillery unit and was taken prisoner at the Belgian front in 1940. He escaped in 1941 according to one source (Gautheret, 1977), or was released due to family hardship according to another (Pierre Jacquet, a chapter in press in *Orchid Biology*, *Reviews and Perspectives* volume IX). On returning to the INRA, Morel was soon appointed "*chef de travaux*." In 1943 Morel joined Gautheret's laboratory (Lecoufle, 1974*a*, 1974*b*) and worked there towards his doctorate. Times must have been difficult under Nazi occupation (Paris was liberated on August 25, 1944), but Morel was successful in his research and even presented a major paper to the Academy of Sciences on January 4, 1944, 8 months before liberation. (Gautheret, 1977, who wrote an appreciation and obituary rather than a detailed biography, reported these events, but provided no details.) Whether he was released early from the POW camp or escaped from it, accepting a fairly visible appointment in a government institution in occupied France was probably not easy and fraught with danger. Therefore, one must admire Morel's bravery. Morel received his doctorate in 1948, went to the USA during the same year, and worked until 1951 with Professor Ralph W. Wetmore (1892–1989) in the biological laboratories at Harvard University. They worked on tissue culture of monocotyledonous plants (Morel and Wetmore, 1951*a*) and ferns (Morel and Wetmore, 1951*b*). One of Morel's tasks during the visit was to establish a plant tissue culture laboratory (Wetmore and Wardlaw, 1951; Wetmore, 1954; Torrey and Thimann, 1972). During that time he also forged lasting friendships at Harvard with several American scientists including the late John Torrey (1922–1993), a noted plant scientist (Arditti, 1985; Torrey, 1985*a*, 1985*b*), and the late Howard A. Schneiderman (1927–1990), an entomologist and developmental biologist. Schneiderman, who held Morel in high regard (and told Joseph Arditti about it), became Dean of Biological Sciences at the University of California, Irvine (UCI) during the late 1960s and the 1970s but his friendship with Morel did not make him a supporter or even a friend of orchid research and the plant sciences at UCI.

Morel also became friends and collaborated with Armin C. Braun (1912–1986) of the Rockefeller Institute in New York City on studies dealing with habituation and hormone autonomy (Braun and Morel, 1950). Braun, a distinguished researcher

on plant tumorigenesis induced by the crown-gall bacterium *Agrobacterium tumefaciens*, has come to be regarded as one of the founding fathers of modern day plant genetic engineering (Braun, 1982). Several techniques rely heavily on the use of the Ti plasmid from that bacterium as a vector for inserting new genetic information (Bevan and Chilton, 1982). On his return to France, Morel was appointed *Maître de recherches* (in 1951 or 1952) and in 1956 *Director de recherches* of the Station Centrale de Physiologie Végétale of the Centre National des Recherches Agronomiques, Ministiére de l'Agriculture (Lecoufle, 1974*a*, 1974*b*).

Dr. Morel's first paper on shoot-tip culture of *Cymbidium* (Morel, 1960) resembles a news release or newspaper notice rather than a scientific paper. It reported sketchily on what was done, described minimally the excision process and culture conditions, and referred to a nutrient medium ("Knudson III") which does not exist. The report concluded by stating "that it is relatively easy to free a *Cymbidium* from the mosaic virus . . . each bud will give several plants so the stock of a rare or expensive variety can be increased . . . [and that] experiments of the same kind are now being conducted with . . . *Cattleya*, *Odontoglossum*, and *Miltonia*, contaminated with different viruses" (Morel, 1960).

This paper (Morel, 1960) introduced a new term into orchid terminology and the English language, "protocorm-like body" (generally abbreviated as PLB), to describe the "small flat bulblet looking exactly like [a] protocorm" (Fig. 1-37) which was formed by the *Cymbidium* stem tips he cultured and preceded plantlet formation (Fig. 1-38). The term protocorm itself was coined by the long-time director of the Bogor (Buitenzorg during Dutch colonial period) Botanic Gardens in Indonesia, Melchior Treub (1851–1910; for photographs see Arditti, 1990, 1992) to describe a stage of lycopod development. Noël Bernard (1874–1911; for a photograph see Arditti, 1990, 1992) applied "protocorm" to the early corm-like stage of orchid seed germination. Bernard did not coin the term as Dr. Phillip J. Cribb stated erroneously (Cribb, 1999). Protocorm-like body, or PLB, should be applied to bodies produced by explants (see Arditti, 1990, 1992; Arditti and Krikorian, 1996 and elsewhere in this volume for further discussions), not to those derived from seeds. And the bodies produced by seeds should not be called PLBs. Neither should they be referred to as "spherules."

The first paper (Morel, 1960) includes only two literature citations. One pertains to the viral mosaic disease (Jensen, 1951). The other deals with freeing plants from virus through stem-tip culture (Morel and Martin, 1955b). It would have been very difficult for anyone to repeat Morel's work because this article did not present sufficient details. Plant scientists who took the trouble to study all of Morel's previous work might have been able to reconstruct the procedures and medium or media. Hobbyists or commercial growers would have had more serious problems in doing that since many of them were looking for a detailed and ready-made magic "formula." However, the orchid firm of Vacherot and Lecoufle "La Tuilerie," Boissy-Saint Leger (Seine-et-Oise) had enough information to start commercial micropropagation of "rare or expensive" orchids before any other establishment. They moved quickly enough to have a clonally propagated plant of *Vuylstekeara* Rutiland "Colombia" bloom in December 1965 (Vacherot, 1966; Lecoufle, 1967), but a recent report suggests that the first plants to be cultured were "some of [their] finest cymbidiums" (Vacherot, 2000).

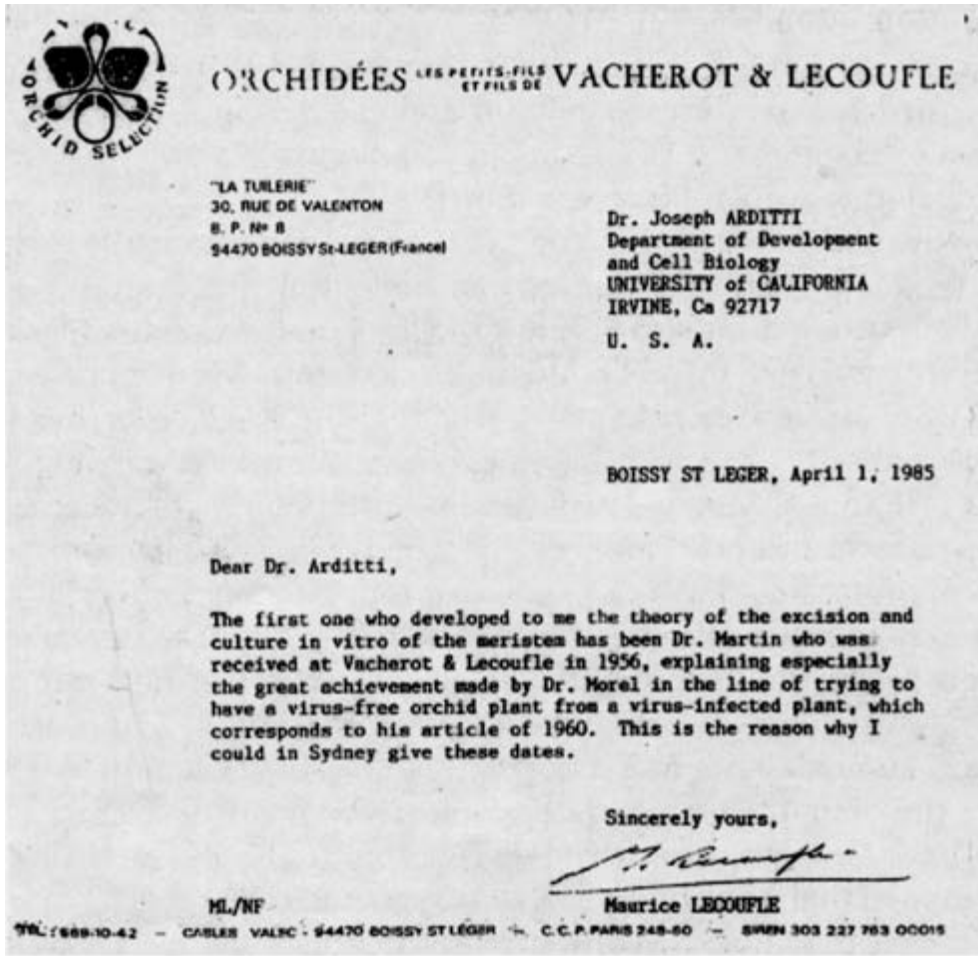
Clonal propagation of *Vuylstekeara* (a hybrid genus) started at Vacherot and Lecoufle (V&L) 24 years before the publication of a specific method for this genus (Kukułczanka et al., 1989) and only 2 years after: (1) January 1963, the reported excision date of the stem tips (Vacherot, 1966); and (2) the development of culture methods (which were not published in detail at the time) for stem tips of the parent genera (Morel, 1963). A report that ". . . at 'La Tuilerie' our first mericlone to flower [was] . . . *Vuylstekeara* Rutiland 'Colombia' . . . in December, 1965 . . ." (Lecoufle, 1967) suggests that "mericloning" started at V&L before or at about the time Morel's first paper was published because ". . . it will take just as long to grow the plants produced from meristem tissue as it takes to grow a new hybrid from seed" (Scully, 1964). As a rule, orchid plants grown from seed require at least 3 years to flower (excluding some recent *Phalaenopsis* hybrids which can be considerably faster), but there are also reports of hybrids which flowered only after 10 or more years (Goh et al., 1982; Goh and Arditti, 1985). Two years from protocorm-like body to flowering appears to be very fast growth and development (but perhaps not impossibly so) for this *Vuylstekeara* hybrid and especially for hybrids available at that time. Altogether it seems that V&L had access to appropriate methods long before they were published.

There is also a suggestion that some "meristem-cultured plants may mature more quickly than plants raised from seeds" (Lecoufle, 1967). One example is plantlets of *Odontonia* Boussole "Blanche" and *Odontonia* Moliere "Lanni" which were removed from their flasks on April 30, 1965 and "flowered ten to eleven months later and in blocks of hundreds, less than two years after being deflasked" (Lecoufle, 1967). If the *Odontonia* plantlets were "deflasked" on April 30, 1965, the cultures were probably started in 1964 or 1963 which is before the publication of culture procedures for this hybrid genus and its parent genera (*Odontoglossum* and *Miltonia*), but after Morel seems to have developed appropriate methods for these orchids without publishing them.

An early advertisement by V&L ". . . to carry out the new method of asexual reproduction . . ." (Scully, 1964) is also an indication that V&L had considerable and early experience with shoot-tip cultures. Such experience could have been gained only through extensive practice and/or access to procedures and media and/or advice from an expert.

Altogether the facts in the three preceding paragraphs lead to a reasonable assumption that there was a close association between Georges Morel "a close friend of" [Michel Vacherot's] father (Vacherot, 2000) and V&L, as well as an exchange of unpublished information well ahead of publication. This assumption is supported by the following:

1. A report that Morel became interested in orchids as early as 1955 or 1956 (Lecoufle, 1971) and "in 1956 started to apply the techniques of meristem culture . . . previously developed to free potatoes, dahlias and carnations from viruses, to various Orchids [sic]" (Morel, 1965*a*).
2. A statement that in 1956, the year "meristem culture was achieved by Dr. Morel, . . . the first who developed to [Marcel Lecoufle] the theory of the excision and culture in vitro has been [sic] Dr. Martin who was received at Vacherot & Lecoufle in 1956, explaining especially the great advancement made by Dr. Morel in the line of trying to have a virus-free orchid plant from a virus infected plant which



ORCHIDÉES LES PETITS-FILS ET FILS DE VACHEROT & LECOUFLE

"LA TUILERIE"
30, RUE DE VALENTON
B. P. N° 8
94470 BOISSY St-LEGER (France)

Dr. Joseph ARDITTI
Department of Development
and Cell Biology
UNIVERSITY of CALIFORNIA
IRVINE, Ca 92717
U. S. A.

BOISSY ST LEGER, April 1, 1985

Dear Dr. Arditti,

The first one who developed to me the theory of the excision and culture in vitro of the meristem has been Dr. Martin who was received at Vacherot & Lecoufle in 1956, explaining especially the great achievement made by Dr. Morel in the line of trying to have a virus-free orchid plant from a virus-infected plant, which corresponds to his article of 1960. This is the reason why I could in Sydney give these dates.

Sincerely yours,

ML/NF

Maurice LECOUFLE

TEL. : 569-10-42 — CABLES VALSC - 94470 BOISSY ST LEGER — C.C.P. PARIS 248-60 — SWIFT 303 227 763 OCO15

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FIG. 1-41. **Letter from the late Maurice Lecoufle, co-owner of the French firm of Vacherot and Lecoufle, giving his version of history.**

corresponds to his article of 1960" (letter dated April 1, 1985 from the late M. Lecoufle, Vacherot and Lecoufle, 30, Rue de Valenton, 94470 Boisy St. Leger, France; Fig. 1-41).

3 A short conversation Joseph Arditti had at the end of April 2002 with Phillipe Lecoufle, current owner of V&L during the 17th Orchid Conference in Shah Alam, Malaysia. When asked if Georges Morel gave V&L unpublished information, his somewhat huffy reply was an assertion that it was proper for Morel to give them such information because "he worked together with us."

This information suggests that Morel and Martin may have been successful in culturing orchid shoot tips at approximately the time they published their paper on

potatoes. However Morel's first paper on orchids was not published until 1960. This delayed publication, the nature of the first paper (Morel, 1960), and subsequent publications (Morel, 1963, 1964*a*, 1964*b*, 1965*a*, 1965*b*, 1970, 1971*a*, 1971*b*, 1971*c*, 1974) pose a number of interesting questions.

One of these questions pertains to the first paper on *Cymbidium* which showed an 18-month-old explant and included the statement that "some plants that are . . . 10 cm high" (Morel, 1960). A *Cymbidium* plant in vitro or in a pot would certainly grow more than 10 cm in 4–5 years (from 1955 or 1956 to 1959 or 1960 when the paper was submitted and published). Therefore it is by no means clear whether the statements in the paper are accurate (Morel, 1960), or if the report is about plants which were produced specifically for that article.

In a subsequent paper, written in French, Morel added anatomical details regarding the protocorm-like body mentioned earlier and mentioned attempts to extend the *Cymbidium* method to *Odontoglossum*, *Miltonia*, and *Phaius* (Morel, 1963). However he did not provide additional information about excision and culture conditions. This paper actually added to the confusion about a medium, which those seeking to duplicate his results might employ by reporting the use of "Knop's Solution" supplemented with 2% glucose (Morel, 1963). The exact composition of the medium was not given. Morel used a modification of "Knop's Solution" for potato stem tips, but the paper on this method (Morel and Martin, 1955*a*) was published in a journal not widely read outside France and is not cited in the orchid article. Therefore, it would have not been easy for orchid scientists, and even more difficult for horticulturists, to find the paper or the recipe. It is not clear if the omissions (exact composition, no citation) were intentional or inadvertent. What is very clear is a consistent practice of omitting critical information or presenting incompletely and/or in a confusing manner.

Altogether the content (or lack of it) of the first and second papers (Morel, 1960, 1963) tends to support the view that Morel held back information and/or was trying to give V&L an advantage. It is also not clear whether Morel withheld information because he planned to apply for a patent: "Morel . . . was the originator [of micropropagation]. Later, I asked him whether he intended to apply for a patent on his discovery. But by this time the process of meristem culture had become a widely practised technique, so he did not" (Vacherot, 2000). In any case, these papers (Morel, 1960, 1963) do not provide enough details to allow for easy (if any) repetition of the work. In the first paper the medium is described as "Knudson III" and in the second it is given as "la solution de Knopp [sic]". There is no Knudson III medium and one can only assume that the reference is to Knudson C in some form. This medium is very different from Knop's solution, which may or may not be suitable for shoot-tip cultures of *Cymbidium*, *Miltonia*, *Odontoglossum*, *Cattleya*, and *Phaius* that were cultured by Morel (Morel, 1963).

A noted (and now deceased) American orchid specialist has suggested that the paucity of details may have been due to the preliminary nature of the first paper. Perhaps, but even a preliminary paper (Morel, 1960) must include more information. Moreover, the second paper (Morel, 1963) was not preliminary. Another suggestion is that Morel may have been "sloppy." If so, one can expect his other papers to be sloppy, but they are not. Morel was a first rate scientist who wrote excellent papers that were anything but sloppy.

In 1958 Frederika Quak from the Institute of Plant Virology in Wageningen, the Netherlands presented a paper at the International Horticultural Congress in Nice organized by Pierre Cornuet (one of the plant pathologists who suggested shoot-tip cultures to Morel) and Claude Martin (one of Morel's collaborators), of INRA, Versailles. Georges Morel and his wife were listed as attendees at the conference, but there is no evidence that he presented a paper. In a presentation (which did not get published until 1961), Quak focused on her work with potato and the use of White's medium (White, 1954) supplemented with "10 p.p.m. thiouracil, 0.1 p.p.m. 2,4-D or 0.1 p.p.m. IAA" (Quak, 1961), but there was no mention of orchids (Arditti and Krikorian, 1996).

Quak and a colleague (Baruch and Quak, 1966) do not cite Morel's paper on *Cymbidium* as an example of an apical meristem culture that could yield virus-free plants. In connection with their work on *Iris* meristems they state that "best results were obtained with media based on that of Morel. Therefore the formula of that medium only is presented here: ½ concentration Knop solution 1000 ml; Berthelot solution 0.5 ml; cystein 1 mg; adenine 5 mg; hydrolysate of casein 200 mg; saccharose [sucrose] 20 g; agar 6 g; vitamin solution (containing calcium panthothenate 1 mg, inositol 100 mg, biotin 10 mg, nicotinic acid 1 mg, pyridoxin 1 mg, distilled water 100 ml. The media were adjusted to pH 6" (Baruch and Quak, 1966).

Baruch and Quak started their experiment in January 1963 and used about 700 meristems of *Iris* "Wedgewood". The abstract of the paper (in English) draws attention to the fact that the "medium of Morel (pers. comm.) gave the best results." The Dutch summary repeats it. So at least by January 1963 Morel did divulge his nutrient medium recipe and it was published in full by Quak. This was 3 years after Morel's initial publication on orchids and 1 year before the French orchid firm of Vacherot and Lecoufle announced that they could propagate orchids via shoot-tip cultures. Whether anyone could or did make a connection between a paper on *Iris* in a Dutch journal on plant pathology and orchids is open to speculation (Arditti and Krikorian, 1996).

Even if orchid scientists could find the composition of the potato or *Iris* media there were no indications that they would be suitable for orchids. In fact, the potato medium is quite different from the one subsequently used for orchids by Morel. It is also interesting to note that Georges Morel was very familiar with Knop's solution and the modified Berthelot trace elements formulation because he used them routinely in his doctoral dissertation work (Morel, 1948). Those trying to learn more about the media used by Morel for orchids could have learned much from this paper (Morel, 1948), but: (1) the connection was not obvious; (2) his published dissertation is not well known; (3) the journal is relatively obscure; and (4) the language, French, is neither read nor spoken by many scientists.

Modifications of Knop's solution have been used for the culture of vegetative axis nodes of *Dendrobium* and *Bletilla* (Yam, 1989), and flower scape nodes of *Phalaenopsis* (Ball et al., 1974/75), but there is no indication that these, or any, modifications of this medium would be suitable for shoot tips of other orchids. The available evidence suggests that at present there is no single solution which is suitable for all orchids.

A third paper (the second published in English) appeared a year later (Morel, 1964*b*). It was longer, had more illustrations, added the results of more work with three

genera (*Cattleya*, *Miltonia*, *Phaius*) to those that were being cultured, and described the culture conditions. It added confusion rather than clarification regarding the culture medium because it was listed as "Knudson III" again. This paper left no doubt that the culture of shoot tips could be used for mass rapid clonal multiplication, but it did not provide enough information for others to use the technique. In retrospect it is clear that even those who were familiar with all three papers (Morel, 1960, 1963, 1964*b*) would have had to guess which medium to use and how to modify it.

It would be interesting to know whether a written request for the recipe of the nutrient medium would have elicited a positive response. There is no information whether such requests were made regarding any of Morel's orchid media. No such requests would have been necessary for the potato medium because it was published. A paper on potato meristems (Kassanis, 1957) states that "the apical meristems were excised as described by Morel and Martin (1955*a*). The medium in which the meristems were cultured was suggested by Dr. G. Morel, but differs from the one which was described by him (Morel and Martin, 1955*a*). It consists of $^1/_2$ concentration of Knop solution, 10 drops of Berthelot solution (Morel, 1948)."

Basil Kassanis spent a few months with Morel at Versailles in 1954 (Hirst and Harrison, 1988). At least one British grower made "arrangements . . . to visit Professor Morel's laboratory in May of 1964 [and] found Professor Morel and his staff extremely helpful and they taught [him] the technique, giving [him] details of the formula used to produce plants from meristematic tissue." Late in 1964 Morel also visited McBean [McBeans Orchids Ltd., Cooksbridge, Lewes, East Sussex, UK] and [the grower] "was privileged" to work with him (Bilton, 1985). It is not clear whether these reciprocal visits with the British growers were made on a voluntary basis or as a consulting arrangement (Arditti and Krikorian, 1996). By May 1964 the French orchid firm of Vacherot and Lecoufle had in effect established its monopoly (in fact it is also not clear if Morel's association with Vacherot and Lecoufle was voluntary, based on friendship, or based on paid consultancy).

Guessing would not be conducive to success, especially for commercial and hobby growers. Development of another suitable medium would have required time (i.e., caused delays for other investigators and/or growers) and delayed knowledge of the "right" formulation would have decisively secured for Vacherot and Lecoufle the lead they already enjoyed. This is an important point since for a long time the only procedures in the literature for *Miltonia* and *Phaius* were the ones published (albeit unclearly) by Morel (the flower stalk method for *Phaius* in this volume was never published due to the tragic death of its author in a traffic accident in Bogor, Indonesia). However, it is not known whether the medium is pivotal. Several procedures and media are currently available for *Cattleya* and other orchids (see appropriate sections in this volume or in Arditti and Ernst, 1993). The same may be true for *Miltonia* and *Phaius*.

A trio of additional papers appeared within the next 3 years (Morel, 1965*a*, 1965*b*, 1966). They included media recipes. Some of these media did but others did not resemble the Knudson C medium (more than likely Morel's "Knudson III"), Knop's solution, or the potato substrate (Morel and Martin, 1955*a*) enough to be called a modification of any of them. One medium for *Cymbidium* was actually described as "potato meristem medium" (Morel, 1966). Therefore, one is left wondering

about the listing of media (Morel, 1960, 1963, 1964*a*, 1964*a*, 1965*b*), especially since the Knudson and Knop solutions were described as suitable for *Miltonia* and *Cymbidium* in a subsequent paper (Morel, 1970). That paper and an earlier one (Morel, 1966) also contain additional information about the micropropagation of *Cattleya*. Information about Vandaceous and European orchids and *Dendrobium* was published between 1966 and 1970 (Morel, 1966, 1970).

Two reviews (Morel, 1971*a*, 1974) were Morel's final contributions in English on the micropropagation of orchids. Both are excellent and contain a considerable amount of basic information. His last review (Morel, 1974), like some of the previous papers (Morel, 1965*a*, 1966), covers culture media and their components in some detail. The discussion is both interesting and enlightening. Media recipes and details about culture conditions are unambiguous. However, at that point in time the information was much less important and useful than it would have been in 1960. This is so because by 1965, 1966, and 1974, that is 5, 6, and 14 years after the initial publication, (1) the French firm of Vacherot and Lecoufle which was co-owned by a son of a "close friend" of Morel (Vacherot, 2000) had established a monopoly, and (2) as a result of research carried out throughout the world several culture media and procedures for the micropropagation of orchids were formulated and published. Publication of a suitable medium in 1960 would have made the technique available to all who wanted to use it even if (1) the medium used by Morel was not pivotal and (2) several media were later shown to be suitable for some orchids (see Arditti, 1977*b*; Arditti and Ernst, 1993; and the present volume for lists and media recipes).

With one exception (Morel, 1963), the initial orchid papers and several subsequent ones were published in periodicals aimed at hobbyists and commercial growers (Morel, 1960, 1964*b*, 1965*a*, 1965*b*, 1966, 1970) and in proceedings of meetings (Morel and Champagnat, 1969; Morel, 1971*a*, 1971*b*, 1971*c*), rather than peer-reviewed scientific journals. One reason for this could have been a laudable intent to make the procedures available to growers. But if this was so, important information (e.g., culture media recipes, details about techniques, etc.) should have been included in each of them. It was not. Another conspicuous deficiency in these papers is the lack of literature citations. Previous papers by others which may have been the source of ideas, media, and methods were not cited. This is not in line with the accepted standards of scientific publication. Lack of citations creates the erroneous impression that the ideas are original. Peer-reviewed scientific journals would have probably rejected most of these papers due to insufficient information about methods, media, and citation of previous work. Yet there is no question whatever that the caliber of Morel's research was high enough to justify papers which could have been published in peer-reviewed scientific journals. Thus, the inevitable conclusion is that the avoidance of scientific journals and their publication standards was not accidental.

A key question, which is nearly impossible to answer at present, is why was there only a single early paper in English, in a non-reviewed journal, and only an incomplete one at that, namely the one on *Cymbidium* (Morel, 1960)? Subsequent papers on *Cymbidium* (Champagnat, 1965; Champagnat et al., 1966, 1968), *Cattleya* (Champagnat and Morel, 1969; Champagnat et al., 1970), *Neottia nidus-avis* (Champagnat, 1971), and *Ophrys* (Champagnat and Morel, 1972) were published in reviewed French journals. Despite its prominence before World War II, French is,

in fact, a language which has long since lost its scientific and international importance and one with which most orchid scientists and growers in the world were/are not familiar. These papers did contain more details than the early one, but by this time the importance of the information was much reduced because a detailed procedure, complete with a medium recipe, had already been published by Wimber (1963).

Additional murkiness to this already muddled bit of orchid history was added recently by statements that "It was here [the firm of Vacherot and Lecoufle], around the end of 1959 . . . an American student in botany . . . Walter Bertsch . . . rushed into my laboratory . . . quite excited [and] said 'are you aware what Dr. Morel is doing?' " (Vacherot, 2000). Morel, a close friend of Mr. Vacherot's father (Vacherot, 2000), was working on potatoes at the time, but "he applied the same techniques he was using on his potatoes to . . . *Cymbidium*" (Vacherot, 2000). This lead Mr. Michel Vacherot to cut "some young growth" on the "following morning." After that he "carefully dissected each growth, excising the meristem tips . . . and transferred [them] into test tubes on a Knudson sowing medium" (Vacherot, 2000). Curiously this is the same medium mentioned (but incorrectly as "Knudson III") by Morel, but published after 1959 (Morel, 1965*a*, 1965*b*, 1970). And, interestingly Mr. Michel Vacherot's account differs significantly from the one by his partner Mr. Maurice Lecoufle (see above). Conflicting and/or unclear reports are not new for those who have attempted to unravel the history of this episode in orchid micropropagation. One can only wonder why those who participated in the events befuddle rather than clarify. With some of the participants (George Morel, Walter Bertsch, Maurice Lecoufle) dead, others getting on in years, and the current generation of V&L owner not forthcoming with information, the truth may never become known.

Mr. Michel Vacherot's account may differ from the one by Mr. Maurice Lecoufle but it does agree with a report that "the possibility of producing unlimited numbers of plants from any single orchid clone" drew the attention of the late Dr. Walter Bertsch who lived in Paris at that time and described himself as being "involved with the breeding program at Vacherot and Lecoufle" (Bertsch, 1966). Bertsch suggested that Vacherot and Lecoufle enter the field. They did and were successful immediately (Bertsch, 1966, 1972). As a result "Vacherot and Lecoufle became the first nursery to develop, on an industrial basis, the meristemming of orchids. For ten years they held the monopoly" (Lecoufle, 1995). This monopoly started in the early 1960s or late 1950s (Orchid Digest Staff, 1995). To announce it Vacherot and Lecoufle published a full page advertisement in the *American Orchid Society Bulletin* for June 1964 which included a photograph of a flask containing plantlets of *Laeliocattleya* Chine "Bouton D'Or" and stated "we do it" (Fig. 1-42). The fact that this cross was registered in 1962 (Royal Horticultural Society, 1961–1963) and the size of the plantlets suggest that the cultures were started before publication of culture media for this hybrid genus or its parent genera (*Cattleya* and *Laelia*).

Vacherot and Lecoufle's "we do it" advertisement was followed in December of that year with a photograph of technicians performing aseptic manipulations in what appears to be a sophisticated laboratory for the time (Fig. 1-43). The two advertisements appeared approximately 1 year before Morel first published extensive details about his procedure and the composition of some of his culture media. It is possible to speculate that the close friendship between Georges Morel, the Vacherots (Vacherot, 2000), and the Lecoufles prompted him and/or one of his associates to

FIG. 1-42–1-45. **Advertisements by and for the French firm of Vacherot and Lecoufle (from various catalogs).**

teach Vacherot and Lecoufle the technique and then to delay publication and withhold information for a while (Arditti and Arditti, 1985). Morel's friends bristle at the suggestion that he was associated with a commercial venture: "More important . . . is the need to dispel the notion that Morel's horticultural contacts may have interfered with the publication of his findings . . ." (Torrey, 1985*b*). In actual fact Morel did not shy away from horticultural contacts. He was willing to publicly endorse specific commercial establishments as is clear from a letter which was published in a mail order catalog below a picture of Morel examining a culture vessel (Orchids Orlando, no date; Figs 1-10, 1-44, and 1-45).

In spite of the information above, it is not possible at present to unravel the situation. While still alive Maurice Lecoufle of Vacherot and Lecoufle refused to answer two very direct question: "Did Morel delay publication or not?" and "If he did delay, why did he do so?" Another person who could throw some light on this question, ". . . Dr. [C.] Martin . . . University of Dijon . . . the closest collaborator [who was] going to write to you [Joseph Arditti] and] enlighten . . . several points" (M. Lecoufle, pers. comm.), never did write. Thus, questions still remain. The best service Morel's friends can provide to his memory is to set the record straight openly and clearly.

Yet another aspect in the first papers which requires an explanation is the lack of citations or credit to Loo, Ball, Limasset, Cornuet, and others. Two visitors to Morel's laboratory in the mid 1960s (one a student and the other a sabbatical year researcher) have suggested that this was not ". . . unusual for French scientists – it was a way of life." One of them ". . . in particular, said that he [Morel] spent very little time in the library." This is not consistent with a report by one of the visitors mentioned here that Morel ". . . had a habit of claiming that other people left significant papers out of their reviews." One has to read, or at least see the reviews before making such a statement. Further, many of Morel's other papers contain adequate and detailed citations (Champagnat et al., 1966, 1970) and even uncomplimentary statements about his predecessors (Morel, 1974). These are clear indications that he was familiar with the literature. Our experience also indicates that Morel followed the literature closely and regularly. He commented on one of our papers (Churchill et al., 1971a) a very short time after it was published.

When asked after one of his lectures at a meeting about Ball's contribution to his work Morel replied: "Ah, Ball" and was angry. This "reply," and a description of Morel as a "glory seeker" by someone who knew him, suggest that he did not cite others as a means of reserving credit and priority for himself. Of course, this suggestion is open to questions since Morel has also been described as (1) ". . . one of the pioneers in the study of shoot meristem culture as well as an early advocate for its practical use in multiplication of virus free plants . . . interested in the free exchange of scientific information and discoveries [who] 'did not take any patent because I feel that a scientist does not have to do this . . .'" (Torrey, 1985*b*), and (2) a very nice, kind, and modest man.

The only point left to consider at this junction is whether Morel appreciated the potential of shoot-tip culture as a means of propagation early enough. He did write that ". . . very often the protocorm-like body divided into a clump of four or five identical structures, each of them producing a new plant . . . usually each bud will give several plants so the stock of rare or expensive variety can be increased at the

same time" (Morel, 1960). This is certainly not such a clear and forceful statement as Thomale's (Thomale, 1957). It suggests that he did not realize the full implication of shoot-tip culture as a means of mass rapid clonal propagation, or that he did but preferred not to call attention to this aspect of his work. However, in his third paper (and the second one in English) on orchid shoot-tip cultures Morel wrote: "We have now discovered a new phenomenon which will make it possible to produce many hundreds 'seedling' plants in one year from a single bud. This is a distinctly new technique of clonal propagation by meristem culture" (Morel, 1964a). This quote is clear and forceful, but it states ". . . we have *now* discovered . . ." (emphasis added). The "now" is 4 years after his first announcement (Morel, 1960). Moreover, it was published after Professor Donald E. Wimber (Fig. 1-46)

FIG. 1-46–1-51. **Orchid micropropagation, people and medium components. 46. Professor Donald E. Wimber [photographed in 1988 in Hiroshima by Joseph Arditti (JA), signature from a letter to JA]. 47. Samuel Mosher, founder-owner of the Does Pueblos Orchid Company, Goleta, California. 48. Protocorm-like bodies in liquid culture (Wimber, 1963). 49. Plantlets in vitro (Wimber, 1963). 50. Everest McDade (photograph courtesy Everest McDade, signature from a letter to JA). 51. Electron microscope photographs of charcoal (courtesy Dr. Maureen Weatherhead).**

had independently discovered shoot-tip cultures (Figs 1-48 and 1-49) and pointed to their potential (D. E. Wimber, 1963, 1965, pers. comm.).

Many consider Morel's orchid work to be highly original and innovative. However a somewhat different picture emerges from a critical evaluation of the historical facts. None of the work that Morel did with potatoes, *Dahlia*, and orchids was original. Media for plant tissue culture in general and stem tips of orchids in particular existed (Loo, 1945*a*, 1945*b*, 1946*a*, 1946*b*, 1946*c*; Knudson, 1946; Rotor, 1949; Mayer, 1956; Thomale, 1956, 1957) before Morel formulated his own by modifying existing ones. Several explant types (shoot tips, buds, nodes) from monocotyledonous plants in general (Robbins, 1922a; Segelitz, 1938; Kikuta and Parris, 1941) and orchid in particular (Rotor, 1949; Thomale, 1956, 1957) were cultured before Morel did it (Morel and Wetmore, 1951*a*). And, a number of procedures were published following established scientific publication protocol prior to his. Shoot tips were used to free plants of virus infection before Morel's work with dahlias, potatoes, and orchids (see above). Even Morel's work on potatoes and dahlias was suggested by others, namely P. Limasset and P. Cornuet (Gautheret, 1983:402, 1985:42).

Georges Morel's major and significant achievement was to produce protocorm-like bodies which were sustainable via subculture and this made true mass rapid clonal propagation possible. He did that by cleverly combining existing procedures and culture techniques into a very useful new application. Having done that, he was also able to generate publicity for an advance whose time had come. He should be credited with imaginatively applying existing knowledge and technology to a new application. Indeed, in this he played a decisive role. However, Morel should not be given the accolades normally reserved for those who originate novel ideas, make basic discoveries, and formulate new principles (Arditti, 2001; Easton, 2001).

In the course of more recent historical reminiscences, it has been claimed that "Ball is really the father of the so called micropropagation method" (Gautheret, 1985:16–17), but if so the same can be said about LaRue and Loo (LaRue, 1936; Loo, 1945*a*, 1945*b*, 1946*a*, 1946*b*, 1946*c*; Ball, 1946). Perhaps Gautheret felt justified in crediting Ball because he showed that stem tips can be cultured in vitro (still, LaRue and Loo did the same). But, Ball does not seem to have appreciated and certainly did not express in print (Ball, 1950) the practical potential of his work. The same can be said of others (LaRue, 1936; Loo, 1945*a*, 1945*b*, 1946*c*; Wetmore, 1954; Krikorian, 1982).

Ball was interested in the basic aspects of growth and development from meristems. Therefore, he is perhaps better viewed as more of an "uncle" than a "father." The same can be said of Loo for his work at CalTech, and LaRue who succeeded in growing "a short typical meristem [of *Naturtium officinale*] into a whole plant" (LaRue, 1936). If Ball is not the father, then Morel could have been, except that: (1) Gavino Rotor Jr. first thought of and implemented in vitro clonal propagation; (2) Hans Thomale was the first to culture orchid tuber explants – he also drew special attention to the mass propagation potential of his work, and (3) Donald Wimber was the first to publish a detailed shoot "meristem" culture procedure and to follow established scientific publication practices in doing it. Still, Morel is considered the father because of the "widely publicized view" (Easton, 2001) which he fostered while at the same time managing to appear modest and unassuming (Arditti, 2001, 2002).

The Fourth Aseptic Culture of an Orchid Explant

Samuel Mosher (1893–1970; Fig. 1-47), a wealthy oilman, grew orchids and eventually established the Dos Pueblos Orchid Company in Goleta, California. Mosher's enterprise included what was described as "the world's largest establishment for the breeding and growing of Cymbidium orchids" (Anonymous, no date). Mr. Mosher was an enlightened and earnest grower and student of orchids, in many ways a throwback to the great British firms of yesteryear like Sanders, Veitch, Black and Flory McBean, Charlesworth, and others (Arditti, 1990). He established a modern and well-equipped laboratory and hired a cytogeneticist, Dr. Donald E. Wimber (Fig. 1-46), to study orchid chromosomes and to manage a large, modern laboratory.

Wimber was born on January 2, 1930 and died of a heart attack in 1997. He received his B.S. from San Diego State College in 1952 and his M.S. and Ph.D. from Claremont College in 1954 and 1956 respectively. He carried out his graduate work under Professor Lee W. Lenz at the Rancho Santa Ana Botanic Garden, became associated with the Dos Pueblos Orchid Company and worked there until 1957. After a period (1958–1960) as a postdoctoral fellow at the Brookhaven National Laboratory, 2 years (1960–1961) at the Royal Cancer Hospital in London, and another stint at Brookhaven (1961–1963), Wimber accepted (in 1963) an appointment at the Biology Department, University of Oregon, remained there and became a distinguished and honored (American Orchid Society Gold Medal) scientist (Ernst, 1992).

While associated with the Dos Pueblos Orchid Company Wimber studied cytology and engaged in seed germination. He was introduced to the technique by Emil Vacin, co-formulator of the Vacin and Went medium (Ernst, 1992). Observing young plants and seedlings led Wimber to the tissue culture of orchids. His first attempt was never published, but it pre-dated both Thomale's and Morel's work. The following account is based on a letter he wrote to Joseph Arditti on December 13, 1976.

Research with embryonic leaves was carried out in the summer of 1955 while Wimber was still a graduate student. It involved several immature shoots from a *Cymbidium lowianum* clone. The shoots were 4–5 cm long. They were surface sterilized with a 10% dilution of the laundry bleach Clorox after a few of the outside scale leaves were removed. Several additional leaves were removed before the last four to six embryonic leaves were broken off and placed on semisolid Vacin and Went nutrient medium. In addition Wimber made several thin transverse sections through the shoot axis after removing many of the covering leaves. Protocorm-like bodies developed at the bases of the embryonic leaves and along the thin sections.

When some of the protocorm-like bodies were quartered and spread on agar, the sections produced plantlets. Wimber showed his results to Sam Mosher and Kermit Hernlund, manager of Dos Pueblos at the time. They were not impressed because the tissues grew slowly. By Christmas of that year the plantlets were only 2–3 mm tall. In 1957 Wimber had a dozen plants in 10–15 cm (4–6 inch) pots. He concluded his letter by stating "I knew I had something, but was rather fearful that some sort of chromosomal change might have occurred so that a faithful reproduction of the parent might not occur." If the cytogeneticist in Wimber had been less persuasive than the propagator he could have been the one credited with the discovery of mass rapid clonal propagation of orchids.

In 1963, Wimber published his first paper on clonal propagation of *Cymbidium* (Wimber, 1963). Like Morel's first paper on shoot-tip culture of *Cymbidium*, Wimber's report was published in the *American Orchid Society Bulletin* (Fig. 1-48, 1-49), but the similarity ends there. Wimber followed standard scientific practice and provided full procedural details, provided the recipe of his medium (modified Tsuchiya), and carefully described the culture conditions (continuous illumination of 100 foot-candles or less, constant temperature of 22°C, rotary shaker, 125-ml Erlenmeyer flasks sealed with rubber stoppers). Also, Wimber was very clear in calling attention to the propagation potential of shoot-tip cultures.

This wealth and clarity of details presented by Wimber is especially remarkable in view of the fact that the procedure was developed while he was employed by a commercial concern which had every right to keep the details secret. By contrast, Morel worked in a government laboratory and at one point received funding from the American Orchid Society. Anyone with the appropriate training or experience with orchid seed germination and the needed facilities could repeat Wimber's work immediately. A subsequent paper elaborated on the initial procedures (Wimber, 1965). Indeed, it could be argued that Wimber was the first to publish on clonal propagation of orchids through stem-tip culture because his was a scientific (albeit non-reviewed) paper (Wimber, 1963), rather than what can be called a public relations announcement or news bulletin (Morel, 1960).

Who Pioneered Micropropagation?

Three lines of research, two short and direct and one long and branched, lead to four separate and independent discoveries of orchid propagation by means of tissue culture. The first discovery, that by Dr. Gavino Rotor in 1949, can be traced to Knudson's work [through the medium (Knudson C) used to culture the *Phalaenopsis* flower stalk buds] and teaching. The scientific and historical line in this case was very short: from Knudson, the famed orchidologist, to a graduate student who had a good idea and made it work (Rotor received his degree for research with Dr. Lawrence McDaniels and Dr. Kenneth Post on the control of flowering in orchids which is still an important work).

Dr. Donald Wimber, chronologically the third discoverer, developed a shoot-tip culture method as a result of his own work with orchid protocorms and seedlings at the Dos Pueblos Orchid Company. His was the shortest line of all since he originated the idea on the basis of his own research (D. Wimber, pers. comm.). He made an interesting observation and followed up on it with innovative research.

The work of Hans Thomale (chronologically the second discoverer) and Georges Morel (the fourth and last discoverer) is based on the line of research which started with Haberlandt and culminated with Loo and Ball. A well-read practical horticulturist, Thomale derived his culture method from a branch of this line established by Dr. L. Mayer. Morel's procedure is based on Limasset's and Cornuet's suggestion, Knudson's and/or Knop's media, and Ball's and Loo's research.

Rotor's approach was the most original since it was not based on any previous or similar work. However, he did not excise the buds from the flower stalks and obtained only one plant per explant. Wimber's is a close second in terms of

originality since it is derived from observations of seedlings growth. He excised shoot tips and obtained multiple plantlets. Thomale's and Morel's methods are the least original since they are based on previous work of the same nature by others with different plants. The differences between them are that (1) Thomale's publication preceded Morel's by 3 years, and (2) he credited the source of his method (". . . a propagation method . . . based on . . . work by Dr. L. Mayer . . .") whereas Morel did not do that (except for misnaming one of the media he used and listing another).

Wimber's and Morel's methods are the most practically useful (immediately after publication and too many years after an initial announcement respectively). Rotor's was used sporadically for a while, but was not very successful or practical. There is no evidence that Thomale's method was used by horticulturists at any time.

Neither Rotor nor Thomale received much if any credit for their discoveries and their contributions are seldom if ever mentioned in the literature. Wimber received some credit, but much less than he deserves. Morel received the lion's share of the credit (as well as adulation, personal publicity, fame, glory, and funding), but deserved much less.

In correspondence with Joseph Arditti, Rotor indicated that the lack of recognition was not a matter of concern for him. After their contributions were made known both Thomale and Mayer wrote to express gratitude for being put on record. Wimber was not disturbed by the lack of recognition and stated so clearly in letters and during a conversation with Joseph Arditti while attending a world orchid conference in Japan. Given Morel's pursuit of glory it is safe to assume that he was pleased by his fame.

Altogether credit should be given to:

1. Dr. Gavino Rotor Jr. for developing the first tissue culture (or in vitro) clonal propagation method for orchids or any other plant even if he did not use an explant as the term is understood at present. The Cornell University Department of Horticulture website reported him as deceased in its update on April 12, 2002.
2. Hans Thomale for: (a) the first clonal propagation method of orchids involving a bud or tip explant; and (b) the earliest clear suggestion that tissue culture has the potential of being used for mass rapid clonal propagation.
3. Professor Donald E. Wimber for being the first to publish a detailed method for the micropropagation of orchids through the culture of shoot-tip explant.
4. Dr. Georges Morel for: (a) suggesting that shoot-tip culture can be used to free orchid plants of viruses; (b) generating considerable publicity for mass rapid clonal propagation through tissue culture; (c) calling the attention of commercial growers to the method; (d) coining the term "protocorm-like body."
5. The firm of Vacherot and Lecoufle for the first commercial use of shoot-tip cultures for mass rapid clonal propagation (on their own and/or with the advice of Dr. Georges Morel and/or Dr. Walter Bertsch).

With all that in mind, it is necessary to keep in mind that the owner(s) of a commercial orchid establishment in the USA claimed to have invented the process (see below).

Root Cultures

B. M. Duggar was trained as a mycologist, received his Ph.D. from Cornell University in 1898 and became a plant physiology pioneer at his alma matter (Krikorian, 1975). There he influenced two young plant physiologists, Lewis Knudson (Fig. 1-25) and William J. Robbins (Fig. 1-19), who utilized aseptic culture methods in their research on roots (Krikorian, 1975). Knudson first used aseptically cultured roots to investigate enzyme secretion and carbohydrate metabolism (Knudson 1916; Krikorian and Berquam, 1969; Krikorian 1975, 1982). Later he worked with root cap cells and showed that they slough off while still alive and can live for several weeks in culture, however they failed to divide and eventually died (Knudson 1919*b*; Gautheret, 1985). That was before plant hormones and vitamins became available. Knudson's studies of carbohydrate metabolism and aseptic culture experiments led him to the asymbiotic germination of orchid seeds (Knudson, 1921, 1922).

Robbins followed a different path. He wanted to test a hypothesis advanced by Jaques Loeb in 1907 that a hormone produced by leaves affected root development in the leaf notches of *Bryophyllum* (Krikorian and Berquam, 1969). To do that he proposed to compare the growth of excised root tips in salt solutions with and without sugar (Loeb, 1907; Krikorian and Berquam, 1969). His idea was that growth in a sugar-containing medium ". . . would demonstrate that sugar was the 'hormone' furnished by the leaf and necessary for the growth of roots in the leaf notches" (Robbins, 1957, cited by Krikorian and Berquam, 1969). Later he succeeded in culturing corn roots and maintained them for long periods (Robbins, 1922*a*, 1922*b*; Krikorian and Berquam, 1969; Krikorian, 1975, 1982; Gautheret, 1983, 1985).

Also in 1922 W. Kotte (Fig. 1-20), who worked in Haberlandt's (Fig. 1-2) laboratory, cultured very short root-tip explants of peas and corn on several media based on Knop's solution and containing glucose, alanine, asparagine, and Justus Liebig's meat extract. The latter was especially effective in supporting normal growth (Kotte, 1922*a*, 1922*b*; Krikorian, 1975, 1982; Gautheret, 1983, 1985). Kotte's purpose was clearly to study the growth of meristematic tissues since ". . . isolated meristematic tissues have not yet been cultured" (Kotte, 1922*a*, translated by Krikorian and Berquam, 1969).

A number of other investigators attempted to culture root tips, but could only obtain limited growth and development. The first successful "indefinite" cultures of root tips were those of tomato in 1934 (White 1934*a*). White's experiments were encouraged by Nobel laureate Wendell Stanley who needed a system for plant virus studies and multiplication. White failed with tobacco roots, but succeeded with tomatoes and obtained virus reproduction in his cultures (White, 1934*b*; Gautheret, 1985). Several years later James Bonner, Robbins, and White demonstrated (separately and independently) the importance of thiamine or its components thiazole and pyrimidine in root cultures (Bonner, 1937; Robbins and Bartley, 1937; White, 1937; Gautheret, 1985). Interestingly, similar findings were made with *Cymbidium* seedlings in the California Institute of Technology (where Bonner spent his entire scientific career) laboratory of Professor Frits W. Went (Fig. 1-5), the discoverer of auxin (Hijner and Arditti, 1973). Numerous investigators worked on root cultures after that with H. E. Street being among the most prominent (Street, 1973, 1977, 1979; Krikorian, 1982; Gautheret, 1983, 1985).

The idea of culturing orchid root tips probably originated independently several times. What may be the first printed suggestion that it could and should be done appeared in a theoretical article which did not report research findings (Beechey, 1970). At the same time our laboratory initiated a research project involving the culture of *Epidendrum* root tips and modified a medium originally developed for the culture of wheat root tips (Ojima and Fujiwara, 1962). Mary Ellen Farrar (later Churchill), an undergraduate student, did most of the work. The roots grew in length only, became thinner, and after 2 years lost their chlorophyll (Churchill et al., 1972b). *Phalaenopsis* roots, which sometimes produce plantlets spontaneously in nature (Anonymous, 1885; Reichenbach, 1885; Fowlie, 1987), proved difficult to culture initially, but were cultured eventually (Tanaka et al., 1976). Roots of *Neottia nidus-avis* (Champagnat, 1971) and other orchids (for a review see Churchill et al., 1973) which also produce buds and/or plantlets in nature seem not to have been cultured.

During the last 20 years roots of *Catasetum* (Kerbauy, 1984*a*; Colli and Kerbauy, 1993; Vaz et al., 1998; Peres et al., 1999), *Cattleya* (Kerbauy, 1991), Crimean orchids (Popkova, 2000), *Cymbidium* (Pindel and Miczyński, 1996*a*, 1996*b*), *Cypripedium yatabeanum* (Jo et al., 2001), *Cyrtopodium* (Sanchez, 1988), *Doritaenopsis* (Tsukazaki et al., 2000; Park et al., 2001), *Rhynchostylis* (Sood and Vij, 1986; Vij et al., 1987), and other orchids (Vij, 1993) have been cultured successfully and used to produce plantlets. It is safe to assume that the future will bring an increase in the number of orchids whose roots can be cultured and used for clonal propagation in vitro.

Rhizome tips have also been cultured, with the earliest success being reported from the laboratory of Professor H. Torikata at the University of Nagoya in Japan (Ueda and Torikata, 1972; for a review see Rao, 1977). The first report of tuber explant culture is that of *Pachystoma senile* (Vij et al., 1983). Other orchids propagated from rhizome explants are *Cymbidium aloifolium* (Nayak et al., 1998), *Cymbidium ensifolium* (Paek et al., 1993; Chang and Chang, 1998, 2000b), *Cymbidium ensifolium* × *Cymbidium kanran* Dougiu (Paek et al., 1993), *Cymbidium goeringii* (Paek and Kozai, 1998), *Cymbidium kanran* Namkuk (Paek and Kozai, 1998), *Cymbidium kanran* Toja (Paek et al., 1993), *Cymbdium kanran* Jeju × *Cymbdium goeringii* (Paek et al., 1993), *Cymbdium niveo-marginatum,* which is viewed by some as being a synonym of *Cymbidium ensifolium* (Paek and Kozai, 1998), *Cymbidium sinense* (Chang and Chang, 2000*a*), *Geodorum densiflorum* (Sheelavantmath et al., 2000), and other orchids.

Leaf Cultures

A number of the early attempts to culture plant cells and tissues by Haberlandt and others were made with leaf explants. These attempts failed because the cells were differentiated (Krikorian and Berquam, 1969; Krikorian, 1975, 1982; Steward and Krikorian, 1975; Gautheret, 1983, 1985). However attempts to culture mature differentiated palisade parenchyma of some plants were successful (Joshi and Ball, 1968*a*, 1968*b*).

At least one orchid [*Hammabrya* (*Malaxis*) *paludosa*] produces bulbils at its leaf tips (Ray, 1724; Godfery, 1933; for reviews see Arditti et al., 1971; Ball et al., 1971; Churchill et al., 1971*a*, 1971*b*, 1971*c*, 1972*a*, 1973). Leaf cuttings can be made of

Restrepia species (Webb, 1981). However these phenomena did not lead to the development of tissue culture procedures for leaf explants. The tendency of juvenile leaves on protocorms to produce protocorm-like bodies lead to the development of micropropagation methods through culture of leaf bases (Champagnat et al., 1970). A claim that these procedures were developed even earlier (Morel, 1960, 1965*b*, 1966, 1970) is not supported by the available evidence ("*Keine Angabe vorliegend*" in Zimmer, 1978).

The first unambiguous and well-documented report that leaves can produce protocorm-like bodies was made in cultures derived from *Cymbidium* shoot tips (Wimber, 1965). An earlier observation in 1955 that embryonic leaves of *Cymbidium lowianum* placed on Vacin and Went medium formed protocorm-like bodies was not published (D. E. Wimber, pers. comm.; Arditti, 1977*a*).

Leaf tips were first used to propagate orchids (*Epidendrum* and *Laeliocattleya*) as a result of unsuccessful attempts to culture foliar explants similar to those taken from peanuts (Joshi and Ball, 1968*a*, 1968*b*). In 1968/1969 – shortly after Professor Ernest A. Ball moved from North Carolina State University to the University of California, Irvine – Ball's laboratory and our laboratory initiated a joint project to culture orchid mesophyll cells. After these explants failed to grow we attempted to culture leaf tips and succeeded almost immediately. The work was carried out by Mary-Ellen Farrar-Churchill who was then an undergraduate student.

A major advantage of leaf-tip cultures is that removal of explants does not endanger the donor plant. Because of that orchid growers and propagators were interested in these methods. To make them widely available they were published in a number of journals and several languages (Arditti et al., 1971; Ball et al., 1971; Churchill et al., 1971*a*, 1971*b*, 1971*c*, 1972*a*, 1973).

Success with these procedures depends on removal of explants before the leaf tips differentiate fully and lose their ability to form callus. If the tips are not taken at the proper stage (i.e., while the tip is still pointed and before the formation of a notch) they die rather than develop when placed in culture. For this reason these methods require attention to detail and are not always easily reproducible. This lead to questions following their publication. However the doubts were resolved following reports that the leaves of *Acampe praemorsa* (Nayak et al., 1997*a*), *Aerides maculosum* (Murthy and Pyati, 2001; Murthy et al., 2001), *Aranda* (Loh et al., 1975; Fu, 1978*a*, 1979*b*; Manorama et al., 1986), *Ascocenda* (Fu, 1978*a*, 1979*b*), *Cattleya* (Fu, 1978*a*, 1979*b*), *Cymbidium* (Gopalan et al., 1992; Pindel and Miczyński, 1996*a*, 1996*b*), *Dendrobium* (Manorama et al., 1986), *Laeliocattleya* (Matos and de Garcia, 1991), *Mokara* (Abdul Ghani and Haris, 1992), *Oncidium* (Chen et al., 1999; Chen and Chang, 2001), *Papilionanthe* (*Vanda*) *teres* (Pathak and Vij, 2001), *Phalaenopsis* (Tanaka et al., 1974; Tanaka and Sakanishi, 1977; Tanaka, 1992; Park and Paek, 1999; Park et al., 2002), *Renantanda* (Goh and Tan, 1982), *Renanthera imschootiana* (Seeni and Latha, 1992; Fukui et al., 2001), *Rhynchostylis retusa* (Vij et al., 1984), terrestrial species (Allenberg, 1976), *Vanda* (Tanaka et al., 1974), *Vanda coerulea* (Seeni and Latha, 2000), *Vanda cristata* (Sharma and Vij, 1997), *Vanda teres* (Niraula and Rajbhandary, 1988), and other orchids (Vajrabhaya and Vajrabhaya, 1976*a*; Chaturvedi and Sharma, 1986) were cultured successfully (for reviews see Arditti, 1977*a*, 1977*b*, 1978; Rao, 1977; Zimmer, 1978, 1980; Fast, 1979; Arditti and Goh, 1981; Czerevczenko and Kushnir, 1986).

Stems

The culture of *Arundina* stem sections was first mentioned in 1966 at the 5th World Orchid Conference in Long Beach, California, but only limited information was presented at the time (Bertsch, 1966; for a review see Zimmer, 1978). Details (from a procedure developed independently of other investigators) became available following the publication of an interesting paper based on a comprehensive investigation of the culture of seeds, shoot tips, and stem disks of this orchid (Mitra, 1971). *Dendrobium* nodes were cultured in 1973 (Arditti et al., 1973; Mosich et al., 1973, 1974*a*, 1974*b*). Stem sections of other orchids have also been cultured.

Flower Buds, Flowers, Floral Segments, and Reproductive Organs

Excised ovaries were the first orchid flower segments to be cultured. This was carried out by Professor I. Ito at the Kyoto Prefectual University in Japan (Ito, 1960, 1961). In an earlier paper Ito reported on another first: the culture of immature *Dendrobium* seeds (Ito, 1955). Subsequent reports regarding the culture of immature seeds (often and erroneously called ovules) are of *Vanilla* (Withner, 1955), *Phalaenopsis* (Ayers, 1960), *Dendrobium* (Niimoto and Sagawa, 1961), *Vanda* (Rao and Avadhani, 1964), and *Paphiopedilum* (Ernst, 1982; for reviews see Withner, 1959; Arditti, 1977*b*; Rao, 1977; Zimmer, 1978; Czerevczenko and Kushnir, 1986). Immature seeds of many additional orchids have been cultured since then. In some cases this is the preferred method of sexual propagation since it saves time and facilitates the germination of several species. This is not a method of micropropagation as such – it is a method of sexual (seed) propagation. However since the contents of ovaries are scraped onto a culture medium it is entirely possible that some of what are presumed to be seedlings may be plantlets produced by ovary tissue and/or cells.

The first young flower buds or inflorescences to be cultured were those of *Ascofinetia*, *Neostylis*, and *Vascostylis* (Intuwong and Sagawa, 1973). Those of *Cymbidium* (Kim and Kako, 1984; Shimasaki and Uemoto, 1991) *Phalaenopsis*, *Phragmipedium* (Fast, 1980*b*), and other orchids were cultured subsequently.

Inflorescences

In anointing "fathers" and giving credits to others for the discovery/invention of micropropagation, a self-appointed arbiter (Gautheret, 1983, 1985) did not even mention Dr. Gavino Rotor's culture of *Phalaenopsis* flower stalk nodes. Nevertheless, Rotor's work pointed the way and others followed by culturing inflorescence explants of several orchids including *Aranda* (Goh and Wong, 1990), *Dendrobium* (Singh and Sagawa, 1972), *Doritaenopsis* (Tokuhara and Mii, 1993; Yamazaki et al., 1997), *Mokara* (Abdul Ghani et al., 1992), *Oncidium* (Chen and Chang, 2000*a*), and *Phalaenopsis* (Ichihashi, 1992*a*, 1992*b*; Tanaka, 1992; Tokuhara and Mii, 1993, 1998, 2001; Chen and Piluek, 1995; Duan and Yazawa, 1995*a*, 1995*b*; Ichihashi and Hiraiwa, 1996; Jiménez and Guevara, 1996; Park et al., 1996; Yamazaki et al., 1997; Islam et al., 1998; Ichihashi et al., 2000; Tokuhara and Mii, 2001; Park et al., 2002).

A Patent

Orchids have always been associated with legends (Lawler, 1984), unusual claims, flamboyance, and interesting attempts to turn a profit. Micropropagation has not been spared some of these.

Perhaps the most interesting and imaginative incident was the attempt to control orchid propagation by means of tissue culture through a patent. This was not a plant patent to cover an exceptional cultivar which is an ethical and accepted practice (Kock, 1967). A claim was made by Mr. Everest McDade (ca. 1916 to ca. 2000; Fig. 1-50) that he developed and used the process as early as 1950 and kept it a secret. He obtained a patent (No. 3,514,900) at least in part on the basis of this assertion (Bergman, 1971). Morel's publications and a publication by the late Professor Harry Kohl (one of Joseph Arditti's professors at UCLA; Kohl, 1962) and Wimber were also used to bolster the application (Torrey, 1985*b*) despite (or perhaps because of) the fact that neither of these scientists nor Michel Vacherot and Walter Bertsch tried to patent the process (Vacherot, 2000).

After the patent was issued an attempt was made to hinder further research. Shortly after publishing our papers on the culture of leaf tips (Churchill et al., 1970, 1971*a*; Ball et al., 1971), we received a letter informing us that our research constitutes an infringement on the patent. The letter also stated that the patent was for sale and offered it to us. We referred the matter to the University of California attorney, who did whatever was necessary. We were never contacted again. In 1972 the patent was sold to the National Orchid Grower's association for $40,000 and placed in the public domain (Easton, 2001).

Details about this bizarre story were sketchy for a while. Now there are two accounts of it. One of these accounts (Arditti and Krikorian, 1996) was pieced together from the literature, conversations with several people, and a letter from Mr. Everst McDade (Asheville, North Carolina) following publication of the first edition of *Micropropagation of Orchids* (Arditti and Ernst, 1963). The second account was published recently (Easton, 2001). The two accounts do not fully agree with each other.

According to the letter from McDade, an electronic engineer, science teacher, and co-owner of Rivermont Orchids, Signal Mountain, Tennesee (the firm no longer exists) until ca. 1949 (or 1950 according to the second account), a "photo of a *Cymbidium* bulb, with a cluster of buds at its base" in an article from ca. 1946 or 1947 (which McDade did not have and claimed he was trying to find again) gave him the idea. It "was a very sudden clear message to us [McDade]. Just what we had been looking for: a renewable source of 'Ramets.' . . . We wanted to use the process for *Cattleya* types. . . . I adapted the *Cymbidium* idea to cattleyas."

The second account tells the story differently: "From early June 1950 [McDade] concentrated on liquid embryo orchid culture, which developed into clonal propagation . . . James Gentry . . . an engineer . . . an assistant [in the] process . . . recalls his work with orchid seeds in 1950 where seedling leaf development was chemically inhibited and each seed developed into a mass of tissue like cancer." When "separated and placed on solid sterile medium . . . they . . . developed . . . into plants." This interesting account brings into the picture embryo culture which was developed several years after 1950 and chemical inhibition of growth, a process which would have required considerable knowledge of plant physiology. It is hard to accept that

two engineers (McDade and Gentry) were far enough ahead of orchid and plant scientists to do all that. Be all this as it may, protocorms often proliferate naturally. Also propagation involving protocorms is sexual. Plants produced in this manner "were in bloom Easter Sunday 1957."

According to McDade, his secretary Dorothy Smith (who was not reported to have plant science training) "made the first meristem cultures in 1950." And, the second account contains an undated photograph of Ms. Smith with a caption that states that she "excised the first meristem tissue to be subsequently multiplied." But the second report also states that Gentry, the engineer, was working with half inch tissue "cubes from the heart of cattleya pseudobulbs" and later with dormant buds from rhizomes which began to multiply in 1951 and flowered in "the late 1950s."

McDade claims that he "wrote scores of letters . . . to authors . . . botany and genetics journals [but] Only a few people took them seriously, or even guessed that we had discovered cloning in [the] year 1950." He also claims that "in October, 1952 [he] actually gave the cloning process paper to the [American Orchid Society Bulletin] . . . for publication and *demonstrated a growth developing from a Cattleya flower stem, a flask, and community pot of a clone*" (emphasis by McDade). According to the second report "In November 1953, Everest was asked by Gordon Dillon, editor of the *A*[*merican*] *O*[*rchid*] *S*[*ociety*] *Bulletin*, to write an article, which he titled "Clones, a new method for the vegetative multiplication of an individual plant." For unknown reasons, the article was never published, and finally was returned to the author in the 1960s.

This statement raises two questions. First, the *American Orchid Society Bulletin* published Morel's first article in 1960 (Morel, 1960) and one wonders why an invited article which describes a revolutionary process would remain unpublished. Second, there are many orchid publications, all constantly in search of articles. Orchid growers, amateur and professional alike, are well aware of this. Therefore the question is why did not a person who "wrote scores of letters . . . to authors . . . botany and genetics journals" submit the returned article to another publication.? There can be very little doubt that it would have been published even after Morel's 1960 article. Wimber's papers were published by both the *American Orchid Society Bulletin* and the *Cymbidium Society News*.

McDade's claims are also not borne out by: (1) his paper in the *American Orchid Society Bulletin* (McDade, 1952); (2) Rivermont advertisements from that period which did not offer for sale any orchids described as being clonally propagated; (3) the patent itself which "relied on" publications by Morel, Kohl and Wimber (Torrey, 1985*a*, 1985*b*); and (4) the chronology involving an idea, which supposedly originated in 1946 or 1947, cultures that were presumably first made in 1950 or 1951, work published between 1955 and 1963, and a patent issued in 1970.

At least three additional arguments can be made against the claim. The first is that the development of tissue culture procedures is not a simple matter. It requires considerable knowledge of plant science. Therefore it is very unlikely that a person or persons not trained and known in plant sciences could have discovered it. This is perhaps the reason why an effort was made to base the claim at least in part on the work of others.

The second is that the process had (and still has) considerable potential for financial gains. Those who used it after 1960 did benefit from it financially. The person

who patented it was clearly aware of (and interested in) financial gains. It is therefore surprising that he did not obtain his patent and publicize the method in 1950. He did so only after 1960 and this suggests that the idea for the patent originated following Morel's first paper in English (Morel, 1960), sketchy as it was. McDade contacted a patent attorney in 1952 and expected that "it would probably take four years (1956) before the orchids he was propagating . . . would flower and be ready for patenting" (Easton, 2001). Even if the plant took longer to flower, and "ultimately [*Cattleya*] Bow Bells 'Edith McDade' would flower in the late 1950s" (Easton, 2001), a patent could have been applied for before Morel publicized his work in 1960.

Third is the fact that evidence usually associated with priority claims is not available. Such evidence generally consists of scientific papers published in peer-reviewed journals, patents, presentations at scientific meetings, and public demonstrations.

By 1974 the furor died down (Arditti, 1977*b*) and not much was heard about the patent for almost 30 years after that (Easton, 2001). The reason for this is undoubtedly the sale of the patent. This episode is unfortunate and points to lax patent laws and lack of careful scrutiny by those who approve patents.

Doubtful Claims

Paphiopedilum species and hybrids have been very popular with orchid growers for a long time. Unfortunately, this genus is difficult to culture. An attempt to capitalize on this difficulty started in the mid 1970s with a content-free article on the tissue culture of *Phragmipedium* (Stokes et al., 1975), another diandrous genus. The authors of this paper were employed by a commercial tissue culture laboratory in the UK.

At about the same time the same laboratory announced that it had developed a tissue culture method for *Paphiopedilum* and would, for a fee, undertake to propagate plants for growers. This claim received some attention at the time. Probing questions regarding the technique were dismissed as being due to professional jealousy. Details about the tissue culture procedure were withheld with the explanation that the method was "proprietary and developed by a commercial laboratory." Eric Young, founder of the orchid foundation which bears his name, was an especially vociferous, loquacious and overbearing promoter and defender of the laboratory which he described as "highly competent and fully equipped."

The laboratory in question promised to display its *Paphiopedilum* cultures at the world orchid conference in Frankfurt in 1975. Joseph Arditti (JA) was invited to see them and visited their booth several times. The purported flasks and photographs were never there having been "borrowed" by someone who "promised" to return them in time for a subsequent next visit, but never did. On a third visit JA waited a long time for the "borrower" to return, but he/she never showed up, and neither did the flasks and photographs. A few months after that the laboratory announced that they would no longer accept *Paphiopedilum* plants for tissue culture.

It is not clear whether any growers submitted *Paphiopedilum* plants for culture and if so what the outcome was. However, the history of research on tissue culture of *Paphiopedilum* suggests that the announcement may have been based on: (1) over-optimism generated by one or a few preliminary successes; (2) an attempt to

capitalize on several procedures (none of them reported to have been repeated) by other researchers which were published at that time (see section on *Paphiopedilum*); (3) wishful thinking; (4) overblown imagination; (5) triumph of hope over reality; and/or (6) simply an attempt to generate business one way or another. One of the individuals associated with this fiasco was apparently associated with another cloning laboratory for a while, but seems to have disappeared from the orchid scene. The other changed employment, and may also no longer be working with orchids. Altogether this is another bizarre chapter in the history of orchid propagation.

Mutations

Since micropropagation is a vegetative means of propagating plants, both horticulturists and scientists assumed at the outset that all offsprings will be true to their parents. This assumption was reinforced by an early report regarding the blooming of "mericlones" which stated that "for each individual cultivar the flowers appeared exactly identical, and in hundreds of them no mutation was observed" (Lecoufle, 1967). Others were equally sanguine (Teo and Teo, 1974; Teo, 1975, 1978, 1978/1979, 1981; Joseph Arditti in conversations) since theoretical considerations suggested that this should be the case.

As experience with micropropagation increased, it became evident that mutations do occur. Among the first to call attention to this were the Thai orchid scientists Professor Thavorn Vajrabhaya and his wife Professor Montakan "Mon" (Vajrabhaya and Vajrabhaya, 1976*a*, 1976*b*; for a review see Vajrabhaya, 1977). Experience since then has shown that mutations do occur during tissue culture (Sahavacharin, 1980; Rentoul, 1981; Teo, 1981; N. Haas-von Schmude, pers. comm.; for additional references see specific procedures in this volume) and are especially prevalent in cases where high concentrations of hormones are used to force excessive proliferation and the production of many plantlets.

Phenotypically these mutations include changes in color, shape and size of blossoms as well as malformed flowers. In some overproliferated clones individual flowers on a raceme may die and render it commercially useless.

It is impossible at present to prevent mutations from occurring or even to predict their nature. However it is possible to reduce their incidence by not overproliferating plants during micropropagation, and not micropropagating plants obtained through tissue culture. Unfortunately, it is impossible to prevent unscrupulous or ignorant propagators from engaging in these practices. *Caveat emptor* is the only protection. Growers can protect themselves by: (1) purchasing plants only from known and reliable sources; (2) keeping each other informed regarding laboratories which produce good or bad plants; and (3) demanding guarantees. However, the best protection is for hybridizers and growers to propagate their own plants.

Theft in Vitro

It is not uncommon for growers and breeders to have their most promising hybrids propagated by commercial micropropagation laboratories. Several of these laboratories

illegally and unethically set aside some of the plants and sell them if a demand develops for a particular clone. This (1) floods the market, (2) reduces prices and the value of the orchid in question, and/or (3) makes it impossible for hybridizers to control their crosses or for cut flower growers to become the sole source of a desirable variety. As with overproliferation there is not much growers can do about this problem except select laboratories carefully and spread information about reliable or unreliable propagators. Another possible approach would be for growers and hybridizers to establish well-supervised laboratories for the purpose of propagating their own plants.

Darkening of Culture Media

Media used for orchid seed germination and micropropagation are sometimes darkened with charcoal to improve growth and development. Professor John T. Curtis (Fig. 1-12) at the Botany Department, University of Wisconsin was the first to darken a nutrient medium for orchid seedlings in vitro. He used lampblack, which is soot produced by the burning of petroleum hydrocarbons. It has very little in common with charcoal except color. Lampblack lacks the large internal surface area, adsorptive properties, and pore structure of charcoal. It has long been used in the production of black inks and paints. Curtis darkened his medium in an unsuccessful effort to simulate natural conditions and thereby bring about the germination of *Cypripedium reginae*, *C. pubescens*, *C. parviflorum*, *C. candidum*, and *C. acaule* seeds (Curtis, 1943).

The charcoal used in orchid media (Fig. 1-51), generally referred to as vegetable charcoal, is made from wood, sawdust, peat, and organic residues which are recovered during the production of pulp (West Virginia Pulp and Paper, no date), carbonized and activated to produce a large surface area. One gram of Nuchar (a commonly used brand) may contain up to 120 billion particles and have a total surface area of 500–2000 m^2. Pore distribution can range from <10 μm to >500 μm (Yam et al., 1990). The pore to volume ratio is 0.9 ml g^{-1}. Charcoal can contain many elements, some in very small amounts (Yam et al., 1990). It is activated through treatment of the carbonized pyrolysis product with steam or carbon dioxide (Yam et al., 1990).

Professor Peter Wekmeister in Germany was the first to darken an orchid culture medium with charcoal (Werkmeister 1970*a*, 1970*b*, 1971). Before that charcoal was employed to darken a medium used to germinate moss spores and grow filamentous algae (Proskauer and Berman, 1970; Krikorian, 1988). Werkmeister darkened the medium to study the growth of roots, gravitropism, and proliferation of clonally propagated plantlets. He died not long after publishing the last of his orchid papers.

Robert Ernst (b. 1916; Fig. 1-55), a surfactant chemist and manufacturer and now a retired Adjunct Professor of Biology at the University of California, Irvine, was the first to add charcoal to practical seedling culture media and found that *Paphiopedilum* and *Phalaenopsis* seedlings grew well on it (Ernst, 1974, 1975, 1976). His findings resulted in the formulation and widespread use of charcoal-containing media for orchid seed germination, seedling culture, and micropropagation (Ernst, 1974, 1975, 1976; for a review see Yam et al., 1990).

FIG. 1-52–1-54. **52. Sharon Spencer (A) and Katie Thurston (B) on their graduation days (source: JA). 53. Jan Cooper on her wedding day (source: JA). 54. Marilyn Cvitanik (A) in New York and Kathy Hill (B) in Irvine (sources: A, www.flyingpug.com/artist.html; B, JA).**

Anticontaminants

The first attempts to formulate media which do not require sterilization involved the use of vanillin and its derivatives (Knudson, 1947; McAlpine, 1947; K. L. McAlpine, pers. comm.; for a short review see Thurston et al., 1979) as well as several antibiotics. All proved to be phytotoxic and could not be used. A number of fungicides and bactericides were screened in our laboratory by several brilliant undergraduates, including Sharon Spencer (Fig. 1-52A), Katie Thurston (Fig. 1-52B), Jan Cooper (Fig. 1-53), Marilyn Cvitanik (Fig. 1-54A), and Kathy Hills (Fig. 1-54B). Several combinations of these substances can be of limited practical use for seed germination (Thurston et al., 1979, 1980) and micropropagation (Spencer et al., 1979/1980; Brown et al., 1982). In general the use of antibiotics and other anticontaminants in orchid micropropagation is limited. A number of phytoalexins and related substances were also screened for phytotoxicity (Hills et al., 1984) and could perhaps prove to be useful if they were to become available commercially. Several antibiotics are being used in tissue culture and micropropagation of other plants. PPM™, a commercial preparation (see Chapter 2), may also prove useful.

Knudson C medium which does not require sterilization and remains free of contaminants has been prepared with 0.1% hydrogen peroxide (Snow, 1987). After a suitable period any hydrogen peroxide which still remains in the medium is decomposed through the use of a sterile catalase solution. The question is whether sterilizing an enzyme (i.e., protein) solution and adding it to the culture under sterile condition is not more complicated than autoclaving the Knudson C medium.

Cell and Protoplast Culture

The first attempts to culture free plant cells utilized mechanically isolated ones. Gottlieb Haberlandt made fairly intensive, but unsuccessful, efforts to culture cells in 1898 and 1902 (Krikorian and Berquam, 1969; Krikorian, 1975, 1982; Steward and Krikorian, 1975). A suggestion (Gautheret, 1985) that Haberlandt failed because he

neglected the findings of a French naval architect, agronomist and student of trees Henri-Louis Duhamel du Monceau (1700–1782) probably has its roots in Gallic chauvinism rather than in scientific reality. Haberlandt failed because: (1) he selected mature, differentiated, specialized non-meristematic (i.e., inappropriate) cells to culture; (2) his ideas were ahead of their time and more advanced than the available plant science technology; (3) not all necessary components were present in the culture media he used ; (4) the plants he chose included monocotyledonous species which can be recalcitrant; (5) there was no previous information to guide him; and (6) his cultures, although clean, were not aseptic (Haberlandt, 1902 translated by Krikorian and Berquam, 1969).

Haberlandt used "tap water, one to five percent sucrose solutions, and Knop's solution with or without sucrose, dextrose, glycerine, asparagine and peptone in various combinations and concentrations." This quote is from Krikorian and Berquam (1969), who raise the question of what might have happened "had coconuts been generally available in Berlin," but it is also interesting to wonder whether they caught his fancy when he saw them (Arditti and Krikorian, 1996) because Haberlandt visited Buitenzorg from November 1891 until February 1892 and spent time in other parts of tropical Asia (Haberlandt, 1910).

Lewis Knudson's attempt to culture sloughed-off root-cap cells of corn and Canada field-pea (Knudson, 1919b) is not as well known as his work with orchids, but is still well ahead of its time. As culture media he employed water and, foreshadowing his work with orchids (for a review see Arditti, 1990), also Pfeffer's Solution, which he modified by replacing dibasic potassium phosphate with the monobasic salt with or without 0.5% sucrose. Some of the Canada pea cells survived for 50 days when roots were also present in the culture medium. The cells lived for an additional 21 days after removal of the roots despite becoming contaminated.

Knudson's experiments suggested the diffusion from roots of growth substances which the cells required, but this research was carried out: (1) 8 years before the discovery of auxins (Went, 1928, 1990; Thimann, 1980); (2) about 20 years before it was demonstrated that vitamin B_1, niacin, and other factors enhance the growth of plant sections in general and excised roots in vitro in particular (Bonner, 1937, 1938, 1940*a*, 1940*b*; Addicott and Bonner, 1938; Bonner and Devirian, 1939); and (3) 35 years before the discovery of cytokinins (Miller et al., 1955*a*, 1955*b*; Miller, 1961, 1977; Skoog, 1994).

Even if Knudson had surmised that his cells needed growth substances, few if any were available or even known at the time and his attempts were doomed to failure. Aseptic techniques were available in Knudson's laboratory by that time even though the culture experiments were carried out 1–2 years before he started work on non-symbiotic germination of orchid seeds (for a review see Arditti, 1990). It is surprising that he did not use them.

The first isolated cells to be cultured successfully were those of tobacco, *Nicotiana tabacum*, and marigold, *Tagetes erecta*. They were grown on filter paper platforms placed on top of proliferating callus masses (for reviews see Muir et al., 1954, 1958; Krikorian, 1975, 1982; Steward and Krikorian, 1975; Gautheret, 1983, 1985). Proof that the colonies on the platforms did not form from cells of callus origin that grew through the paper was obtained by culturing a single cell of *Tagetes erecta* on a platform placed on top of a sunflower callus (Muir et al., 1958). Other research

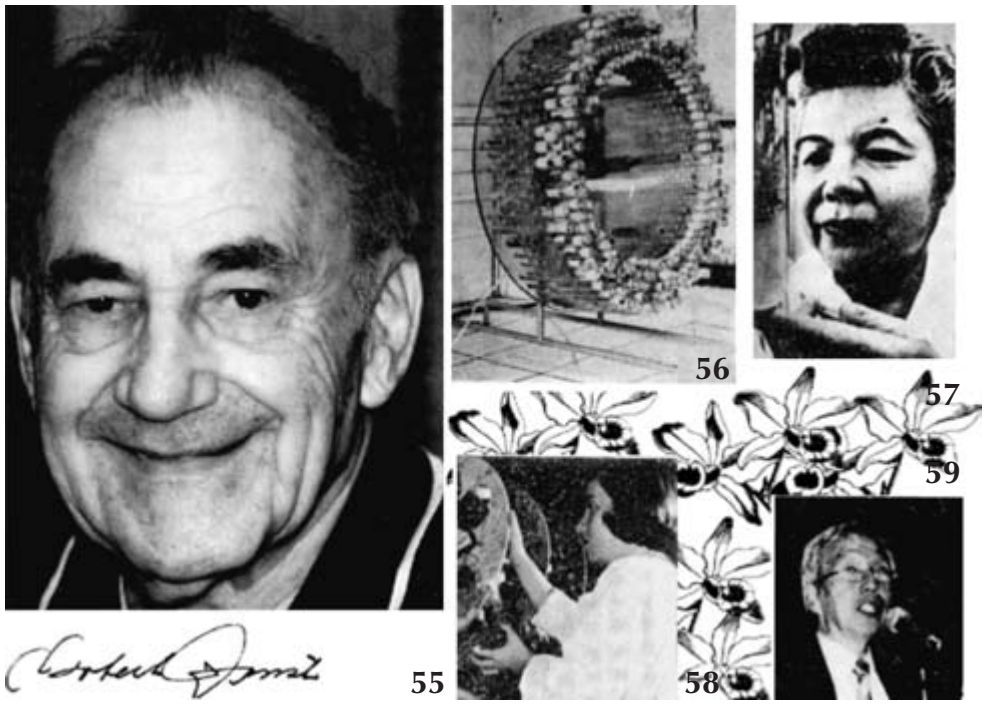


FIG. 1-55–1-59. **Orchid tissue culture specialists and apparatus. 55. Professor Robert Ernst (photograph by JA, signature from JA's files). 56. Rotating shaker for orchid tissue and cell cultures (Morel, 1966). 57. Marion Mapes (courtesy Professor Emeritus Abraham D. Krikorian). 58. Kathryn Mears-Trupin (courtesy Professor Emeritus Abraham D. Krikorian). 59. Professor Yoneo Sagawa (Nagoya International Orchid Conference).**

followed (Bergmann, 1960), and convincing proof that an isolated cell can divide was provided by tobacco cells which divided in drops of medium in microculture (Vasil and Hildebrandt, 1965*a*, 1965*b*). Shortly after that, isolated mesophyll cells of *Arachis hypogea* were prompted to divide in culture and produced what can best be described as protocorm-like bodies or structures which look like them (Joshi and Ball, 1968*a*, 1968*b*).

Using an apparatus that slowly (1 rpm) rotates "nipple culture flasks" around a horizontal axis (Fig. 1-56), Frederick Campion Steward (1904–1994; Fig. 1-9a) and his associates at Cornell University, Russell C. Mott (Fig. 1-9b), Marion O. Mapes (1913–111981; Fig. 1-57), and Kathryn Mears-Trupin (Fig. 1-58), obtained suspension cultures of carrot cells and eventually regenerated plants from them (for reviews see Krikorian, 1975, 1982, 1989*b*; Steward and Krikorian, 1975; Gautheret, 1983, 1985; Arditti and Ernst, 1993). *Cymbidium* cell cultures were established using the same system. Plants were regenerated from these cells subsequently (Steward and Mapes, 1971*a*). Two decades later *Phalaenopsis* plants were regenerated from embryoids derived from a loose-celled callus (Sajise et al., 1990) in Professor Yoneo Sagawa's (Fig. 1-59) laboratory at the University of Hawaii. Other orchid cells have also been cultured (see specific procedures in Chapter 3).

The first protoplast preparations were obtained in 1892 through surgical release from plasmolyzed cells of water aloe, *Stratiotes aloides*. Eventually digestion of cell walls became the method of choice (for historical surveys see Steward and Krikorian, 1975; Krikorian, 1982). What may well be the first preparation of orchid protoplasts resulted from work with leaves (i.e., mesophyll cells) of *Cymbidium* Ceres and "virus free protocorms of *Cymbidium pumilum*, *Brassia maculata* and *Cattleya schombocattleya*" (Capesius and Meyer, 1977). The protoplasts were used for the isolation of nuclei but apparently no effort was made to produce callus masses or regenerate plants from them. Regardless of the research for which these protoplasts were used, it is necessary to point out that there is no "*Cattleya schombocattleya*." It is not clear if what was meant is "*Cattleya*, *Schombocattleya*," "*Cattleya* or *Schombocattleya*," "*Cattleya* and *Schombocattleya*," or "*Cattleya* × *Schombocattleya*."

Production of orchid protoplasts and subsequent fusion between and within genera was first reported in 1978, but the ultimate fate of the fusion products has not been described in the literature (Teo and Neumann, 1978*a*, 1978*b*, 1978*c*). Early isolations of orchid protoplasts have been reported from Portugal (Pais et al., 1982, 1983), the USA (Price and Earle, 1984; Kuehnle and Nan, 1990; Sajise et al., 1990), Singapore (Loh and Rao, 1985; Hew and Yip, 1986; Hew, 1987; Koh et al., 1988), Japan (Yasugi et al., 1986; Yasugi, 1986, 1989*a*, 1989*b*, 1990; Kobayashi et al., 1993; Belarmino and Mii, 2000; Tokuhara and Mii, 2001), India (Seeni and Abraham, 1986; Gopalakrishnan and Seeni, 1987), Taiwan (Chen et al., 1990*a*, 1991), Philippines (Belarmino and Mii, 2000), and elsewhere (see Chapter 3).

More recently, 4×10^6 protoplasts were obtained per gram of young leaf tissue of *Phalaenopsis* (Chen et al., 1995). Their average diameters were 31.2 μm. The diameters of protoplasts from root tips and petals were 36.4 μm and 31.1 μm respectively. Approximately 90% of the protoplasts were viable. Some of the protoplasts divided after 5 days. Only a few divided twice after 10 days. Very few clusters were formed after 21 days, and they eventually died (Chen et al., 1995). However, Yoneo Sagawa (b. October 11, 1926; Fig. 1-59) and his co-workers at the University of Hawaii have reported regeneration of *Phalaenopsis* plants from protoplasts (Sajise et al., 1990). Research with Sagawa's callus is also being carried out by Professor Syoichi Ichihasi in Japan.

A direct electrical current pulse of 2500 V cm^{-1} of 2 milliseconds duration was sufficient to cause fusion in 10% of *Phalaenopsis* protoplasts to form hybrid cells. The fate of these fusion products was not described, but the context of the paper (Chen et al., 1995) suggests that they did not survive. Studies of transgenic *Phalaenopsis* are also in progress (Chen et al., 1995).

Flowering in Vitro

After Professor Lewis Knudson published his method of asymbiotic orchid seed germination, J. Costantin (1857–1936), Noël Bernard's mentor, attacked him bitterly by questioning his veracity. When he could not sustain the smear Costantin challenged Knudson to prove that an asymbiotic orchid can flower. Knudson proved that this is possible by growing a *Laeliocattleya* plant asymbiotically from seed to bloom

FIG. 1-60–1-64. **The first in vitro flowering by orchid plants produced through micropropagation. The work with *Cymbidium* was done by Dr. Xiong Wang at the Shanghai Institute of Plant Physiology in the late 1970s and early 1980s. 60. Dr. Xiong Wang (photograph courtesy Dr. Xiong Wang, signature from a letter to Joseph Arditti). 61. *Cymbidium goeringii* flowering in a small vial (courtesy Dr. Xiong Wang). 62. *Cymbidium* plants produced through micropropagation flowering in pots. These plants also flowered in vitro (courtesy Dr. Xiong Wang). 63. Flower (a), bud (b), and shoot (c) of *Cymbidium ensifolium* in a large vial. 64. Professor Tet Fatt Chia (www.ntu.edu/birc/people.htm).**

(for reviews and photographs see Arditti, 1984, 1990, 1992). This was in 1930. Since then there have been sporadic reports of orchid seedlings which flowered in vitro (for a review see Chia et al., 1999).

The first in vitro flowering by clonally propagated plants was reported by Xiong Wang (Fig. 1-60), an investigator working on micropropagation of *Cymbidum ensifolium* and *Cymbidium goeringii* (Fig. 1-61–1-63) at the Shanghai Institute of

Plant Physiology (Wang, 1984, 1986, 1988*a*, 1988*b*, 1990, no date *a*, no date *b*; Wang et al., 1981, 1988*a*, 1988*b*; Wu et al., 1987). Clonally propagated *Oncidium varicosum* was reported to flower in Brazil at the same time (Kerbauy, 1984*b*). Next to flowers were plantlets of *Phalaenopsis* hybrids in Japan (Tanaka et al., 1988b; Duan and Yazawa, 1994*a*, 1994*b*, 1994*c*, 1995*a*, 1995*b*). *Cymbidium ensifolium*, *Cymbium gyokuchin*, *Cymbidium kanrang*, and *Cymbidium niveo-marginatum* were reported to flower in vitro in Korea (Paek et al., 1990; Kostenyuk et al., 1999). *Doriella* flowered after that, also in Japan (Duan and -Yazawa, 1994*a*, 1994*b*, 1994*c*, 1995*a*, 1995b). *Cymbidium ensifolium* was also reported to flower in vitro. Cytokinins promote the flowering of *Cymbidium ensifolium* var. *misericors* (Chang and Chang, 2003).

Flowering of *Dendrobium* plantlets was induced for the first time at the National University of Singapore by Professor Tet Fatt Chia (Fig. 1-64) and his associates (for a review see Chia et al., 1999). Professor Chia followed this feat with the introduction of a bioluminescence gene into orchids.

A report on flowering of *Dendrobium* in vitro published in 1996 is devoid of worthwhile scientific content and has no useful information about techniques and procedures (Goh, 1996). It is essentially a (self-written) paean and ode to its author (Goh, 1996). Methods used to bring about in vitro flowering of *Dendrobium* Sonia and *Dendrobium* Madame Thong In are not described. The medium used to culture the plantlets is only described as a "translucent chemical" and "unnamed potion" (Anonymous, 1995). Two students who did the actual work are only referred to obliquely without being mentioned by name, let alone credited. Only papers which list the writer (Goh, 1996) as the first author are cited. Access to two theses which describe the work is prohibited. A considerable amount of sleuthing was required to find sources and establish the identities of those who did the actual work (Chan, 1991; Ng, 1997; Sim, no date). This is yet another bizarre episode in the history of orchids in general and orchid micropropagation in particular.

One possible conclusion at present is that flowering of orchids in vitro may be spontaneous or induced by the state of the explant, nutritional conditions, medium components, intangibles, and/or by environmental factors (Chia et al., 1999). However there is also evidence which suggests that cytokinins, and more specifically benzyladenine (BA), N^6-(2-isopentenyl) adenine (2iP), and thidiazuron (TDZ) can induce flowering (see Chang and Chang, 2003, for a short review). Development of a method which can induce flowering of orchids in vitro can prove to be of commercial importance.

The Future

Attempts to predict the future must always be tempered by recalling past predictions that flight in heavier than air aircraft and ventures into space would not be possible. Still, several predictions are possible regarding orchid micropropagation. They must be considered in the context of the prognostications made in the first edition.

The first prediction in the previous edition was that existing tissue culture and clonal propagation methods will be improved. This did happen. Therefore it is safe to predict again that the future holds more and better methods.

Our second prediction in the first edition was that new methods will be developed for genera and species which have not been cultured as yet, either due to difficulties (*Cypripedium* and *Phragmipedium*, for example) or lack of interest (*Habenaria*, for instance). Methods for *Cypripedium* (Jo et al., 2001) and *Habenaria* (Latha, 1999a) were developed. Therefore, it is safe to predict that in the future methods will be developed for other recalcitrant and/or less popular orchids including a procedure for *Phragmipedium*.

Procedures were developed for cell and protoplast cultures of a few orchids. Therefore the third prediction that "it is almost certain that with time cell and protoplast culture methods will be developed for many orchids" has also come to pass, at least partially. This suggests the development of additional and improved methods in the future.

The fourth prediction was assumed to be "safe" – that parasexual (i.e., cell fusion) hybrids of orchids will come into existence and the Eric Young award will be given to a deserving scientist or a group of scientists (in an amount that will probably be much larger than the initial sum due to accumulated interest). This prediction was not safe at all. There are still no parasexual hybrids of orchids and chances are that cell fusion will not play a role in orchid hybridization because as a technique it does not have much if any future.

Another bioengineering procedure which does not seem to have much of a future with orchids is electrophoretic insertion of genes (Griesbach, 1994) despite multiple publications (Griesbach et al., 1989; Griesbach, 1993, 1994; Griesbach and Klein, 1993; Griesbach and Kadzimin, 1994), because: (1) "protocorms had difficulty surviving certain buffer concentrations and were subject to desiccation"; (2) the actual number of transgenic plants which were obtained was not stated; (3) "evidence for genomic integration of *gus*A is lacking"; and (4) transformation was assessed by histochemical GUS staining only (for a review see Kuehnle, 1997).

Insertion of genes through biolistic methods is a much more promising technique and has actually been applied to orchids. Bioengineering is beyond the scope of this book, but it is safe to predict that bioengineered orchids will become a reality. The most notable achievements is this area to date are the insertion of: (1) firefly bioluminescence genes and "*gus*A/*neo* fusion gene portions of pBI426" into orchids by Professor Tet Fatt Chia in Singapore; (2) "the plant expressible NOS-NPT II (for antibiotic resistance) encoded by the gene *neo* and papaya ringspot virus coat protein genes" by Professor Adelheid (Heidi) Kuehnle and her associates at the University of Hawaii; and (3) the *bar* gene which codes for herbicide resistance by H. Anzai and associates in Japan (for a review see Kuehnle, 1997). Additional advances in this area can be expected in the future. Some of these advances will result from work by W. H. Chen and his associates in Taiwan.

"A foregone conclusion," the development of computerized flow systems for orchid tissue culture, has also not gone very far even if robotization, bioreactors, flow systems, computerization, new equipment, and automation are coming into play (Koch, 1974*c*; Kuhn, 1981*a*, 1981*b*; Tisserat and Vandercook, 1985, 1986; Hew et al., 1987; Okamoto, 1996; Paek et al., 2001).

Investigations on low temperature storage of orchid callus (Sivasubramanian et al., 1987) and protocorm-like bodies suggest that in the future orchid germ plasm, seedlings, and tissues will be stored cryogenically.

Isolated orchid petal cells have been used in plant physiology research (Hew and Yip, 1986). This suggests that in time isolated orchid organs, tissues, and cells will be used as model systems for research on plants.

Past bizarre claims, questionable publications, unusual episodes, flamboyant individuals, people with overblown egos, and talented con-men/-women associated with orchid tissue culture suggest that similar eccentricities will be part of the future.

Perhaps the safest prediction for this, the second edition of a book first published in 1993 (Arditti and Ernst, 1993), is that Joseph Arditti (72 as this revision nears completion) will not participate actively if at all in a possible third version because of age or involuntary departure to the big orchid garden in the sky.

Klio, the muse of history, contemplating an orchid. Needless to say, the orchid was added to an ancient statue through computer magic.

CHAPTER TWO

General Outline of Techniques and Procedures

Methods for the in vitro culture of isolated plant cells, tissues, and organs or of seeds are not difficult or complex, but they do require some equipment and certain skills and knowledge. The general outline of these skills as well as the list of methods, media, and apparatus in this chapter in the first (Arditti and Ernst, 1993) and present editions of this book are taken from the appendices in *Orchid Biology, Reviews and Perspectives* Volumes I and II (Arditti, 1977*c*, 1982*a*), *Tissue Culture of Taro* (Arditti and Strauss, 1979), other reviews (Butcher and Ingram, 1976; Pierik, 1987; Vij and Pathak, 1990; Attawar, 1992; Arditti and Ernst, 1993; Prakash and Pierik, 1993; Vij, 1993; Ichihashi, 1997; Prakash et al., 1996; Kishi and Tagaki, 1997*a*, 1977*b*; Tisserat and Jones, 1999; Vij et al., 2000*a*; Bautista, 2002; among others), the general literature, and the World Wide Web.

Media Components

Media used for orchid tissue culture and seed germination may reflect both the special requirements of each species, hybrid, plants(s), and/or seeds used originally and the preferences of the investigators who carried out the initial research. Therefore, when preparing media it is important to follow instructions carefully and strictly as recommended by each procedure and given in every formulation and to measure and weigh all compounds accurately. Every effort should be made to use exactly the substances listed in the recipe tables. To make this possible it is advisable to start preparations by ordering all necessary chemicals and apparatus well in advance. Supply houses (see Appendix 2) issue informative and detailed hard copy catalogs which are free on request, but are usually mailed by bulk and/or surface mail. Therefore requests for catalogs should be made several weeks or months before the anticipated need. Even if a vast amount of information is available at present on the World Wide Web, catalogs can be very useful especially in laboratories where there are only a few computers or if connections are slow and unreliable.

Suppliers usually have well-illustrated web sites (see Appendix 2) and detailed on-line catalogs that simplify ordering and eliminate the wait for mailed information. Shipping can be faster too, but there is a direct relationship between speed and

cost of delivery. Therefore, to reduce costs and prevent disappointment it is still advisable to order well in advance of need.

Chemicals must be stored in accordance with instructions on the package. If there are no instructions, it is best to store organic substances in a refrigerator at 4°C in properly closed containers under dry conditions, or in a freezer. Some chemicals can absorb water from the air and may solidify or become liquefied as a result. The problem can be especially acute in humid climates. To prevent this from happening, such chemicals should be stored under vacuum, in incubators, or in cabinets at a temperature that is high enough to keep humidity very low (but without damaging the compound). Several light bulbs which are constantly on can raise the temperature of such enclosures to a level that is sufficient to reduce the relative humidity. To reduce fire hazard the cabinets should be fireproof. The illumination itself is without effect. Therefore, fluorescent lamps that produce less heat than incandescent bulbs may not be very effective. However, it is important to keep in mind that some chemicals are light-sensitive. When stored in an illuminated cabinet or incubator such chemicals must be placed in a dark container and/or wrapped in aluminum foil, black plastic, paper, or cloth.

Macroelements

The prefixes *macro-* or *major-* when applied to elements (or nutrients) refer to the fact that these substances are needed in relatively large amounts (Box 2-1). They include calcium (Ca), magnesium (Mg), nitrogen (N), phosphorus (P), potassium (K), and sulfur (S). Depending on the medium, several salts may be used to supply each mineral [for example, potassium can be provided as KNO_3, KH_2PO_4, K_2HPO_4, and KCl among others; nitrogen may be added as KNO_3, NH_4NO_3, $Ca(NO_3)_2$, $(NH_4)_2SO_4$, other salts, and/or urea]. The combinations of salts in each medium are designed to provide an appropriate balance of nutrients in proper concentrations.

BOX 2-1. **Periodic table of the elements.**

B, Boron; Ba, Barium; Br, Bromine; C, Carbon; Ca, Calcium; Cl, Chlorine; Co, Cobalt; Cu, Copper; Fe, Iron; H, Hydrogen; I, Iodine; K, Potassium; Mg, Magnesium; Mn, Manganese; Mo, Molybdenum; N, Nitrogen; Na, Sodium; Ni, Nickel; O, Oxygen; P, Phosphorus; S, Sulfur; Se, Selenium; Si, Silicon; V, Vanadium; Zn, Zinc. Aluminum (Al) is added to some media, but rarely.

<+> H																	He
Li	Be											<–> B	<+> C	<+> N	<+> O	F	Ne
<–> Na	<–> Mg											Al	<–> Si	<+> P	<+> S	<–> Cl	Ar
<+> K	<+> Ca	Sc	Ti	<–> V	Cr	<–> Mn	<–> Fe	<–> Co	<–> Ni	<–> Cu	<–> Zn	Ga	Ge	As	<–> Se	<–> Br	Kr
Rb	Sr	Y	Zr	Nb	<+> Mo	Yc	Ru	Rh	Pd	Ag	Cd	In	Sn	Sb	Te	<–> I	Xe
Cs	<–> Ba	La	Hf	Ta	W	Re	Os	Ir	Pl	Au	Hg	Tl	Pb	Bi	Po	At	Rn
Fr	Ra	Ac															

Rare Earth Elements

Ce	Pr	Nd	Pm	Sm	Eu	Gd	Tb	Dy	Ho	Er	Tm	Yb	Lu
Th	Pa	U	Np	Pu	Am	Cm	Bk	Cf	Es	Fm	Md	No	Lr

Plant nutrients
Not plant nutrients
<–> Microelements
<+> Macroelements

Research with callus tissue of *Aranda* Noorah Alsagoff has shown that at pH 4.5 and 5.0, 2.5–3.0 g of tissue took up a total of 5.2 mmol of nitrate in 20 days of incubation: "Ammonium [from 3.8 mmol of $(NH_4)_2SO_4$] in culture media was consumed in cultures at pH value of 5.0 and 5.5" (Lee et al., 1987). At this pH the callus took up ammonia in preference to nitrate. Maximal "depletion of total nitrogen in culture media containing both ammonium and nitrate was observed at pH 5" (Lee et al., 1987).

On sugar-free Vacin and Went medium, seedlings of *Dendrobium* Multico White took up 64.6% of the ammonium (NH_4^+), 15.7% of the nitrate (NO_3^-), and 21.6% of the phosphate (PO_4^{3-}). In the presence of sugar, uptake was 80.2, 22.1, and 33.3%, respectively (Hew and Lim, 1989). The ratio of ammonium to nitrate uptake was 4.15 : 1 and 3.63 : 1, respectively. These plants clearly prefer ammonium.

Substitutions

Experience in several countries over the last 40 years has shown that it is sometimes necessary to make substitutions and modifications in recipes due to shortages or unavailability of chemicals and/or preferences by some orchids. If this becomes necessary, the changes should be made by experienced workers since what may appear to be small and insignificant alterations can in fact introduce major modifications. For example, 100 mg (0.57 mol) of K_2HPO_4 (dibasic potassium phosphate, MW 174.18, 44.89% potassium, 17.79% phosphorus) contains 1.54 as much potassium as an equal weight (0.74 mol) of KH_2PO_4 (monobasic potassium phosphate, MW 136.09, 28.73% potassium, 22.76% phosphorus). Using 50 mg K_2HPO_4 as a substitute for 100 mg of KH_2PO_4 will result in the same amount of potassium but only 0.78 as much phosphorus. One mole of K_2HPO_4 contains twice as much potassium as an equimolar amount of KH_2PO_4, an equal amount of phosphate (PO_4^{2-}) and half as much hydrogen (H^+).

Substituting one salt for another may be even trickier since if, for example, KCl is used to replace one of the potassium phosphate salts, phosphorus will be eliminated entirely and the chloride content may become supraoptimal. In such cases another salt may have to be added to supply phosphorus, but this could introduce an added complication. For instance, if ammonium phosphate [$(NH_4)_2PO_4$ or $(NH)H_2PO_4$] is then used to add phosphorus (as PO_4^{2-}) it will introduce additional nitrogen (as ammonium ion, NH_4^+) which must be taken into consideration, and so on.

Some substitutions can be relatively simple: $MgSO_4$ [magnesium sulfate, usually $MgSO_4 \cdot 7H_2O$, formula weight (FW) 246.47, but sometimes anhydrous $MgSO_4$, FW 120.37] can be replaced by $MgCl_2$ (magnesium chloride, usually $MgCl_2 \cdot 6H_2O$, FW 203.30) since other salts provide a sufficient amount of sulfate (SO_4^{2-}). However, caution is necessary because $MgCl_2$ can increase chloride (Cl^-) to levels which may be toxic for some tissues. Also, waters of hydration must be taken into consideration. Therefore substitutions must be made on a molar, not weight, basis.

Another example is that of iron because $FeCl_3 \cdot 6H_2O$ (ferric chloride, FW 270.30) and $Fe(NO_3)_3 \cdot 9H_2O$ (ferric nitrate, FW 404.00) can be used as replacements for $FeSO_4 \cdot 7H_2O$ (ferrous sulfate, FW 278.02). In this instance only the iron (Fe) concentration must be adjusted since it is the important component (Table 2-1). The levels of sulfate (SO_4^{2-}), chloride (Cl^-), or nitrate (NO_3^-) must also be taken into consideration in this case since they are usually provided by other salts in the medium

TABLE 2-1. **Substitutions of salts in culture media**[a]

Compound	Molecular or formula weight	Number of atoms or ions[b]		Amount added liter^{-1}			
				Original			
		Cation	Anion	Weight	Moles[c]	Substitute[d] weight[e]	Remarks[f]
Aluminum							
Aluminum chloride, $AlCl_3$	133.34	1*	3*	31 µg	0.23 µmol	0.15 µg	Both 31 and 30 µg represent 0.23 µmol and are therefore the same for practical purposes. This salt may be explosive at higher concentrations and should be handled with care
Aluminum chloride, $AlCl_3$	133.34	1*	3	30 µg	0.23 µmol	0.15 µg	
Aluminum sulfate, $Al_2(SO_4)_3{\cdot}16H_2O$	630.39	2*	3	–	–	72.50 µg	
Boron							
Boric acid, H_3BO_3	61.83	3	1*	1.01 mg	0.02 mmol	1.71 mg	
Boric acid, H_3BO_3	61.83	3	1*	10 mg	0.16 mmol	17.1 mg	
Boric acid, H_3BO_3	61.83	3	1*	6.2 mg	0.12 mmol	10.6 mg	
Boric acid, H_3BO_3	61.83	3	1*	1.5 mg	0.03 mmol	2.6 mg	
Boric acid, H_3BO_3	61.83	3	1*	1.01 mg	0.02 mmol	1.72 mg	
Boric acid, H_3BO_3	61.83	3	1*	0.6 mg	0.01 mmol	1.03 mg	
Boric acid, H_3BO_3	61.83	3	1*	56 µg	0.91 µmol	95.81 µg	
Boric acid, H_3BO_3	61.83	3	1*	30 µg	0.47 µmol	51.33 µg	
Boric acid, H_3BO_3	61.83	3	1*	20 µg	0.32 µmol	34.22 µg	
Sodium borate, $Na_2B_4O_7{\cdot}10H_2O$	382.37	2	4*	–	–	–	Use 0.25 mol of this for every mole of boric acid because its molecule contains four atoms of boron
Calcium							
Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	236.15	1*	2*	500 mg			It is preferable not to make substitutions since they may alter the medium. If it is necessary to use calcium nitrate with a different number of waters of hydration; please refer to Table 2.2. Such substitutions can be made because the levels of calcium and nitrate remain the same; waters of hydration make no difference
Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	236.15	1*	2*	400 mg			
Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	236.15	1*	2*	136.7 mg			
Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	236.15	1*	2*	100 mg			
Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	236.15	1*	2*	1 g			
Calcium nitrate, $Ca(NO_3)_2$	164.11	1*	2*	200 mg			
Calcium chloride, $CaCl_2{\cdot}2H_2O$	147.03	1*	1	440 mg			This salt is interchangeable with calcium sulfate provided molarity is the same. To calculate orig. wt × 172.17/147.02
Calcium chloride, $CaCl_2{\cdot}2H_2O$	147.03	1*	1	75 mg			
Monocalcium phosphate, $Ca(H_2PO_4)_2$	234.05	1* + 2	2*	100 mg			May form insoluble di and tri basic calcium phosphate. Calcium chloride can also be substituted for calcium phosphate but high level of chlorine may be toxic. A good substitute would be calcium nitrate provided care is taken to balance the ions in the medium. 488 mg of monocalcium phosphate hydrate are the equivalent to 200 mg dihydrate, anhydrous phosphate. In making susbstitutions with other calcium salts the concentrations should be equimolar in respect to Ca^{2+}
Tricalcium phosphate, $Ca_3(PO_4)_2$	310.20	3*	2*	200 mg			
Dicalcium phosphate, $CaHPO_4$	136.06	1*	1*	200 mg			
Dicalcium phosphate, $CaHPO_4{\cdot}2H_2O$	172.09	1*	1*				
Calcium sulfate, $CaSO_4$ (gypsum)	136.14	1*	1				These salts are sparingly soluble in water (2–3 g l^{-1}) and may not be very useful except in emergency situations
Calcium sulfate, dihydrate, $CaSO_4{\cdot}2H_2O$	172.17	1*	1				
Cobalt							
Cobaltous nitrate, hexahydrate, $Co(NO_3)_2{\cdot}6H_2O$	291.04	1*	2				78.58 µg = 0.27 µmol; 34.93 µg = 0.12 µmol; 32.01 µg = 0.11 µmol. Use 70 µg of cobaltous chloride to substitute for the cobaltous nitrate. 45 µg of the cobaltous chloride can be used as a substitute for 30 µg of the cobaltous chloride; 20 µg will replace 25 µg of the latter. For substitutions due to waters of hydration see the appropriate table
Cobaltous nitrate, $Co(NO_3)_2$	183.03	1*	2	50 µg	0.27 µmol		
Cobaltous chloride, $CoCl_2{\cdot}6H_2O$	237.93	1*	2	30 µg	0.12 µmol		
Cobaltous chloride, $CoCl_2{\cdot}6H_2O$	237.93	1*	2	25 µg	0.11 µmol		

Copper							
Cupric sulfate, $CuSO_4 \cdot 5H_2O$	249.68	1*	1	25 µg	0.10 µmol	13.46 µg	
Cupric sulfate, $CuSO_4 \cdot 5H_2O$	249.68	1*	1	19 µg	0.08 µmol	10.23 µg	
Cupric sulfate, $CuSO_4 \cdot 5H_2O$	249.68	1*	1	1 µg	0.004 µmol	0.54 µg	
Cupric sulfate, $CuSO_4 \cdot H_2O$	177.62	1*	1	50 µg	0.28 µmol	37.86 µg	
Cupric sulfate, $CuSO_4 \cdot H_2O$	177.62	1*	1	40 µg	0.23 µmol	30.28 µg	
Cupric sulfate, $CuSO_4 \cdot H_2O$	177.62	1*	1	30 µg	0.13 µmol	22.71 µg	
Cupric sulfate, anhydrous, $CuSO_4$	159.60	1*	1		0.08–0.28 µmol		As a substitute
Cupric chloride, $CuCl_2 \cdot 2H_2O$	170.49	1*	1	54 µg	0.32 µmol	100 µg $CuSO_4 \cdot 5H_2O$	
Cupric chloride, $CuCl_2 \cdot 2H_2O$	170.49	1*	1	10 µg	0.06 µmol	8.57 µg $CuSO_4 \cdot 5H_2O$	
Iodine							
Potassium iodide, KI	166.01	1	1*	0.75 mg	4.51 µmol	1.15 mg	
Potassium iodide, KI	166.01	1	1*	0.83 mg	5.0 µmol	1.27 mg	
Potassium iodide, KI	166.01	1	1*	99 µg	0.6 µmol	151.36 µg	
Potassium iodide, KI	166.01	1	1*	30 µg	0.18 µmol	45.87 µg	
Potassium iodide, KI	166.01	1	1*	20 µg	0.13 µmol	30.58 µg	
Potassium iodide, KI	166.01	1	1*	10 µg	0.06 µmol	15.29 µg	
Sodium iodide, NaI	149.89	1	1*				The amount used should be equimolar with potassium iodide. Because the concentrations being added are tiny, the iodine requirements, if any, are minute and the weight differences are very small the amount of NaI added to a solution can be equivalent to that of KI. The weight can be calculated as follows: orig. wt. × 149.89/166.01
Iodine	253.81	1*					Calculate as: orig. wt. × 253.81/166.01. Pharmaceutical tincture of iodine can also be used. The volume used would depend on the concentration of the available preparation
Iron							
Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	278.02	1*	1	27.9 mg	0.1 mmol		These should be used with 37.3 mg of Na_2EDTA. It is preferable not to replace them with the organic iron salts in this list. $FeCl_3 \cdot 6H_2O$ can be used as a substitute at the level of 27.03 mg. See text for details
Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	278.02	1*	1	27.8 mg	0.1 mmol		
Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	278.02	1*	1	25 mg	0.9 mmol		
Ferric chloride, $FeCl_3 \cdot 6H_2O$	270.30	1*	3	1 mg	3.7 µmol	1.03 mg	Add Na_2EDTA as above
Ferric chloride, $FeCl_3 \cdot 6H_2O$	270.30	1*	3	0.5 mg	1.85 µmol	0.51 mg	
Ferric citrate, $Fe(C_6H_5O_7)_x \cdot 5H_2O$		_*	–	10 mg			These compounds are hard to find and/or not very good as sources of iron as a mixture of 27.8 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$ and 37.3 Na_2EDTA per liter of medium
Ferric citrate, $Fe(C_6H_5O_7)_x \cdot 3H_2O$		_*		5.4 mg			
Ferric sulfate, $Fe_2(SO_4)_3$	399.80	2*	3	1 mg	2.50 µmol		See text for additional details. Or, they can be replaced with approximately, 0.5, 1, 15, and 13 mg respectively of $FeCl_3 \cdot 6H_2O$. The exact ratios of iron and citrate or tartrate are not always given
Ferric tartrate, $Fe_2(C_4H_4O_6)_x \cdot H_2O$	573.94	_*	X	30 mg			
Ferric tartrate, $Fe_2(C_4H_4O_6)_3$	573.94	2*	3	28 mg			
Ferric ammonium sulfate, $(NH_4)_2Fe(SO_4)_2 \cdot 12H_2O$	482.19	1 + 1*	2				The amount used must be equimolar in respect to iron with the salt being replaced. Can be used to substitute for all salts in the iron list. It is preferable to chelate them through the addition of equimolar amounts of EDTA (0.1 mmol or 37.2 mg). See text for details
Ferric ammonium sulfate, $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$	392.14	2 + 1*	2	39.2 mg	0.1 mmol		
Ferrous chloride, $FeCl_2 \cdot 4H_2O$	198.81	1*	2	19.88 mg	0.1 mmol		
Ferric nitrate, $Fe(NO_3)_3 \cdot 9H_2O$	404.00	1*	3	40.40 mg	0.1 mmol		

TABLE 2-1. (***Continued***)

Compound	Molecular or formula weight	Number of atoms or ions[b]		Amount added liter^{-1}			Remarks[f]
		Cation	Anion	Original Weight	Original Moles[c]	Substitute[d] weight[e]	
Magnesium							
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	246.48	1*	1	400 mg	1.62 mmol	329.94 mg	
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	246.48	1*	1	370 mg	1.50 mmol	305.20 mg	
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	246.48	1*	1	250 mg	1.01 mmol	206.21 mg	
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	246.48	1*	1	240 mg	0.97 mmol	197.97 mg	
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	246.48	1*	1	125 mg	0.50 mmol	103.11 mg	
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	246.48	1*	1	120 mg	0.49 mmol	98.98 mg	
Magnesium chloride, $MgCl_2 \cdot 6H_2O$	203.31	1*	2	–	1 mmol	203.31 mg	
Magnesium nitrate, $MgNO_3 \cdot 6H_2O$	256.41	1*	1	–	1 mmol	256.41 mg	
Manganese							
Manganous sulfate, $MnSO_4 \cdot 4H_2O$	223.06	1*	1	25 mg	0.11 mmol	18.9 mg	These salts can be interchanged, but molarities must remain constant
Manganous sulfate, $MnSO_4 \cdot 4H_2O$	223.06	1*	1	22.3 mg	0.1 mmol	16.9 mg	
Manganous sulfate, $MnSO_4 \cdot 4H_2O$	223.06	1*	1	7.5 mg	33 µmol	5.7 mg	
Manganous sulfate, $MnSO_4 \cdot 4H_2O$	223.06	1*	1	68 µg	0.31 µmol	51.5 µg	
Manganous sulfate, $MnSO_4 \cdot 4H_2O$	223.06	1*	1	10 µg	0.05 µmol	7.6 µg	
Manganous sulfate, $MnSO_4 \cdot 2H_2O$	187.02	1*	1	5.7 mg	30.5 µmol	5.2 mg	
Manganous sulfate, $MnSO_4$	151	1*	1	4.5 mg	29.8 µmol	5.0 mg	
Manganous chloride, $MnCl_2 \cdot 4H_2O$	197.91	1*	2	2 mg	10.1 µmol	1.7 mg	
Manganous chloride, $MnCl_2 \cdot 4H_2O$	197.91	1*	2	1 mg	5.05 µmol	0.9 mg	
Manganous chloride, $MnCl_2 \cdot 4H_2O$	197.91	1*	2	0.4 mg	2.0 µmol	0.3 mg	
Manganous chloride, $MnCl_2 \cdot 4H_2O$	197.91	1*	2	36 µg	0.18 µmol	30.76 mg	
Manganous sulfate, $MnSO_4 \cdot H_2O$	169.02	1*	1	–	10.0 µmol	1.7 mg	
Manganous nitrate, $Mn(NO_3)_2 \cdot 4H_2O$	251.01	1*	2	–	10.0 µmol	2.5 mg	
Molybdenum							
Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	241.98	2	1*	250 µg	1.03 µmol	1.3 mg	
Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	241.95	2	1*	50 µg	0.21 µmol	255.4 µg	
Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	241.95	2	1*	25 µg	0.10 µmol	127.7 µg	
Ammonium molybdate, $(NH_4)_2 \cdot Mo_7O_{24} \cdot 4H_2O$	1235.95	6	7* (MoO_4^{2-})	20 µg	0.016 µmol	3.9 µg	
Molybdenum trioxide, MoO_3	143.94	1*	1* (MoO_4^{2-})	16 µg	0.11 µmol	137.4 µg	
Molybdenum trioxide, MoO_3	143.94	1*	3		0.22 µmol	32 µg	
Molybdenum trioxide, MoO_3	143.94	1*	3		0.015 µmol	2.1 µg	
Molybdenum trioxide, MoO_3	143.94	1*	3		1 µmol	144 µg	
Molybdic acid, $H_2MoO_4 \cdot H_2O$	179.98	1*					Toxic, irritant
Molybdenum oxide, MoO_3	143.94	1*					
Nickel							
The two chloride compounds listed below as possible substitutes are irritants and suspected carcinogens. Sine there is no firm evidence that the addition of nickel salts is necessary for orchid cultures it may be wise to omit them altogether. If the addition proves necessary caution should be excercised when handling these or similar salts							
Nickel chloride, $NiCl_2$	129.65	1*	2	17 µg	0.13 µmol	38.1 µg	
Nickel chloride, $NiCl_2 \cdot 6H_2O$	237.71	1*	2	30 µg	0.13 µmol	36.7 µg	
Nickel nitrate, $Ni(NO_3)_2 \cdot 6H_2O$	290.81	1*	2		0.13 µmol	37.8 µg	
Nickel sulfate, $NiSO_4 \cdot 6H_2O$	262.86	1*	1		0.13 µmol	34.2 µg	

Nitrogen							Substitutions are not advisable and usually not necessary
Ammonium[g]	Commonly used nitrogen salts include potassium nitrate, calcium nitrate, and ammonium sulfate. Salts potentially useful as substitutes include ammonium phosphate and ammonium nitrate. Other salts like sodium, magnesium, and nitratre may introduce toxic levels of Na^+ and Mg^+						
Nitrate[g]							
Zinc							
Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	287.54	1*	1	10 mg	34.8 µmol	10.35 mg	These salts can be interchanged, but molarities must remain constant
Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	287.54	1*	1	9 mg	31.3 µmol	9.31 mg	
Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	287.54	1*	1	7 mg	24.3 µmol	7.24 mg	
Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	287.54	1*	1	1 mg	0.35 µmol	1.04 mg	
Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	287.54	1*	1	0.565 mg	0.2 µmol	0.55 mg	
Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	287.54	1*	1	0.331 mg	0.1 µmol	0.34 mg	
Zinc sulfate, $ZnSO_4 \cdot H_2O$	179.48	1*	1	50 µg	0.3 µmol	82.87 µg	
Zinc sulfate, $ZnSO_4 \cdot H_2O$	179.48	1*	1	30 µg	0.2 µmol	49.72 µg	
Zinc sulfate, $ZnSO_4 \cdot H_2O$	179.48	1*	1	20 µg	0.1 µmol	33.15 µg	
Zinc sulfate, $ZnSO_4$	161.47	1*	1	1.5 mg	0.93 µmol	2.76 mg	
Zinc chloride, $ZnCl_2$	136.28	1*	2	3.93 mg	28.84 µmol	8.58 mg	
Zinc chloride, $ZnCl_2$	136.28	1*	2	0.152 mg	1.1 µmol	0.33 mg	
Zinc nitrate, $Zn(NO_3)_2 \cdot 6H_2O$	297.47	1*	2	–	0.1 µmol	29.75 µg	

[a]Compounds which are used in published media are preferable. In some cases these compounds can be substituted for each other, but concentrations may have to be recalculated. Substitutes are usually provided for the first compound and concentration in each list, but in some instances for additional compounds and concentrations. Sample calculation: in aluminum, for example, the substitute is aluminum sulfate, $Al_2(SO_4)_3 \cdot 16H_2O$, MW 630.39, and the amount and calculation for the substitution are 0.147 mg and 1/2[(31 × 630.39)/133.34], respectively. Substitutions are generally not advisable, but there are instances in which they may be unavoidable. This table was prepared for such eventualities. In cases where substitutions have been made the modified media should be tested with a standard clone before being used for rare and/or expensive ones. Some of the chemicals listed here may be toxic, explosive (aluminum chloride), or otherwise dangerous. For this reason chemicals must be used with care and in accordance with instructions on the label. The authors and the publisher assume no responsibility for any damages, injury, or losses which may result from the use of chemicals. This responsibility rests entirely and solely with the user. See text for more details. This table is more detailed than strictly necessary to facilitate its use for instructional purposes and to clarify the approach taken to prepare it. Well-equipped laboratories have most of the necessary chemicals and the need to make substitutions may arise seldom or never. However chemicals may be lacking in smaller or somewhat isolated laboratories and substitutions could become necessary. The auther has had such experiences during travels and they led to this table. Further, the information in this table may be useful for those with more limited laboratory experience.

[b]Atoms of the relevant elements (i.e., usually those which head each list: Al, aluminum; B, boron; Ca, calcium; Co, cobalt; Cu, copper; I, iodine; Fe, iron; K, potassium; Mg, magnesium; Mn, manganese; Mo, molybdenum; Ni, nickel; and Zn, zinc) are marked with* (see text for details).

[c]Abbreviations: *M*, molar; mol, mole; mmol, millimole (1/1000th of a mole); m*M*, millimolar; µmol, micromole (1/1000 of a millimole and 1/1,000,000 of a mole); µ*M*, micromolar (see text for details). The terms *M*, m*M*, and µ*M* are not interchangeable with mole, mmole, and µmole.

[d]All substitutions are for the main elements (iron, Fe; magnesium, Mg; potassium, K; and zinc, Zn). Substitutions must always be on an equimolar basis because the molecular weights of chemicals vary. The same weight of two chemicals will not necessarily provide equivalent amounts of the the main element. For example, 250 mg of $MgSO_4 \cdot 7H_2O$ will provide 1.01 mmol (250/MW = 246.48) whereas 250 mg of $MgCl_2 \cdot 6H_2O$ is equivalent to 1.23 mmol (250/MW = 203.31). The difference is 0.22 mmol or one-fifth of the original amount.

[e]All calculations are for substitutions of the relevant elements (see footnote *b* above). The formula used for the calculations is: [(original amount × molecular or formula weight of the substitute/molecular or formula weight of the original substance)/number of atoms of relevant element per molecule]. Figures taken from other sources are as listed originally. With very few exceptions figures obtained through calculations have been rounded off to two decimal places. See text for more details.

[f]Relevant element (see footnote *b* above). Some of the salts listed here may not be available commercially and are used as examples (see text for details).

[g]Ammonium and nitrate are used in several forms and often as part of compounds where more than one of the ions in the molecule are necessary (KNO_3, for example) and/or provided by other substances [for instance, a medium may contain KNO_3, KH_2PO_4, and $(NH_4)_2SO_4$] in a delicate balance. For example, replacing ammonium sulfate with ammonium phosphate will eliminate sulfur from the medium and increase phosphorus levels. Using calcium sulfate instead of ammonium sulfate will retain the sulfur, but eliminate ammonium. Replacing calcium nitrate with potassium nitrate will eliminate calcium and increase the potassium concentration. Doing the reverse may raise calcium and reduce or eliminate potassium. Utilizing calcium chloride in place of calcium nitrate may not change calcium levels but it will remove the nitrate. Partial substitutions will change the concentrations, and balance of elements. Therefore, it is not advisable to make substitutions of major elements without careful considerations and calculations; none are listed here.

and their concentrations can change. However, differences will be minor. Still, caution is needed to prevent supraoptimal levels of any one element and to take waters of hydration into consideration. Again, substitutions must be made on a molar, not weight, basis.

Substitutions between salts that contain a different number of waters of hydration ($\cdot xH_2O$) are not complicated because adjustments must be made only for the salt itself. For example, 250 mg of $MgSO_4 \cdot 7H_2O$ contain as much magnesium and sulfate as 122.09 mg of $MgSO_4$ (see Table 2-2). The difference in the amount of water in the molecule is not important. This is true for a number of salts that are used in culture media (Table 2-2). A formula to use for calculating substitution involving the same salt with different waters of hydration is:

$$\frac{OW \times FWS}{FWO} = SW \qquad (I)$$

where:

FWO = formula weight [molecular weight of the salt plus the weight of the water(s) of hydration] of the original substance;
FWS = formula weight of the substitute;
OW = weight [in grams (g), milligrams (mg), or micrograms (μg)] of the original substance;
SW = weight of the substitute substance to use (in g, mg, or μg; the units must be the same as those of OW).

If $MgSO_4$ is to be used in place of 250 mg of $MgSO_4 \cdot 7H_2O$ (Table 2-2) this expression becomes:

$$\frac{250 \text{ mg} \times 120.37}{246.47} = 122.09 \text{ mg} \qquad (II)$$

Substitutions and replacements of salts should never be made on an equal weight or percentage basis, but only on the premise of equivalent molarities. For example, 27.8 mg (0.14 mol) of ferrous chloride ($FeCl_2 \cdot 4H_2O$, MW 198.81) is not an appropriate substitute for the same weight (0.1 mol) of ferrous sulfate ($FeSO_4 \cdot 7H_2O$, MW 278.02) since the molecular (and formula) weights of these salts are different. To provide the same amount of iron (Fe) the substitution must be based on molarity [number or fractions of moles, millimoles, or micromoles (weight in g, mg, or μg equivalent to or a fraction of the molecular weight)]. An equal weight of chloride salt (27.8 mg) will provide 40% more iron than the sulfate. The chloride ($2Cl^-$ per molecule), sulfate ($1SO_4^-$ per molecule), percentage of iron in the salt (28.09% in the chloride and 20.09% in the sulfate), and the molecules of water (4 waters of hydration per molecule in the ferrous chloride and 7 in the ferrous sulfate) are irrelevant in this instance. A simple formula for calculation of equivalent molarities is:

$$\frac{OW \times MWS}{MWO} = SW \qquad (III)$$

where:

MWO = molecular weight of the original substance;

TABLE 2-2. **Use and substitution of chemicals whose molecules contain waters of hydration**

Compound	Formula	MW or FW[a]	Weight of waters of hydration	Weight of compound	Amount of original compound to add			Amount of substitute to add[c]	
					Weight	Fraction of MW or FW[a]	Molarity[b]	Molarity[b]	Weight
Calcium chloride, dihydrate	$CaCl_2 \cdot 2H_2O$	147.02	36.03	110.99	440 mg	2.99	2.99 mmol	–	–
Calcium chloride, anhydrous	$CaCl_2$	110.99	0	110.90	–	–	–	2.99 mmol	329.21 mg
Calcium nitrate, quadrihydrate	$Ca(NO_3)_2 \cdot 4H_2O$	236.15	72.06	164.09	1 g	0.00424	4.24 mmol	–	–
Calcium nitrate, anhydrous	$Ca(NO_3)_2$	164.09	0	164.09	–	–	–	4.24 mmol	694.86 mg
Cobaltous chloride, hexahydrate	$CoCl_2 \cdot 6H_2O$	237.93	108.09	129.84	30 µg	0.12	0.13 µmol	–	–
Cobaltous chloride, anhydrous	$CoCl_2$	129.84	0	129.84	–	–	–	0.13 µmol	16.37 µg
Cobaltous chloride, hexahydrate	$CoCl_2 \cdot 6H_2O$	237.93	108.09	129.84	25 µg	0.11	0.11 µmol	–	–
Cobaltous chloride, anhydrous	$CoCl_2$	129.84	0	129.84	–	–	–	0.11 µmol	13.64 µg
Cobaltous nitrate, anhydrous	$Co(NO_3)_2$	183.03	0	182.94	50 µg	0.27	0.27 µmol	–	–
Cobaltous nitrate, hexahydrate	$Co(NO_3)_2 \cdot 6H_2O$	291.03	108.09	182.94	–	–	–	0.27 µmol	79.54 µg
Cupric chloride, dihydrate	$CuCl_2 \cdot 2H_2O$	170.48	36.03	134.45	50 µg	0.29	0.29 µmol	–	–
Cupric chloride, anhydrous	$CuCl_2$	134.45	0	134.45	– –	–	0.29 µmol	39.43 µg	
Cupric chloride, dihydrate	$CuCl_2 \cdot 2H_2O$	170.48	36.03	134.45	10 µg	0.06	0.06 µmol	–	–
Cupric chloride, anhydrous	$CuCl_2$	134.45	0	134.45	–	–	–	0.06 µmol	7.89
Cupric sulfate, pentahydrate	$CuSO_4 \cdot 5H_2O$	249.68	90.08	159.61	0.026 mg	0.10013	0.1 µmol	–	–
Cupric sulfate, anhydrous	$CuSO_4$	159.61	0	159.61	–	–	–	0.1 µmol	0.017 mg
Ferric chloride, hexahydrate	$FeCl_3 \cdot 6H_2O$	270.30	108.09	162.21	1 g	0.0037	3.7 µmol	–	–
Ferric chloride, anhydrous	$FeCl_3$	162.22	0	162.21	–	–	–	3.7 µmol	0.6 mg
Ferric chloride, hexahydrate	$FeCl_3 \cdot 6H_2O$	270.30	108.09	162.21	0.5 mg	0.00185	1.85 µmol	–	–
Ferric chloride, anhydrous	$FeCl_3$	162.22	0	162.21	–	–	–	1.85 µmol	0.3 mg
Magnesium sulfate, heptahydrate	$MgSO_4 \cdot 7H_2O$	246.47	126.11	120.37	250 mg	1.01	1.01 µmol	–	–
Magnesium sulfate, hexahydrate	$MgSO_4 \cdot 6H_2O$[d]	228.46	108.09	120.37	–	–	–	1.01 mmol	252.01 mg
Magnesium sulfate, pentahydrate	$MgSO_4 \cdot 5H_2O$[d]	210.44	90.07	120.37	–	–	–	1.01 mmol	213.45 mg
Magnesium sulfate, quadrihydrate	$MgSO_4 \cdot 4H_2O$[d]	192.42	72.05	120.37	–	–	–	1.01 mmol	195.18 mg
Magnesium sulfate, trihydrate	$MgSO_4 \cdot 3H_2O$[d]	174.41	54.03	120.37	–	–	–	1.01 mmol	176.91 mg
Magnesium sulfate, dihydrate	$MgSO_4 \cdot 2H_2O$[d]	156.39	36.01	120.37	–	–	–	1.01 mmol	158.63 mg
Magnesium sulfate, monohydrate	$MgSO4 \cdot H_2O$[d]	138.38	18.02	120.37	–	–	–	1.01 mmol	140.36 mg
Magnesium sulfate, anhydrous	$MgSO_4$	120.37	0	120.37	–	–	–	1.01 mmol	122.09 mg
Manganese sulfate, quadrihydrate	$MnSO_4 \cdot 4H_2O$	223.06	72.06	150.99	7.5 mg	0.034	33.62 µmol	–	–
Manganese sulfate, monohydrate	$MnSO_4 \cdot H_2O$	169.01	18.02	150.99	–	–	–	33.62 µmol	5.68 mg
Manganese sulfate, anhydrous	$MnSO_4$	151	0	150.99	–	–	–	33.62 µmol	5.08 mg
Sodium molybdate, dihydrate	$Na_2MoO_4 \cdot 2H_2O$	241.95	36.03	205.92	0.25 mg	0.001	1 µmol	–	–
Sodium molybdate, anhydrous	Na_2MoO_4	205.96	0	205.92	–	–	–	1 µmol	0.206 mg
Sodium molybdate, dihydrate	$Na_2MoO_4 \cdot 2H_2O$	241.95	36.03	205.92	50 µg	0.207	0.21 µmol	–	–
Sodium molybdate, anhydrous	Na_2MoO_4	205.92	0	205.92	–	–	–	0.21 µmol	42.554 µg
Sodium molybdate, dihydrate	$Na_2MoO_4 \cdot 2H_2O$	241.95	36.03	205.92	25 µg	0.103	0.10 µmol	–	–
Sodium molybdate, anhydrous	Na_2MoO_4	205.92	0	205.92	–	–	–	0.13 µmol	21.277 µg
Nickel chloride, anhydrous	$NiCl_2$	129.61	0	129.61	17 µg	0.131	0.13 µmol	–	–
Nickel chloride, hexahydrate	$NiCl_2 \cdot 6H_2O$	237.71	36.03	201.68	–	–	–	0.13 µmol	31.187
Nickel chloride, hexahydrate	$NiCl_2 \cdot 6H_2O$	237.71	36.03	201.68	30 µg	0.13	0.13 µmol	–	–
Nickel chloride, anhydrous	$NiCl_2$	129.61	0	201.68	–	–	–	0.13 µmol	16.357
Zinc sulfate, heptahydrate	$ZnSO_4 \cdot 7H_2O$	287.54	126.11	161.43	8.6 mg	0.02991	29.91 µmol	–	–
Zinc sulfate, quadrihydrate	$ZnSO_4 \cdot 4H_2O$	233.5	72.06	161.43	–	–	–	29.91 µmol	6.97 mg
Zinc sulfate, anhydrous	$ZnSO_4$	161.43	0	161.43	–	–	–	29.91 µmol	4.83 mg

[a]FW, formula weight; MW, molecular weight. This table is more detailed than strictly necessary to facilitate its use for instructional purposes and to clarify the approach taken to prepare it. Well-equipped laboratories have most of the necessary chemicals, and the need to make substitutions may arise seldom or never. However, chemicals may be lacking in smaller or somewhat isolated laboratories and substitutions could become necessary.

[b]One mole (abbreviated mol) is the weight in grams equal to the molecular weight of a compound; common fractional parts of the mole are the millimole (mmol; 1000 mmol = 1 mol) and the micromole (µmol; 1000 µmol = 1 mmol; 1,000,000 µmol = 1 mol).

[c]Calculation: Original amount to be added (in g or mg) × Molecular or formula weight of substitute/Molecular or formula weight of original compound. Figures taken from other sources are as listed as given originally. With very few exceptions figures obtained through calculations have been rounded off to two decimal places. Always make substitutions on an equimolar basis because the molecular weights of chemicals vary; the same weight of two chemicals will not necessarily provide equivalent amounts of the the main element. For example, 250 mg $MgSO_4 \cdot 7H_2O$ will provide 1.01 mmol (250/MW = 246.48) whereas 250 mg of $MgCl_2 \cdot 6H_2O$ is equivalent to 1.23 mmol (250/MW = 203.31). The difference is 0.22 mmol or one-fifth the original amount.

[d]This compound either does not exist or is not commonly available but is presented here as an example.

MWS = molecular weight of the substitute;
OW = weight (in g, mg, or μg) of the original substance;
SW = weight of the substitute (in g, mg, or μg; the units must be the same as those of OW).

In the example above this expressions becomes:

$$\frac{27.8 \text{ mg} \times 278.02}{246.47} = 122.09 \text{ mg} \qquad \text{(IV)}$$

This formula (III) was used to calculate a number of possible substitutions (Table 2-1).

Storage

Stock solution of macroelements (except those containing nitrogen) can be stored at room temperature (usually about 22°C), but higher or lower temperatures will have no deleterious effects. Nitrogen-containing stock solutions can become contaminated even in a refrigerator and should be stored in a freezer. Frozen stock solution can be thawed under elevated temperatures to accelerate the process. Stock solutions should be stored in containers that are capped tightly to prevent evaporation of the water because this will increase their concentrations. All macroelement salts can be sterilized by autoclaving. And, in conclusion, it is important to repeat the warning that macroelement salts should be substituted only if strictly necessary and with great care. Because of its importance this information will be repeated elsewhere in the book.

Microelements

Culture media vary widely in the use and content of micro- or minor elements or nutrients (the terms are based not on their importance, but on the small amounts which are required; Box 2-1). The reasons for this are: (1) utilization of existing formulations; (2) imprecise information regarding the requirements of orchids; and (3) the presence of many of these elements as impurities in other media components. Their concentrations (within reasonable limits) and even presence or absence (in some instances) do not seem to be critical since shoot tips and other explants from one and the same genus can be cultured on media with different levels and formulations of microelements. Substitutions and changes in microelements are simpler to make because their concentrations are lower and therefore differences in the non-relevant part of the molecule (usually, but not always, sulfate or chloride) are not important (Table 2-1). Formulae I and III for calculating macroelement substitutions can also be used for microelements. However, it is very important to keep in mind that many microelements can be toxic at higher levels [the difference between 1 and 10 mg may appear small (9 mg), but the increase is tenfold (i.e., an order of magnitude)]. Such a large increase can result in toxicity. Microelement stock solution can be stored and thawed like those of macroelements. Also, like macroelements, all microelement salts are heat-stable and can be autoclaved.

Iron

Until the advent of chelating agents, the incorporation of an available form of iron in culture media presented a problem. Many iron salts are not sufficiently soluble. Others are soluble initially, but in solution the iron is oxidized to an insoluble or sparingly soluble form. Ferric chloride ($FeCl_3$), ferrous sulfate ($FeSO_4$), and a number of other salts as well as the citrate and tartrate of iron were all used in culture media at one time or another. When ethylene diamine tetraacetic acid [free acid (EDTA, MW 292.25), disodium salt (Na_2EDTA, MW 336.02) and disodium dihydrate ($Na_2EDTA{\cdot}2H_2O$), MW 272.24] became available as chelating agents (sometimes under a trade name such as Sequestrene) they found widespread use in culture media including, of course, the well-known Murashige–Skoog (MS) medium (Murashige and Skoog, 1962). The amounts used in MS are 37.3 mg Na_2EDTA l^{-1} and 27.8 mg $FeSO_4{\cdot}7H_2O$ l^{-1}. These amounts were widely assumed to be equimolar until careful recalculations showed that this is not the case (Singh and Krikorian, 1980). The actual concentrations turn out to be 100 μmol l^{-1} (100-μ*M* solution) of the iron salt and 111 μmol l^{-1} (111-μ*M* solution) of the chelating agent. This leaves an excess of 11 μmol of chelating agent per liter of medium.

What the effects of the excess EDTA may be is not clear, but it is possible that "this excess will affect availability of other divalent micronutrient cations such as copper, zinc, manganese, etc. depending on the stability of their chelate with EDTA. The effect of the excess EDTA remains to be elucidated . . ." (Singh and Krikorian, 1980). The concentrations of microelements given in the original MS paper (Murashige and Skoog, 1962) are still used in that medium and several other media. This combination is effective regardless of the EDTA concentration used to chelate the iron and there does not seem to be a compelling reason to make changes. A number of other microelement formulations are used in several media. All seem to be unaffected by the EDTA concentration.

Auxins and Anti-auxin

The most commonly used auxins in orchid tissue culture media (Table 2-3) are the naturally occurring auxin, indoleacetic acid (IAA), and the synthetics naphthaleneacetic acid (NAA), indolebutyric acid (IBA), and 2,4-dichlorophenoxyacetic acid (2,4-D). Other auxins and occasionally auxin–amino acid conjugates are also used in some media. Both the auxin(s) and the concentration(s) being used are usually a result of trial and error and it is best not to make changes and substitutions without careful thought and good reasons. When changes are made it is necessary to keep in mind that auxins may differ from each other both qualitatively and quantitatively. This means that the effects of one auxin on a certain species may be different from those of other auxins, and may differ with the orchid. The effects of different concentrations of the same auxin may differ in respect to one species and may not be the same with another orchid. As a rule, synthetic auxins are generally more stable and remain active longer than the naturally occurring substances. Auxins should never be substituted and their concentrations should never be changed without prior tests.

TABLE 2-3. **Some hormones, antihormones, and hormone inhibitors used in orchid micropropagation media**[a]

Hormone	Molecular weight	Concentration[b]	
		Weight (mg l^{-1})	Molarity (µmol l^{-1})
Anti-auxin			
trans-cinnamic acid	148.20	1.5	0.01
		15	0.10
		150	1.01
Auxins			
2,4-Dichlorophenoxyacetic acid (2,4-D)	221.04	1	4.52
		2	9.04
		5	22.60
		10	45.24
Indoleacetic acid (IAA)	175.19	1	5.71
		2	11.42
		5	28.54
		10	57.08
		25	142.70
		50	285.40
		100	570.81
Indolebutyric acid (IBA)	203.24	1	4.92
		2	9.83
		5	24.60
		10	49.20
		25	123.01
		50	246.02
		100	492.03
Naphthaleneacetic acid (NAA)[b]	186.21	1	5.37
		2	10.74
		5	26.85
		10	53.70
		25	134.26
		50	268.51
		100	537.03
Cytokinins			
Benzyladenine (benzylaminopurine, BA)	225.6	1	4.44
		2	8.88
		5	22.20
		10	44.39
		25	110.98
		50	221.97
		100	441.93
		250[c]	1109.83
		500[c]	2219.66
		1000[c]	4439.32
6-Dimethylaminopurine (DMAP)	163.18	1	6.13
		2	12.26
		5	30.64
		10	61.28
		25	153.21
		50	306.41
		100	612.82
Kinetin (6-furfurylaminopurine)	215.21	1	4.65
		2	9.29
		5	23.23
		10	46.47
		25	116.17
		50	232.33
		100	464.66
N^6-(2-isopentenyl adenosine), hemihydrate	344.48	1	2.90
		5	14.52
		10	29.04
		25	72.59
		50	145.19
		100	290.38

TABLE 2-3. (***Continued***)

Hormone	Molecular weight	Concentration[b]	
		Weight (mg l^{-1})	Molarity (µmol l^{-1})
Thidiazuron (*N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea, TDZ)	220.25	1	4.54
		2	9.08
		5	22.70
		10	45.40
		25	113.51
		50	227.02
		100	454.03
Zeatin (4-hydroxy-3-methyl-*trans*-2-butenylaminopurine)	219.25	1	4.56
		5	22.81
		10	45.61
		25	114.03
		50	228.05
		100	456.10
Ethylene as ethephon (2-chloroethylphosphonic acid)	144.50	1	6.92
		2	13.84
		5	34.60
		10	69.20
Ethylene inhibitor (silver thiosulfate, STS)	242.2	1	4.12
		2	8.23
		5	20.58
		10	41.16
Gibberellin			
Gibberellic acid (GA_3)	346.38	1	2.89
		2	5.77
		5	14.44
		10	28.89
		25	72.78
		50	144.35
		100	288.70
GA synthesis inhibitor (antigibberellin)			
Ancymidol [α-cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidinemethanol]	256.30	1	3.90
		2	7.80
		5	19.51
		10	39.01
		25	97.54
		50	195.08
		100	390.17

[a]Not all concentrations listed here are in general use and some levels which may be employed in certain media are not included in this table. Hormone concentrations can be expressed in milligrams per liter (mg l^{-1} or the less frequently used mg/l), parts per million (ppm), rarely parts per billion (ppb), millimoles per liter (mmol l^{-1}) and micromoles per liter (µmol l^{-1}); or they can be given as millimolar (m*M*) or micromolar (µ*M*) solutions. Thus, 10 mg kinetin per liter can be written as 10 mg kinetin l^{-1}, 10 mg kinetin/liter (this format should not be used), 0.05 mmol l^{-1} [the actual number is 0.045661, but in the scientific literature numbers are usually rounded off to two and rarely three significant figures to the right of the decimal point; the use of more digits does not make a number more accurate – it makes it sillier except under special circumstances because most laboratory balances cannot weigh with such accuracy (it is not necessary anyway)], 45.61 µmol l^{-1}, 0.05-m*M* solution, or 45.61-µ*M* solution. One mole (abbreviated mol and not *M* or M) is the number of grams equal to the molecular weight of a compound. It consists of 1000 mmoles (mmol) or 1,000,000 µmoles (µmol); i.e., 1 mmol = 1000 µmol. Example: 1 mol of zeatin = 219.25 g, 1 mmol = 219.25 mg , and 1 µmol = 219.25 µg. Auxins, cytokinins, gibberellins, and ethylene have different functions and cannot be substituted for each other. Substitutions can sometimes be made within a group (i.e., one auxin for another, or a specific cytokinin for a different one), but this is not advisable because hormone functions or tissue responses to substances may be specific. Unlike salts and sugars, hormone substitutions within a group need not always be equimolar because hormone activities may vary. Hormone inhibitors (as for example ancymidol) or antagonists e.g., *trans*-cinnamic acid are used for specific purposes and must not be eliminated from a medium or substituted. Gibberellins, ethylene, and abscisic acid are used seldom if ever. Morphactins are added to a few media very rarely and are not included in this table.

[b]Both α-NAA and β-NAA may be used but this is not always indicated clearly in original research papers. The α form should be employed unless specified otherwise.

[c]These extremely high concentrations are generally found only in pastes used to induce plantlet formation on *Phalaenopsis* flower-stalk nodes. Some pastes may also contain *trans*-cinnamic acid.

Most auxins are not destroyed by autoclaving at 110–120°C for 50–60 min especially if the pH is non-acidic. However, autoclaving at low pH and in the presence of other factors may destroy IAA (Posthumus, 1971). This finding was confirmed more recently in a study which also showed that IBA is more stable than IAA (Nissen and Sutter, 1990). For this reason heat sterilization (autoclaving) for auxins is not recommended without prior determination that there would be no detrimental effects. Initially, at least, it is best to sterilize auxins by filtration (cold sterilization) or by dissolving them in ethyl alcohol (see culture media tables in Chapter 3). Once it is clear that a medium is appropriate it can be autoclaved after the auxin has been added and tested with explants. In many instances orchid explants and tissues grow and develop well on media that are autoclaved following the addition of auxin. This suggests that the auxin is not destroyed during autoclaving, or that it may be destroyed fully or in part, but the explants and/or tissue do not require it at least in the initial levels. It is also possible that whatever (if any) auxin remains in the medium after destruction during autoclaving is sufficient and/or that the heat denaturation product(s), should there be any (with or without auxin remnants), satisfy whatever requirements the orchid may have.

Illumination provided by cool white fluorescent tubes (Nissen and Sutter, 1990) and of unspecified nature (unpublished result by John Finer, finer.1@osu.edu posted on plant-tc@lists.umn.edu) causes the degradation of both IAA and IBA in both liquid and solid media. IBA is more stable than IAA under these conditions (Nissen and Sutter, 1990). Charcoal can absorb up to 97% of IAA and IBA in MS medium (Nissen and Sutter, 1990).

Some media contain the anti-auxin *trans*-cinnamic acid (*t*CA). Its purpose is to break bud dormancy. This compound is not interchangeable with any of the auxins and should not be used in media other than those specifically formulated to include it. Light may affect *t*CA and convert some of it to *cis*-cinnamic acid (*c*CA), but this does not seem to affect it usefulness. It is not possible to use *c*CA as a substitute for *t*CA. Heat sterilization of *t*CA is not advisable without prior determination that this will not affect its usefulness.

Cytokinins

The synthetics kinetin (6-furfuryl aminopurine), benzyladenine (N^6-benzylaminopurine, N^6-benzyladenine, BA, BAP), dimethylaminopurine (DMAP), thidiazuron (TDZ), and the naturally occurring zeatin are used most commonly in orchid culture media (Table 2-3). As with auxins, the cytokinins being used and their concentrations are based on empirical findings. Changes should be avoided. The effects of different cytokinins and their concentrations differ like those of auxins. Experiments with aqueous solutions of kinetin, zeatin, and isopentenyladenosine have shown that they are not broken down when autoclaved for 1 h at 120°C (Dekhuijzen, 1971). The effects of autoclaving at low pH and/or in the presence of other media components are less clear. It also not clear how autoclaving in culture media affects BA and DMAP. For these reasons, heat sterilization of cytokinins is not advisable without prior testing. In general cytokinins should be treated like auxins in respect to sterilization.

Thidiazuron (*N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea, TDZ) is a relatively recent addition to the list of cytokinins which are used in orchid micropropagation. It was introduced as a cotton defoliant named Dropp and was assumed to be a cytokinin antagonist. However, when tested it proved to be a "very potent cytokinin" (Mok et al., 1982, 1987; Mok and Mok, 1985; Machteld C. Mok, pers. comm.). It was used in the tissue culture of a few woody and herbaceous species within a short time of its discovery (for example see Meyer and Kerns, 1986; Fellman et al., 1987) and additional plants after that (Chalupa, 1988; Badzian et al., 1989; Gribaudo and Fronda, 1991; Bates et al., 1992; Huetteman and Preece, 1993). TDZ was first used for micropropagation of orchids, specifically *Phalaenopsis* (Ernst, 1994), a dozen years after its cytokinin-like properties were discovered and following its use with other plants.

TDZ is soluble in dimethylsulfoxide (DMSO), *N*,*N*-dimethylformamide (DMF), 0.1 or 0.01N KOH or NaOH (forming a light-tan-colored solution), 0.5N HCl, and 50% or more ethanol (Huetteman and Preece, 1993; and K. M. K. Bhatti, kmkbhatti@yahoo.com; T. Chandrasekhar, chandrasekhart2k@yahoo.com; Gregory Franklin, gfranklin7lin@yahoo.com; J. E. Preece, jpreece@siu.edu; M. Rigby, vitrotec@hotmail.com; Thimmappaiah, thim12@yahoo.com; Z. Zhang, zhangzh@missouri.edu, all pers. comm.). It can be autoclaved (M. Compton, mcompton@uwplatt.edu; J. E. Preece, jpreece@siu.edu; Thimmappaiah, thim12@yahoo.com; Z. Zhang, zhangzh@missouri.edu, all pers. comm.), but there is also a report that autoclaving may cause some loss of activity. Therefore several investigators prefer to filter sterilize it (A. Zelcer, zelcer@volcani.agri.gov.il, pers. comm.). It can also be dissolved in 70–95% ethanol and added to media after autoclaving.

Gibberellins and Antigibberellin

Gibberellins are used very seldom in culture media for orchids. When used, GA_3 is the most common form (Table 2-3). Autoclaving reduces gibberellin activity by more than 90% (van Bragt and Pierik, 1971). Therefore gibberellins must be cold-sterilized through filtration or by dissolving them in ethanol.

The antigibberellin ancymidol is used in at least one orchid medium. It should be dissolved in 50–95% ethanol and added to media after autoclaving.

Abscisic Acid

This is a growth inhibiting hormone that is not used in orchid culture media. Autoclaving of "dilute solutions of the [2-*cis* and 2-*trans*] isomers at various pH values" does not affect them (Wilmar and Doornbos, 1971). However the effects of media components during autoclaving are not known. Therefore, should abscisic acid (ABA) be added to culture media, it should not be heat-sterilized without prior testing. The cold sterilization procedures used for auxins and cytokinins would be suitable for ABA. Light causes a number of changes and interconversions between the isomers of ABA (Wilmar and Doornbos, 1971).

Ethylene and Ethylene Inhibitor

The only gaseous plant hormone, ethylene is rarely if ever used in culture media. Should it become necessary to add this hormone to a culture medium the ethylene-generating solid chemical ethrel (ethephon) is the most convenient form (Table 2-3). Heat sterilization is not advisable. Ethylene itself can be sterilized by passing it through sterilizing filters.

Silver thiosulfate is an ethylene inhibitor. It is made as follows:

Step 1. A 0.1-*M* solution of sodium thiosulfate is prepared by dissolving 1.58 g of the salt in 100 ml of distilled water.

Step 2. A 0.1-*M* solution of silver nitrate ($AgNO_3$) is prepared by dissolving 1.7 g of the salt in 100 ml of distilled water. This solution must be stored in the dark until it is used.

Step 3. Silver thiosulfate, 0.02 *M*, is prepared by slowly pouring 20 ml of the 0.1-*M* stock $AgNO_3$ into 80 ml of the 0.1-*M* sodium thiosulfate solution. The solution can be stored for up to a month.

Amino Acids

The most commonly used amino acid in orchid culture media is glycine because it is a component of the MS medium (Murashige and Skoog, 1962). Other amino acids are also used in some media. Amino acids can not be substituted for each other and should be added as listed in each recipe. It is possible that their omission may not have major effects but this must be tested carefully before trying it with a valuable clone. The effects of autoclaving on amino acids may vary and it is best to follow the original procedures in each case. If there are doubts, both autoclaving and cold sterilization (filter or dissolving in ethanol) should be tested before deciding which sterilization method to use.

Polyol

The only polyol used in orchid culture media is *myo*-inositol (other names for it are *meso*-inositol, *i*-inositol, inositol, cyclohexitol, inosite, meat sugar, and bios I; chemically it is hexahydroxycyclohexane, $C_6H_{12}O_6$) because it is part of the MS medium (Murashige and Skoog, 1962). Its function is not clear and it may not even be required, but *myo*-inositol should not be removed from a medium without prior testing. Inositol is usually sterilized by autoclaving. Hexitols in coconut water have also been shown to have growth promoting effects on plant embryos (van Overbeek et al., 1941; Shantz and Steward, 1952, 1955; see Chapter 1 for additional details and citations). However, these substances are not added to culture media other than as part of coconut water.

Polyamines

All plants contain polyamines, which have been studied for more than five decades. However, their importance in plant development was noted only recently. In plants

polyamines play roles in and affect cell division, embryogenesis, flower development, fruit ripening, root induction, and tuber formation. Putrescine, spermidine, and spermine are the most common polyamines in plants. Some have been used in tissue culture including orchid micropropagation to promote adventitious root initiation, shoot formation, and somatic embryogenesis.

Jasmonates

Initially jasmonic acid was thought to be a plant growth inhibitor. Subsequently jasmonic acid and methyl jasmonate were characterized as compounds that promote senescence and retard growth. In tissue culture jasmonic acid and methyl jasmonate can enhance meristem formation.

Vitamins

Niacin (nicotinic acid), pyridoxine (vitamin B_6), and thiamine (vitamin B_1) are most commonly added to orchid culture media as part of MS medium. Biotin, folic acid, and pantothenic acid (as calcium pantothenate) are also used in some media. It is not clear if all, or any, of them are required, but media formulations should not be changed without preliminary tests. Vitamins are not heat resistant and should not be sterilized by autoclaving (ten Ham, 1971). However media that contain vitamins are often heat-sterilized. This suggests that: (1) vitamins may survive autoclaving fully or in part; (2) explants and tissue do not require vitamins; and/or (3) whatever remains active after autoclaving can satisfy any requirements the explants may have. In practical terms this means that if a medium or media prove suitable without autoclaving the vitamins, tests should be carried out before switching to large-scale heat sterilization.

Nucleotides and Nucleic Acids

Some media include cytidylic and/or guanylic acid and/or other nucleotides as well as nucleic acids. Their functions are not clear and they may not even be required. However, it is advisable not to change media formulations. These substances may be autoclaved, but the high temperature and pressure at the low pH of orchid culture media may change them. For critical work it is advisable to try both autoclaving and cold sterilization (filtering the substances through sterilizing filters or adding them in alcohol solutions if their solubility will permit it) and then use the method which works best.

Organic Acids

Citric acid or tartaric acid were added as solubilizers for iron in several media formulated before the advent of chelating agents like EDTA. The iron salt–organic acid combinations in such media can and should be replaced with chelated iron (usually

a mixture of $FeSO_4 \cdot 7H_2O$ and Na_2EDTA). A few media still include organic acids. Their functions are not clear and it is not known if they are really required. Still, changes in the composition of media should not be made without prior testing. Organic acids can be sterilized by autoclaving.

Banana

The first use of banana in orchid seedling cultures dates back to 1950 (Withner, 1959*b*). All reports since then indicate that banana homogenate enhances the growth of orchid seedlings (for reviews see Withner, 1959*b*, 1974*b*; Arditti, 1968) and carrot root explants (Steward and Simmonds, 1954). These effects could be due to the presence in the pulp of plant hormones and related substances (Steward and Simmonds, 1964; Khalifah, 1966) and/or any number of other compounds (Table 2-4). There is some evidence that the substance(s) which enhance the growth of orchid seedlings is/are insoluble in water and ether but soluble in ethanol (Arditti, 1968). In micropropagation banana homogenate is added to media for plantlet development. It can be sterilized by autoclaving.

TABLE 2-4. **Composition of banana pulp**[a,b]

Component	Concentration	Component	Concentration
Minerals		**Acidity**	
Aluminum	Small amount	NaOH, 1N	4.06–4.46 ml 100 g^{-1} pulp
Boric acid	Some	pH	4.2–4.75; ripe fruit, 6.2; green 7.2
Calcium (as CaO)	0.028–0.37% of pulp DW	**Moisture**	
Calcium	8–24 mg 100 g^{-1} edible portion	Moisture content	74.4–77.4% of fresh pulp
Calcium	5.71 mg 100 g^{-1} pulp	Moisture	70.6–75.9% of FW pulp
Chlorides	0.171–0.38% of pulp DW	Osmotic pressure	7.15–29.06 atm
Chlorine	380 mg 100 g^{-1} edible portion	**Carbohydrates and related substances**	
Copper	0.09 mg 100 g^{-1} pulp	Carbohydrates	15.09 g 100 g^{-1} pulp
Copper	0.61 mg 100 g^{-1} edible portion	Carbohydrates	18.60–21.51% of fresh pulp
Iodine	5–200 ppb in fresh fruit	Fructose	1.45–3.24% of fresh pulp
Iron	1.37 mg 100 g^{-1} pulp	Glucose	2.24–4.21% of fresh pulp
Iron	6 ppm	Glucose	11.81% of just ripe fruit DW
Iron	1.8 mg 100 g^{-1} edible portion	Glycosides	0.23–0.25% of FW
Iron (Fe_2O_3)	0.0064–0.0079% of pulp DW	Maltose	very small amounts
Iron (Fe_2O_3)	0.7 mg 100 g^{-1} edible portion	Non reducing sugars	6.12–13.38% of pulp FW
Magnesium (MgO)	0.18% of pulp DW	Reducing sugars	6.19–10.73 of pulp FW
Magnesium	94 mg 100 g^{-1} edible portion	Soluble sugars	20% of FW
Manganese	1.95 mg 100 g^{-1} edible portion	Starch	0.4–7% of FW
Phosphates (P_2O_5)	0.179–0.304% of pulp DW	Starch	2.93–6.54% of pulp DW
Phosphates (P_2O_5)	26 mg 100 g^{-1} edible portion	Starch	13% of water insoluble fraction
Phosphorus	290 ppm	Sucrose	7.95–12.08% of fresh pulp
Phosphorus	85 mg 100 g^{-1} edible portion	Sucrose	4.50% of just ripe fruit DW
Potassium	251.43 mg 100 g^{-1} pulp	Sucrose : fructose ratio	4 : 3
Potassium (K_2O)	1.21–1.68% of pulp DW	Total sugars	11.5–12.5% FW
Potassium (K_2O)	370–1275 mg 100 g^{-1} edible portion	Total sugars	6.59–13.45% of fresh pulp
Silica (SiO_2)	0.058–0.96% of pulp DW	Total carbohydrates	73 g 100 g^{-1} edible portion
Sodium	0.57 mg 100 g^{-1} pulp	**Cellulose, fibers, and related substances**	
Sodium (Na_2O)	0.201–0.273% of pulp DW	Cellulose	0.13–0.19% of fresh pulp
Sodium (Na_2O)	1–3 mg 100 g^{-1} edible portion	Cellulose	4.8%, water insoluble fraction
Sulfur (SO_3)	0.046–0.053% of pulp DW	Crude fiber	22.82% of just ripe fruit DW
Sulfur	36 mg 100 g^{-1} edible portion	Hemicellulose	0.12–0.21% of fresh pulp
Zinc	28 mg kg^{-1} edible portion	Hemicelluloses	1–2% of fruit DW
Ash		Lignin	0.15–0.85% of fresh pulp
Ash	0.70–0.85% of fresh pulp	Total fiber	0.78–0.88% of fresh fruit
Ash	0.8 g 100 g^{-1} edible portion	Total fiber	0.5–1.5 g 100 g^{-1} edible portion
Ash	0.6% of water insoluble fraction		

TABLE 2-4. (***Continued***)

Component	Concentration
Pectic substances	
Pectin	0.27–0.40% of fresh pulp
Pectin	0.34–0.57% of fruit FW
Pectin	1.3%, water insoluble fraction
Protopectin	0.21–0.56% of fresh pulp
Protopectin	0.29–0.35% of pulp FW
Organic acids	
Acetic	some
Butyric	some
Citric	0.15–0.32%, non-volatile acids
Citric	0.15–0.32%, fruit
Citric	455 mg 100 g^{-1} edible portion
Glycosuccinic	some, only in unripe bananas
Glyoxylic	no units given in original report
α-Ketoisocaproic	no units given in original report
α-Keto, β-Hydroxypyruvic (?)	no units given in original report
α-Keto, β-methylvaleric	no units given in original report
Malic	0.053–0.50%, non-volatile acids
Malic	0.053–0.50%, fruit
Malic	1520 mg 100 g^{-1} edible portion
Oxalic	0.0064%, fruit
Oxalic	19 mg 100 g^{-1} edible portion
Oxaloacetic	no units given in original report
Succinic semialdehyde	no units given in original report
Tartaric acid	some

Acids in ripening fruit on day
μg g^{-1} FW

Day:	0	6	12
β-Hydroxypyruvic	0.79	0.72	0.28
α-Ketoglutaric	1.75	1.00	
α-Ketoisovaleric	1.64	2.16	1.67
Pyruvic	14.02	3.00	1.78
Succinic semialdehyde	0.79	0.90	0.67

Lipids, fats, and fatty acids	
Crude fat	0.30–0.47% of pulp FW
Fat	110 mg 100 g^{-1} pulp
Fat	0.2 g 100 g^{-1} edible portion
Total fat	1.2 g 100 g^{-1} edible portion
Linoleic acid	some
Linolenic acid	some
Lipids	0.12% of FW
Lipids	3.7% water insoluble fraction
Oleic acid	some
Palmitic acid	some
Sterols	
Cholesterol	24 mg 100 g^{-1} edible portion
Phytosterol	some
Sterol	some

Tannins	Standard units 100 $tissue^{-1}$
"Active" tannin	1.99–4.35
Tannin	2.57–4.35
Tannin	0.4% water insoluble fraction
Polyols and related compounds	
Inositol	34 mg 100 g^{-1} pulp
Phytin [Ca/Mg salt of phytic acid (inositol, hexaphosphoric acid)]	0.41–5.11%, oven dry sample
Volatile constituents	
Acetaldehyde	
Amylacetate	
Amylbutyrate	
Ethyl alcohol	
Methyl alcohol	

Component	Concentration
Nitrogen content	
Nitrogen	1.4% of DW
Nitrogenous matter	8.91%, DW, just ripe fruit
Protein	0.81–1.49%, pulp FW
Protein	1.1 g 100 g^{-1} edible portion
Protein	740 mg 100 g^{-1} edible pulp
Protein	9.8% water insoluble fraction
Proteins	3.9 g 100 g^{-1} edible portion
Enzymes	Amylase, ascorbic acid oxydase, carboxylase, catalase, invertase, lipase, oxygenase, phosphatase, peroxydase, polyphenoloxidase, protease

	Percent of N fraction of albumin-globulin protein
Amide nitrogen	10.13
Humin nitrogen	2.92
Monoamino nitrogen	37–64
Nonamino nitrogen	7.65

Amino acids

Kjeldahl nitrogen

	Soluble	Insoluble
Green fruit	50.5	93.5 mg 100 g^{-1} FW
Yellow fruit	53.9	104.8 mg 100 g^{-1} FW
Green fruit	35.1	64.9%, total nitrogen
Yellow fruit	33.9	66.1%, total nitrogen

Soluble nitrogen in ripening fruit
μg amino acid g^{-1} FW

Day:	0	6	12
α-Alanine	10	3.5	2.8
β-Alanine	tr	tr	tr
α-Aminobutyric acid	12	7.2	8.5
Arginine	2.5	1.7	tr
Asparagine	60.0	41.9	56.5
Aspartic acid	13.4	8.6	10.2
Glutamic acid	3.9	2.5	1.4
Glutamine	57.2	33.8	32
Glycine	2.3	2.2	4.3
Histidine	22.5	29.7	42.5
Leucines	1.2	8.7	14.8
Lysine	1.9	1.6	3.1
Pipecolic acid	13.3	9.4	15.0
Proline	2.0	6.7	7.5
Serine	6.5	6.4	12.9
Tyrosine	2.0	1.2	1.8
Valine	1.0	4.3	7.8

	g g^{-1} nitrogen or 100 g^{-1} edible portion[c]
Alanine	0.22
Arginine	0.21
Aspartic acid	0.80
Cystine	0.042
Glutamic acid	1.00
Glycine	0.20
Histidine	0.42
Isoleucine	0.11
Leucine	0.29
Lysine	0.23
Methionine	0.038
Phenylalanine	0.14
Proline	0.19
Serine	0.20
Threonine	0.16
Tryptophan	0.072

TABLE 2-4. (***Continued***)

Component	Concentration			Component	Concentration
Tyrosine	0.072			Methionine sulfoxide	Present
Valine	0.17			Phenylalanine	Present
	Each amino acid as percent of total protein nitrogen in ripening fruit			Proline	5–10%
				Serine	<5%
	Day: 0	6	12	Threonine	<5%
				Tyrosine	<5%
α-Alanine	10	8.9	9.6	Valine	5–10%
Arginine	8.0	6.8	8.9	**Vitamins**	
Aspartic acid	9.0	13.5	9.4	Vitamin A	131.0–131.43 IU mg^{-1}
Cystine	tr			Vitamin A	50–332 IU 100 g^{-1} pulp
Glutamic acid	10.4	11.0	10.8	Vitamin A	50–332 IU 100 g^{-1} edible portion
Glycine	9.0	7.1	8.6	Vitamin A (equivalent)	1.6–2.03 μg g^{-1} β-carotene
Histidine	3.8	5.3	3.3	Vitamin A	5.1% of USDA RDA/100 mg
Hydroxyproline	tr	1.3	1.1	Vitamin C	30 mg 100 g^{-1} edible portion
Leucines	18.0	13.9	15.1	Vitamin D	0–0.2 IU g^{-1}
Lysine	6.3	8.5	8.6		100 g^{-1} pulp or edible portion
Proline	3.4	5.0	4.9	Biotin (vitamin H)	4.4 μg
Serine	6.4	7.1	8.6	Folic acid (vitamin B[c])	95 μg
Threonine	5.3	4.4	4.8	Niacin	40–61 μg/0.7–1.8 mg
Tyrosine	2.8	2.9	2.8	Pantothenic acid	70 μg FW/0.61 mg
Valine	7.4	13.9	15.1	Vitamin B_1 (thiamine)	34–48 μg/40–270 μg
	Ninhydrin reactive substances			Vitamin B_2 (riboflavin)	40–87 μg/87–180 μg
				Vitamin B_6 (pyridoxine)	0.32 mg/0.5 mg
	Percent of total amino acids			Vitamin C	6.86 mg/3–11 μg, 0.1 mg g^{-1}
				Vitamin E (tocopherols)	poor source
Alanine	<5%			Vitamin E	1.1 mg 100 g^{-1} edible portion
α-Aminobutyric acid	5–10%			Vitamin K	none
Asparagine	>15%			Niacin	4.8% of USDA RDA/100
Arginine	<5%				Percent of USDA RDA in 100 mg
Aspartic acid	5–10%			Vitamin B_1 (thiamine)	2.6%
Glutamic acid	<5%			Vitamin B_6 (riboflavin)	5.3%
Glutamine	10–15%			Vitamin C (ascorbic acid)	20%
Glycine	<5%			**Growth promoting substances** (No quantitative data in the original report)	
Histidine	10–15%			"Auxin-like"	
Leucines	<5%			6-(α^6-Isopentenylamino purine)	
Lysine	<5–15%			Ethylene (evolution)	
Pipecolic acid	5–10%			Of same general nature as in coconut water	
Proline	<5%			Purine	
Serine	<5%			Zeatin	
Threonine	<5%			Zeatin riboside	
Tyrosine	<5%			**Pigments**	
Valine	<5%			Carotenes	0.6–1 μg/g FW
Total	*Alcohol insoluble protein hydrolysates* 3.13 μg g^{-1} FW			α-Carotene	31% of carotenes
				β-Carotene	28% of carotenes
	Percent of total amino acids			β-Carotene	1.5–2 ppm FW
				Lutein	33% of carotenes
	Alanine 5–10%			**Energy**	
Arginine	<5%				285 kilocalories
Aspartic acid	>15%				100 g^{-1} edible portion
Glutamic acid	>15%				
Glycine	5–10%				
Histidine	<5%				
Leucines	>15%				
Lysin	5–10%				

[a]The reasons for the growth stimulating effects banana pulp has on orchid seedlings and plantlets are not clear. Information on the composition of banana pulp is presented here for those who may wish to study or speculate about the factor(s) which may be involved. All units used here are those given in the source papers. The different values given in the literature for components are sometimes difficult, if not impossible, to reconcile. That is why multiple entries are included for a number of substances. This table is a modification of a previous one (Arditti, 1968; Arditti et al., 1982). Sources: Anonymous, no date; von Loesecke, 1950; Steward and Simmonds, 1954; Steward et al., 1960*a*, 1960*b*; Diem, 1962; Whatt and Merrill, 1963; Palmer and Roberts, 1967; Tamura, 1970; van Staden and Stewart, 1975.

[b]Abbreviations: DW, dry weight; FW, fresh weight; IU, international units; ppb, parts per billion; ppm, parts per million; RDA, recommended daily allowance; tr, trace; USDA, United States Department of Agriculture.

[c]The original paper (Tamura, 1970) states that amino acids, grams per "gram nitrogen edible portion" or in "100 gram edible portion," were used to indicate the value but does not state which.

Coconut Water

Erroneously called "coconut milk," coconut water (CW), the liquid endosperm of coconut seeds (Table 2-5), was first used in orchid seedling media in 1951 (Mariat, 1951). Despite a number of reports since then (for reviews see Ernst, 1967*b*; Arditti and Ernst, 1984), CW is not used extensively in orchid seed germination and seedling culture. However it does have beneficial effects on some orchid explants. It can also increase proliferation without causing mutations. CW is incorporated in several media because of that. The reasons for the beneficial effects of CW are not clear. Plants hormones and related substances, hexitols (Pollard et al., 1961) and many other substances singly and in combination may be the reason(s) for these effects (Table 2-5). CW from green (unripe) nuts is the most commonly used and recommended form. However CW from ripe nuts can also be used. If frozen, CW can be stored for prolonged periods without loss of activity. CW can be autoclaved.

Hydrolysates and Autolysates

Peptone, tryptone (both hydrolysates), yeast extract (an autolysate), and a number of other complex additives are used in some orchid culture and micropropagation media (Table 2-6).

Sugars

Sucrose (ß-D-fructofuranosyl-α-D-glucopyranoside, kitchen sugar, beet sugar, cane sugar, sugar, saccharose, $C_{12}H_{22}O_{11}$, MW 342.30) is most commonly used in orchid culture media. When sterilized by autoclaving (which is the usual, simplest, and most widely used method) some of it hydrolyzes into its component molecules fructose (ß-D-fructose, levulose, fruit sugar, $C_6H_{12}O_6$, MW 180.16) and glucose (blood sugar, corn sugar, grape sugar, dextrose, $C_6H_{12}O_6$, MW 180.16). Therefore seeds and tissues are usually cultured in a mixture of fructose, glucose, and sucrose. There is some evidence to suggest, at least in respect to the seeds of several species, that cane sucrose is preferable to that obtained from beets (at least in respect to kitchen-grade sucrose). Some orchid seeds and explants may grow better on fructose.

If necessary glucose and/or fructose can be substituted for sucrose in tissue culture media (a number of additional sugars including maltose and trehalose can be used for seeds). However, it is necessary to keep in mind that due to the different molecular weights of sucrose on the one hand and glucose and fructose on the other, substitutions may present some problems (Table 2-7). The commonly used 20 g of sucrose amounts to 0.06 mol (or 60 mmol) whereas the same weight of fructose or glucose is 0.11 mol (110 mmol). As a result the osmolarity (concentration as reflected in osmotic effects) of a solution containing 20 g of sucrose will be different from that of a medium made with 20 g of fructose or 20 g of glucose. Such differences in osmolarity and/or sugar level may have an effect on the survival of newly excised tissues or freshly prepared protoplasts. On the other hand, solutions of 20 g of each of these sugars contain almost the same number of carbons and cutting

TABLE 2-5. **Partial composition of coconut water, copra, and coconut oil (Raghavan, 1966, 1976; Woodroof, 1979)**

Substance	Content
Coconut water	
Inorganic ions	*mg per 100 g*
Chlorine	183
Copper	0.04
Iron	0.10
Magnesium	30
Phosphorus	37
Potassium	312
Sodium	105
Sulfur	24
Nitrogenous compounds	*μmol ml*$^{-1}$
Ethanolamine	0.01
Ammonia	Present[a]
Amino acids and related substances	*μg ml*$^{-1}$
Alanine	312
β-Alanine	12
γ-Aminobutyric acid	820
Arginine	133
Asparagine and glutamine	60
Aspartic acid	65
Glutamic acid	240
Glycine	13.9
Homoserine	5.2
Isoleucine	18
Leucine	22
Lysine	150
Methionine	8
Ornithine	22
Phenylalanine	12
Proline	97
Serine	111
Threonine	44
Tryptophan	39
Tyrosine	16
Valine	27
Cystine	0.97–1.17[b]
Dihydroxyphenylalanine	Present[a]
Histidine	Trace[a]
Hydroxyproline	Trace[a]
Pipecolic acid	Present[a]
Enzyme	
Acid phosphatase	Present[a]
Catalase	Present[a]
Dehydrogenase	Present[a]
Diastase	Present[a]
Peroxidase	Present[a]
RNA polymerases	Present[a]
Organic acids	*meq ml*$^{-1}$[c]
Citric acid	0.37
Malic acid	34.31
Pyrrolidine carboxylic acid	0.39
Shikimic and quinic acids, etc.	0.57 mg ml^{-1}
Vitamins	*mg ml*$^{-1}$
Biotin	0.02
Folic acid	0.003
Niacin (nicotinic acid)	0.64
Pantothenic acid	0.52
Riboflavin	0.01
Pyridoxine	Trace[a]
Thiamine	Trace[a]
Sugars	*mg ml*$^{-1}$
Fructose	5.25
Glucose	7.25
Sucrose	9.18
Sugar alcohols	*Percent*[a]
Mannitol	0.8
Sorbitol	15[d]
myo-Inositol	0.01
scyllo-Inositol	0.05
Plant hormones	
Auxin	0.07 mg ml^{-1}
1,3-Diphenylurea	5.8 mg l^{-1}
Cytokinin	Present[a]
Gibberellin	Present[a]
Miscellaneous	
Leucoanthocyanin	Present[a]
Phyllococosine	Present[a]
Copra	
Protein	*Percent*
Crude	20.30–22.89
Extracted	20–25
Fiber, crude	8.53–12
Lipids	
Crude fat	2.76–7.4
Oil	63–65
Water (moisture)	4.84–11.14
Nitrogen-free: extract	29.15–47.46
Ash	6.82–6.88
Amino acids	
Histidine	Deficient[a]
Methionine	Deficient[a]
Lysine	Deficient[a]
Tryptophan	Deficient[a]
Saturated acids	*Percentage*
Lauric	High
Myristic	High
Palmitic	High
Stearic	High
Other acid: oleic	High
"Meat" or kernel	
Lipid: oil	45–50%
Coconut oil	
Acids	*Percent*
Capric	7
Caprylic	8
Lauric	48
Linoleic	1
Myristic	19
Oleic	6
Palmitic	7
Stearic	4
Crude	*ppm*
Ketones:	
Heptan-2-one	40
Nonan-2-one	73
Pentadecan-2-one	65
Tridecan-2-one	138
Undecan-2-one	290
Lactones:	
Decalactone	97
Dodecalactone	60
Hexalactone	20
Octalactone	51
Tetradecalactone	30
From fresh nuts	*ppm*
Ketones:	
Tridecan-2-one	138
Undecan-2-one	290
Lactones:	
Decalactone	88
Dodecalactone	65
Hexalactone	27
Octalactone	64
Tetradecalactone	20

[a]No units given.
[b]Units: grams per 100 g dried protein.
[c]meq ml^{-1} is milliequivalents per milliter.
[d]Units: mg ml^{-1}.

the concentrations of fructose or glucose in half for the purpose of reducing the osmolarity will also reduce the total carbon. This too may have an effect.

Another point to consider is the length, size, and nature of sugar molecules. Equal weights of different length molecules contain the same amount of carbon, but as indicated before the molarities will be different. However, this is not the only difference. The ability of plants to hydrolyze or take up sugar molecules of different sizes may differ and this could determine the actual amount of sugar that is available to seeds, seedlings, explants, and/or plantlets. For example, plantlets on a medium that contains 20 g glucose l^{-1} are on a 0.11-*M* solution of an easily available sugar. The plantlets can easily take up the glucose molecule. If placed on medium that contains 20 g maltose the plantlets will be on a 0.06-*M* solution of a sugar which can also be taken up, utilized, and/or hydrolyzed easily (Ernst et al., 1971*b*). However if the plants are placed on media that contain 20 g maltotriose (a molecule made of three glucose residues), maltotetraose (four glucoses), maltopentaose (five glucoses), or maltohexaose (six glucoses), the molarities of the solutions decrease (approximately 0.04, 0.03, 0.024 and 0.020 *M*, respectively). The different osmolarities will affect growth, but size itself is not the only relevant factor. Plantlets may not be able to take up and/or hydrolyze the longer molecules as fast and as easily as the shorter ones. As a result plantlets on longer sugars may actually be growing on suboptimal concentrations even if carbon levels are the same. And this is indeed the case (Ernst and Arditti, 1990). This problem may occur even on equimolar concentrations of sugar molecules of different sizes, lengths, and complexities. The molarity/availability/osmolarity factor becomes even more complex if the larger sugars consist of more than one kind of sugar molecule such as raffinose (galactose, glucose, fructose), stachyose (galactose, galactose, glucose, fructose), verbascose (galactose, galactose, galactose, flucose, fructose) and others.

Given these facts, changes in sugars and their concentrations should not be made unless this is strictly necessary. If changes must be made this must be done on a molar, not weight, basis and the media with the new sugars or sugar levels should be tested with an easily obtainable cultivar before using them for a rare and expensive one.

A formula to use for calculating equivalent molarities of sugars is:

$$\frac{\text{OWS} \times \text{MWSS}}{\text{MWOS}} = \text{SWS} \qquad \text{(V)}$$

where:

MWOS = molecular weight of the original sugar;
MWSS = molecular weight of the substitute sugar;
OWS = weight (in g, mg, or μg) of the original sugar;
SWS = weight (in g, mg, or μg) of the substitute sugar.

If glucose is to be used to substitute for an equimolar amount of sucrose, equation V becomes:

$$\frac{20\ \text{g} \times 180.16}{342.30} = 11.11\ \text{g} \qquad \text{(VI)}$$

For the reverse (replace 20 g of glucose with sucrose), equation V becomes:

TABLE 2-6. **Typical analysis of Difco peptones and hydrolysates**[a]

Component	Peptone	Proteose peptone	Proteose peptone no. 3	Tryptone	Tryptose	Neopeptone
Percent						
Ash	3.53	9.61	4.90	7.28	8.44	3.90
Ether soluble extract	0.37	0.32		0.30	0.31	0.30
Total nitrogen	16.16	14.37	13.06	13.14	13.76	14.33
Primary proteose nitrogen	0.06	0.60		0.20	0.40	0.46
Secondary proteose nitrogen	0.68	4.03		1.63	2.83	3.03
Peptone nitrogen	15.38	9.74		11.29	10.52	10.72
Ammonia nitrogen	0.04	0.00		0.02	0.01	0.12
Free amino nitrogen (Van Slyke)[c]	3.20	2.66		4.73	3.70	2.82
Amide nitrogen	0.49	0.94		1.11	1.03	1.23
Monoamino nitrogen	9.42	7.61		7.31	7.46	7.56
Diamino nitrogen	4.07	4.51		3.45	3.98	4.43
Arginine	8.0	6.8	5.9	3.3	5.05	4.7
Aspartic acid	5.9	7.4	6.6	6.4	6.9	6.7
Cystine (Sullivan)[c]	0.22	0.56		0.19	0.38	0.39
Glutamic acid	11.0	12.0	11.2	18.9	15.4	15.2
Glycine	23.0	11.6	8.9	2.4	7.0	6.3
Histidine	0.96	1.7	1.7	2.0	1.8	2.3
Isoleucine	2.0	3.3	3.3	4.8	4.0	4.3
Leucine	3.5	6.4	6.0	3.5	7.4	8.4
Lysine	4.3	5.3	5.1	6.8	6.0	6.4
Methionine	0.83	2.0	1.8	2.4	2.2	2.4
Phenylalanine	2.3	3.3	3.1	4.1	3.7	4.3
Threonine	1.6	3.5	3.2	3.1	3.3	3.7
Tryptophan	0.42	0.72	0.85	1.45	1.08	1.01
Tyrosine	2.3	3.4	0.36	7.1	5.2	5.3
Valine	3.2	4.4	4.0	6.3	5.3	6.0
Organic sulfur	0.33	0.60		0.53	0.57	0.63
Inorganic sulfur	0.29	0.04		0.04	0.04	0.09
Phosphorus	0.079	0.24	0.46	0.75	0.49	0.112
Iron	0.0023	0.0038	0.0044	0.0071	0.0054	0.0021
SiO_2	0.042	0.078	0.019	0.090	0.084	0.18
Potassium	0.22	0.70	0.21	0.30	0.50	0.85
Sodium	1.08	2.84	0.033	2.69	2.76	0.45
Magnesium	0.056	0.118	0.00048	0.045	0.081	0.051
Calcium	0.058	0.137	0.0396	0.096	0.116	0.198
Chlorine	0.27	3.95		0.29	2.77	0.84
Chloride	0.27	3.95	4.15	0.29	2.12	0.84
Parts per million						
Manganese	8.6	5.3	7.8	13.2	9.2	5.8
Lead	15.00	5.00	3.00	6.00	5.50	5.00
Arsenic	0.09	0.25	0.00	0.07	0.16	0.37
Copper	17.00	31.00	9.00	16.00	23.50	19.00
Zinc	18.00	44.00	37.00	30.00	37.00	2.00
Micrograms per gram						
Pyridoxine	2.5	3.0	4.1	2.6	2.8	5.0
Biotin	0.32	0.43	0.24	0.36	0.39	0.73
Thiamine	0.50	3.0	2.7	0.33	1.66	3.4
Nicotinic acid	35.00	131.00	169.00	11.00	71.00	134.00
Riboflavin	4.00	11.00	13.00	0.18	5.59	11.4
Reaction, pH[b]	7.0	6.8		7.2	7.3	

[a]*Sources*: *Difco Manual*, 9th ed., 1953, Difco Laboratories, Detroit, Mich.; H. W. Schoenlein, Difco Laboratories, pers. comm., 1957. Courtesy of E. McDonald, Technical Services, Difco Laboratories, Detroit, Mich. Other peptones are probably similar.

[b]pH of a 1% solution in distilled water after autoclaving 15 min at 121°C.

[c]These are analytical methods.

Component	Protone	Casitone	Cosamino acids (technical grade)	Casamino acids	Yeast extract
Percent					
Ash	2.50	6.66	30.8	3.64	10.1
Ether soluble extract	0.31				
Total nitrogen	15.41	13.00	7.85	11.15	9.18
Primary proteose nitrogen	5.36				
Secondary proteose nitrogen	7.60				
Peptone nitrogen	2.40				
Ammonia nitrogen	0.05				
Free amino nitrogen (Van Slyke)[c]	1.86				
Amide nitrogen					
Monoamino nitrogen					
Diamino nitrogen					
Arginine	3.9	3.2	1.9	3.8	0.78
Aspartic acid	10.8	6.5	4.0	0.49	5.1
Cystine (Sullivan)[c]	0.27				
Glutamic acid	8.1	20.0	12.6	5.1	6.5
Glycine	5.0	2.5	1.3	1.1	2.4
Histidine	5.9	2.1	1.4	2.3	0.94
Isoleucine	0.71	5.0	2.9	4.6	2.9
Leucine	13.6	8.2	4.0	9.9	3.6
Lysine	10.3	7.0	4.4	6.7	4.0
Methionine	1.9	2.6	1.08	2.2	0.79
Phenylalanine	6.8	4.3	2.0	4.0	2.2
Threonine	4.6	4.2	2.2	3.9	3.4
Tryptophan	1.65	1.38	Nil	0.8	0.88
Tyrosine	3.0	2.8	0.52	1.9	0.60
Valine	10.1	6.3	3.8	7.2	3.4
Organic sulfur	0.45				
Inorganic sulfur	0.16				
Phosphorus	0.15	0.72	0.29	0.35	9.89
Iron	0.0099	0.0039	0.0101	00.0006	0.028
SiO_2	0.52	0.073	0.022	0.053	0.052
Potassium	0.06	0.12	0.16	0.88	0.042
Sodium	0.30	0.24	1.05	0.77	0.32
Magnesium	0.057	0.00060	0.0039	0.0032	0.030
Calcium	0.263	0.0913	0.0538	0.0025	0.040
Chlorine	0.38				
Chloride	0.38	0.425	21.34	11.2	0.190
Parts per million					
Manganese	6.0	9.7	5.7	7.6	7.8
Lead	9.00	5.00	3.00	4.00	16.00
Arsenic	0.46	0.32	0.00	0.50	0.11
Copper		10.00	8.00	10.00	19.00
Zinc	13.00	10.00	14.00	8.00	88.00
Micrograms per gram					
Pyridoxine	0.24	1.1	0.025	0.073	20.0
Biotin	0.0021	0.34	0.050	0.102	1.4
Thiamine	0.17	0.48	0.02	0.12	3.2
Nicotinic acid	2.1	24.00	2.5	2.7	279.00
Riboflavin	0.046	0.68	0.019	0.03	19.00
Reaction, pH[b]					

TABLE 2-7. **Sugars in orchid tissue culture media**[a]

Sugar	Number of carbons	Molecular weight	Amount of sugar per liter		
			Weight, g	Percent	Molarity, mmol
Fructose	6	180.16	0.5	0.05	2.78
			1	0.1	5.55
			2	0.2	11.10
			3	0.3	16.65
			4	0.4	22.20
			5	0.5	27.75
			5.26	0.53	29.2
			6	0.6	33.3
			7	0.7	38.85
			8	0.8	44.40
			9	0.9	49.95
			10	**1**	**55.51**
			10.52	1.05	58.4
			15.78	1.58	87.6
			20	**2**	**111.01**
			30	3	166.52
Glucose	6	180.16	0.5	0.05	2.78
			1	0.1	5.55
			2	0.2	11.10
			3	0.3	16.65
			4	0.4	22.20
			5	0.5	27.75
			5.26	0.53	29.2
			6	0.6	33.3
			7	0.7	38.85
			8	0.8	44.40
			9	0.9	49.95
			10	**1**	**55.51**
			10.52	1.05	58.4
			15.78	1.58	87.62
			20	**2**	**111.01**
			30	2	166.52
Sucrose	12	342.30	1	0.1	2.92
			2	0.2	5.84
			3	0.3	8.76
			4	0.4	11.69
			5	0.5	14.61
			6	0.6	17.53
			7	0.7	20.45
			8	0.8	23.37
			9	0.9	26.30
			9.52	0.95	27.8
			10	1	29.21
			19	1.9	55.51
			20	**2**	**58.43**
			30	**3**	**87.64**
			38	3.8	111.01

[a]The most commonly used concentrations of each sugar are given in boldface. Some concentrations that are used seldom if ever are included for comparison purposes. It is important to note that equal weights of fructose and glucose (both 6-carbon sugars) are equimolar and contain the same number of carbons. The same weights of glucose or fructose and sucrose (a 12-carbon sugar) contain nearly the same number of carbons, but represent different molarities. For example, 20 g of glucose are equal in molarity and number of carbons to the same weight of fructose. The same 20 g of sucrose contain nearly as many carbons as an equal weight of glucose or fructose, but half as many moles. Most recipes for orchid tissue culture media list sugar content by weight rather than molarity. Both molarity and weight are given here to allow for comparisons.

Tissues and organs may be less sensitive to molarities of media than are isolated cells and protoplasts, which are affected greatly even by small differences. The true concentration of a solution (i.e., its osmolarity) is determined not by weight but by the number of molecules in it. Therefore, if tissue culture media are to be used for single-cell and protoplast cultures, substitutions of sugars must be made on the basis of molarity, not weight or percentage.

$$\frac{20\text{ g} \times 342.30}{180.11} = 38.01\text{ g} \qquad \text{(VII)}$$

If the number of carbons is to be kept equal the total in each molecule must be taken into account and the expression changes to:

$$\frac{\text{COS}}{\text{CRS}} \times \text{OWS} \times \frac{\text{MWSS}}{\text{MWOS}} = \text{SWS} \qquad \text{(VIII)}$$

where:

COS = number of carbons in the original sugar;
CRS = number of carbons in the replacement sugar;
MWOS = molecular weight of the original sugar;
MWSS = molecular weight of the substitute sugar;
OWS = weight (in g, mg, or μg) of the original sugar;
SWS = weight (in g, mg, or μg) of the substitute sugar.

If glucose (six carbons) is to be used as a substitute for sucrose without a change in the number of carbons in the solution, equation VIII becomes:

$$\frac{12}{6} \times 20\text{ g} \times \frac{180.16}{342.30} = 22.22\text{ g} \qquad \text{(IX)}$$

or

$$2 \times 20\text{ g} \times \frac{180.16}{342.30} = 22.22\text{ g} \qquad \text{(X)}$$

for the reverse it is:

$$\frac{6}{12} \times 20\text{ g} \times \frac{180.16}{342.30} = 19\text{ g} \qquad \text{(XI)}$$

or

$$0.5 \times 20\text{ g} \times \frac{180.16}{342.30} = 19\text{ g} \qquad \text{(XII)}$$

Anticontaminants

In some instances it may not be possible to adequately surface-sterilize tissues or seeds, or a valuable culture may become contaminated. When this happens the incorporation of bactericides or fungicides in the culture medium may save the cultures by either eliminating the contaminant(s) or at least keeping it/them in check.

Efforts to formulate orchid seed and seedling culture media that do not require sterilization or can reduce contamination started shortly after the Knudson C medium was developed (for a short review see Thurston et al., 1979). Vanillin derivatives (Knudson 1947; McAlpine, 1947; and personal communications) and several antibiotics (Schaffner, 1954) were tested as additives for this purpose, but were found to be phytotoxic and unsuitable. More recently, several combinations were formulated (Table 2-8) following the screening of a number of substances (Thurston et al.,

TABLE 2-8A. **Stock solutions of anticontaminants**[a]

Compound	Amount per liter of culture medium (final concentration in culture medium), mg	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
Amphotericin B	10	100 mg 10 ml^{-1} 70% ethanol[b]	1	Keep frozen between uses
Benlate	50	500 mg 10 ml^{-1} distilled water[c] or 70% ethanol	1	Keep frozen between uses
Gentamycin	50	Sterile injectable liquid prepared according to instructions in package[d]	Depends on instructions in package	Keep frozen between uses
Nystatin	25	250 mg 10 ml^{-1} absolute ethanol	1	Keep frozen between uses
Penicillin G	100	1 g 10 ml^{-1} 70% ethanol	1	Keep frozen between uses
Sodium omadine	5	50 mg 10 ml^{-1} 70% ethanol	1	Keep frozen between uses
Vancomycin	50	500 mg 10 ml^{-1} 70% ethanol	1	Keep frozen between uses
Graphite[e]	2000	No stock	No stock	Weigh

[a]To prepare a mixture for use, mix the required compounds in a small vial approximately 1–2 h before needed, add the graphite, and shake well. Add this mixture to the medium after agar has been dissolved. The graphite can also be added before autocloning.
[b]The 70% ethanol solution is prepared by bringing 737 ml of 95% ethanol to 1000 ml with distilled water. Ethanol (95%) can be purchased in drugstores with prescription.
[c]A precipitate will form. Shake well before use.
[d]This step requires a sterile syringe-and-needle combination that can be purchased in drugstores with prescription.
[e]Not an anticontaminant, but used to darken media to prevent photodestruction of light-sensitive compounds.

TABLE 2-8B. **Formulations of anticontaminants for use in culture media for orchid seedlings (Thurston et al., 1979)**

Number	Formulation[a,b]
1	Benlate + nystatin + penicillin G + gentamycin + graphite[c]
2	Benlate + nystatin + penicillin G + gentamycin + sodium omadine + graphite[c]
3	Benlate + nystatin + penicillin G + gentamycin + amphotericin B + vancomycin + graphite[c]

[a]*Concentrations*: amphotericin B, 10 mg l^{-1}; benlate, 50 mg l^{-1}; gentamycin, 50 mg l^{-1}; nystatin, 25 mg l^{-1} (100, 500 units l^{-1}); penicillin G, 100 mg l^{-1} (159, 500 mg l^{-1}); sodium omadine, 5 mg l^{-1}; vancomycin, 50 mg l^{-1}; graphite, 2 g l^{-1}.
[b]*Suppliers*: amphotericin B, gentamycin, nystatin, penicillin G, and vancomycin can be obtained from Sigma Chemical Co.; sodium omadine is available from the Olin Corporation, Agricultural Division, 700 N. Buckeye St., Little Rock, AR 72114, USA; benlate formulations are sold by retail nurseries and plant shops. Graphite may be purchased from the J. T. Baker Chemical Co., 222 Red School Lane, Phillipsburg, NJ 08865, or 995 Zephyr Ave., Hayward, CA 94544.
[c]Not an anticontaminant, but used as a darkening agent to prevent photodestruction of light-sensitive compounds.

1979, 1980; Spencer et al., 1979/80; Brown et al., 1982; Cvitanik and Arditti, 1984; the research was carried out entirely by several hard working and very bright undergraduate students, all except one of them women between the ages of 19 and 21, at the University of California, Irvine; see Figs 1-52–1-54). These formulations cannot be used routinely, but may be useful in special cases.

Medium is prepared as usual for tropical orchids through the step of dissolving the agar. The anticontaminants (Table 2-8) are dissolved or suspended and mixed in a total of 6 ml 70% ethanol (i.e., ethyl alcohol; the control media in the original research suggested that this much alcohol may actually enhance seedling growth).

All culture vessels, funnels, and other glassware used with unsterilized anticontaminant-containing media must be washed with 70% ethanol or rubbing (i.e., isopropyl) alcohol and allowed to dry upside down in clean, dust-free areas. Tools must be washed similarly and flamed before use. Work surfaces must be first washed with soap and water and then with 70% ethanol or rubbing alcohol. Water used for the preparation of media must be boiled for 5 min, allowed to stand in a covered vessel for 24 h, and boiled again for another 5 min.

Work areas must be clean and dust-free. All work must be carried out quickly and efficiently.

The formulations described here (Table 2-8) are not suitable for use in seed germination media and should not be employed for that purpose. They were not tested widely and may be unsuitable for all explants. They are known to be suitable for *Cattleya* and *Stanhopea* seedlings (Thurston et al., 1979) and *Phalaenopsis* flowerstalk-node cultures (Spencer et al., 1979/80). Since plantlets derived from explants are similar to seedlings in their general requirements and responses to media components, these formulations may appear to be potentially useful in orchid micropropagation (see section on *Phalaenopsis*). Still, it is advisable to test each combination with a few cultures prior to large-scale use. On the whole their usefulness will probably prove to be limited and care should be exercised in any attempts to use them.

Amphotericin B (92.7% pure, 10 ppm), nystatin (4020 units mg^{-1}, 25 ppm), and sodium omadine (90% pure, 5 ppm) when used singly delayed the development of *Cymbidium* shoot-tip explants, but had no other deleterious effects (Brown et al., 1982). Penicillin G (1595 units mg^{-1}, 100 ppm) did not affect callus growth but inhibited plantlet formation. Benomyl (50% pure, 50 ppm), Dowicide (97% pure, 5 ppm), gentamycin (from a sterile ampoule, 50 ppm), Quintozene (PCNB; 99% pure, 100 ppm), and vancomycin (97.8% pure, 50 ppm) were inhibitory. All combinations of these compounds inhibited callus formation, growth, and plantlet development (Brown et al., 1982). These findings indicate that except in rare and unusual cases where a single compound may be used to eliminate contamination from a very valuable culture these substances are not suitable for orchid micropropagation.

The orchid phytoalexins, orchinol and loroglossol (Stoessl and Arditti, 1984), a synthetic analog (dehydroorchinol), a possible precursor of orchinol [3,4′-(dihydroxy-5-methoxydihydrostilbene)] and batatasin III (3,3′-dihydroxy-5-methoxydihydrostilbene) reduced the growth of *Cattleya aurantiaca* seedlings (Hills et al., 1984; see Fig. 1-54). This suggests that they may have the same effects on plantlets produced in tissue culture. Therefore these compounds should not be used to combat contamination without prior testing. Whether phytoalexins (those from orchids or other plants) in general may have similar or different effects on orchid seedlings and/or tissue cultures is not clear at present. Therefore their possible incorporation in tissue culture media will require screening in advance. An additional, and critical, problem with phytoalexins is their unavailability.

Several antibiotics have been used in tissue culture with plants other than orchids to control or prevent contamination (Table 2-9). Only a few of these substances were tested with orchids. Therefore they should be used with great caution and only following tests with expendable tissues and cultures.

A potentially very useful preparation which can be used to combat contamination was patented in the United States in 1998 by Dr. Assaf Z. Guri and Dr. Kishor N. Patel. The patent (US Patent 5,750,402) was assigned to Plant Cell Technology in Washington, DC (www.ppm4plant-tc.com). This preparation is a mixture of methylchloroisothiazolinone, methylisothiazolinone, magnesium chloride, and magnesium nitrate, which may also contain potassium sorbate or sodium benzoate, or both.

The preparation is amber-colored to clear liquid at pH 3.0–4.0 which has a mild, inoffensive odor. It is relative safe, but can generate toxic fumes (hydrogen chloride, nitrogen oxides, and sulfur oxides). Inhalation and eye and/or skin contact can cause irritation (Plant Cell Technology, no date *a–d*). Therefore protective gear and breathing masks are recommended by Plant Cell Technology (www.ppm4planttc.com/MSDS.htm).

TABLE 2-9. **Antibiotics, bactericides, and fungicides which can be useful in plant tissue culture media**[a]

Name	Activity[b]	Solubility[c]	Stability at 37°C, days	Storage temperature, °C	Recommended or reported concentration[d]	Remarks[e]
Actinomycin D	B	O, W		2–8	1.0 mg	
Aliette						See Fosetyl-AL
Amphotericin B	F	D, E, O	3	2–8	2.5 mg	T
Amphozone						See Amphotericin B
Ampicillin	B	W	3	2–8	100.0–400.0 mg	Pencillin-like
Apron						See Metalaxyl
Aureomycin	B				40.0 mg	
Banner						See Tilt
Bavistin					10.0–50.0 mg	
Benomyl (benlate)	F	E, W		Room	10.0–100 mg 1.0–2.0 g	T
Bleomycin sulfate		W		2–8	10.0–100 mg	
BMC						See Bavistin
Botrilex						See PCNB
Bravo						See Chlorothalonil
Carbendazim						See Bavistin
Carbendazole						See Bavistin
Carbenicillin	B	E, W		2–8	U 500.0 mg	Penicillin-like
Cefotaxime	B	W		2–8	100.0–1000.0 mg	
Cephalothin	B	W	3	2–8	100.0 mg	
Chloramphenicol	B	E, W	5	2–8	2.5–200 mg	
Chlorothalonil					250.500 mg	
Clotrimazole	F					10.0–50 mg
Ciprofloxacin					5.0–100 mg	
Clinafarm						See Imazalil
Cycloheximide	F	E			10.0 mg	
Daconil						See Chlorothalonil
Desmel						See Tilt
Dihydrostreptomycin	B	W	5	2–8	100.0 mg	
Diniconazole					125.0 µmoles l^{-1}	
Dithane	F				0.5–2 tbsp l^{-1}	Toxic, do not use
Efosite Al						See Fosetyl-Al
Enilconazole						See Imazalil
Erythromycin	B	E, H	3	2–8	100.0 mg	CS
Ethanol	B, F, M				2.5–50 ml	Restricted growth
Ethanol	B, F, M				10 ml	Deadly
Ethirimol					50.0 mg	
FB-5097	F					See Clotrimazole
Fosetyl-AL					1.5 mmoles l^{-1}	
Fungizone						See Amphotericin B
Geneticin		W	8	2–8	100.0–800 mg	
Hygromycin B		W		2–8	200.0–400 mg	
G418						
Gentamycin sulfate	B, M	W	5	2–8	50.0 mg	T, CS
Imaverol						See Imazalil
Imazalil						
Kanamycin monosulfate	B, M	W	5	2–8	100.0 mg	CS
Lincomycin HCl	B	W	4	2–8	100.0 mg	
Lotrimin						See Clotrimazole
MBC						See Bavistin
Metalaxyl					0.5–100 mg	
Miconazole					10.0–50 mg	
Micurb Super						See Ethirimol
Milgo						See Ethirimol
Milstem						See Ethirimol
Mitomycin C		W		2–8	10.0–50 mg	
MK-360						See Thiabendazole
Monostat						See Miconazole
Mycophenolic acid		Methanol		2–8	25.0 mg	
Mycosporin	F					See Clotrimazole
Nalidixic acid					12.5–200 mg	
Neomycin sulfate	B	W	5	2–8	50.0 mg	
Nystatin	F	E, O, SW	3	0	50.0 mg	T

TABLE 2-9. (***Continued***)

Name	Activity[b]	Solubility[c]	Stability at 37°C, days	Storage temperature, °C	Recommended or reported concentration[d]	Remarks[e]
Omadine, sodium					5.0 mg	T
Omnizole						See Thiabendazole
Orbit						See Tilt
Paromomycin sulfate	B	W	5	2–8	100.0 mg	CS
Pentochloronitrobenzene (PCNB)	F	E, O			100.00 mg	T
Penicillin G	B	E, W	3	2–8	100.0 mg	T
					1000.000 u	T
PPM	B, F	W	Extended	Room	2.0 ml	T, proprietary mixture
Polymyxin B sulfate	B	W	5	2–8	50.0 mg	
Propiconazole						See Tilt
Puromycin HCl		W		-0	10.0–100	
8-Quinolinol hemisulfate						
Quintozene						See PCNB
Radar						See Tilt
Ridomil						See Metalaxyl
Rifampicin					10.0–50 mg	
Spectinomycin dihydrochloride	B	W		2–8	7.5–20 mg	
Spotless						See Diniconazole
Streptomycin sulfate	B	W	3	2–8	100.0 mg	
Subdue						See Metalaxyl
Sumi-8						See Diniconazole
TBZ						See Thiabendazole
Termil						See Chlorothalonil
Terraclor						See PCNB
Tetracycline hydrochloride	B	W	4	-0	10.0 mg	
Thiaben						See Thiabendazole
Thiabendazole					10.0–50 mg	
Tibatin	F					See Clotrimazole
Ticarcillin	B	W			300.0 mg	
Tilt					1.0 g	
Timentin	B	W		2–8		
Trimethoprim					15.0 mg	
Trimysten	F					See Clotrimazole
Tylosin tartrate	B	W	3	2–8	8.0 mg	
Vancomycin	B	E, W		2–8	U 40.0–50 mg	T

[a]Most of these substances have not been tested with orchids (see Table 2-8) and their effects on orchid explants, callus, protocorms, protocormike-like bodies, seedlings, plantlets, and plants are not known. Therefore they should not be used without prior testing. In general, routine use of these compounds is not recommended. Their use should be attempted only in cases when rare and valuable cultures are in danger of being lost due to contamination. Technicians who use them should wear gloves, masks that prevent inhalation of the substances, and safety clothing that will not allow penetration through the skin. If this is not done those who come into contact with the substances can become sensitized to the substances or may have allergic reactions which can be dangerous. This precaution was recommended by Dr. Teoh Eng-soon of Singapore. Information in this table was obtained from a preprint by V. C. Pence and J. A. Sandoval (kindly made available by Dr. Valerie C. Pence of the Center for Research of Endangered Wildlife, The Cincinati Zoo and Botanical Garden); *Phyto*Technology Laboratories LLC (www.phytotechlab.com), Sigma (www.sigmaaldrich.com) and Bautista et al., 2002).

[b]Abbreviations: B, antibacterial, F, antifungal (including yeasts and molds); M, anti-mycoplasma.

[c]Abbreviations: D, DMF; E, ethanol; H, 2*M* HCl; M, methanol (probably also ethanol); N, NaOH; O, DMSO; SW, suspension in W, water.

[d]Abbreviations: g, g l^{-1}; ml, ml l^{-1}; mg, mg l^{-1}; u, units l^{-1}; U, up to.

[e]Abbreviations: CS, cold sterilize; T, tested with orchids.

PPM™ is heat-stable (i.e., it can be autoclaved), broad based, and effective against both bacteria and fungi because it "targets and inhibits multiple enzymes" (Plant Cell Technology, no date *b*). It can also be used to decontaminate tissues (Plant Cell Technology, no date *b–d*). Plant Cell Technology has detailed instructions regarding the use of PPM (Plant Cell Technology, no date *b–d*) on its web site (www.ppm4plant-tc.com/MSDS.htm). An important point to keep in mind is that PPM inhibits the growth of contaminants. It does not destroy them. This means that cultures can become contaminated after a while. Should this happen, the explants,

tissues, callus, seedlings, or plantlets should be decontaminated again with PPM (see below) and moved to fresh medium.

There are several reports in the literature regarding the use of PPM in tissue culture (for one example see Niedz and Bausher, 2002; for a longer list of references see Plant Cell Technology, no date *c*). However, information about its use with orchids is limited. Dr. Assaf Guri made the following suggestion: "Skip sterilization with sodium of calcium [hypochlorite] and soak the shoot tips in non-pHed 4% PPM solution in which full strength . . . basal salts are added [this means inorganic salts only of the medium which will be used to culture the explant]. I can't tell you the exact exposure time but I'll suggest from 1 to 4 hours [those who plan to use PPM will have to experiment] during which the tips are very gently agitated without Tween 20. Without rinsing place the tips into the proper medium with 0.1% PPM." Professor Victor M. Jimenez of the University of Costa Rica suggested using 0.2% PPM but pointed out that contaminations may set in after 3 months. Should this happen, his recommendation is to move the plant material to fresh and clean medium. However, if the contamination is excessive it cannot be removed. Therefore cultures should be examined daily and tissues must be moved at the first sign of contamination.

A document by Roger Nick of Spring Orchids Laboratory on the Plant Cell Technology web site (www.ppm4plant-tc.com/ppp.htm) describes the use of PPM for the sterilization of orchid seeds and plants. Unsterilized seeds of *Cattleya*, *Phalaenopsis*, and *Dendrobium* hybrids harvested at the green capsule stage were placed on half-strength MS medium (Murashige and Skoog, 1962) supplemented with 20 g sucrose l^{-1}, 10 mg thiamine l^{-1}, 1 mg pyridoxine l^{-1}, 1 mg niacin l^{-1}, and 100 mg *myo*-inositol l^{-1} solidified with 8 g agar l^{-1} with and without 2 ml PPM l^{-1}. Germination and growth were not affected by the PPM. After 2 months of growth there was no contamination and there were no differences between seedlings on PPM-free and PPM-containing media. This method is effective with seed from capsules which split prematurely.

Seedlings which became contaminated following subculture from flasks where the seeds were germinated initially could be cleaned by placing them in a solution of 20 ml PPM l^{-1} for 24–36 h on a rotary shaker and moving them after that without rinsing to half-strength MS containing 2 ml PPM l^{-1}. The seedlings did not become contaminated again and grew normally.

When *Phalaenopsis* stem sections are washed with a mild detergent and water, submerged in 25% Clorox solution with Tween 20 for 25–30 min, rinsed in sterile distilled water three times, placed in quarter-strength MS for 24 h and cultured on half-strength MS, 25–30% become contaminated. If 20 ml PPM l^{-1} are added to the sterilization process, contamination drops to 5%. Some *Phalaenopsis* hybrids are more sensitive to PPM than others.

An internet inquiry on discussion groups dealing with orchids (Orchid Guide Digest) and tissue culture (plant-tc@tc.umn.edu) elicited several responses:

- Professor Michael E. Compton, University of Wisconsin-Plateville wrote: "I have used PPM at . . . 2 ml/l for meristem cultures of *Oncidium* without ill effects. However, PPM at this rate does not protect meristems from heavy microbial infections."

- Marty Kalin from Plant Cell Technology wrote that when orchid seeds that were surface-sterilized for 10 min with 10% Clorox, suspended in solution of 4 ml PPM l^{-1} and placed on autoclaved medium "without the benefit of a laminar flow hood in a non sterile environment (an open room) approximately 10% (of 255 flasks) became contaminated vs an expected 45–60%." Germination of seeds of "several genera . . . including temperate terrestrial and tropical species" were not affected by the PPM.
- Esteban McGrath, a *Hibiscus* grower from Puerto Rico who used to grow orchids, suggested that PPM should be used to sterilize both seeds and utensils. He reported losses that did not exceed 2%.
- Simon M. Wellinga of SymPhyto in the Netherlands wrote: "Back in 1998 we ordered a couple of 100 ml bottles and tried PPM with our cultures, which at that time consisted mostly of botanical *Cattleya* and *Laelia* species. We did so out of curiosity and to find out whether this mixture would meet expectations. In an initial trial PPM was both used as an ingredient of our standard germination media and as a cure for contaminated seedlings, which after treatment were replated onto media either containing PPM or without it. Although PPM was used strictly according to the recommendations that came with the product and while following our standard laboratory practices (work done in a laminar flow hood, autoclave cycles as usual, etc.), all our experimenting was not planned ahead in what one would call a sound and statistically justifiable setup. Therefore our results cannot be claimed to be scientific evidence, and neither have they been published. The reason that we only tested PPM qualitatively, and not quantitatively, was that we would never have been able to utilise this ingredient on a larger scale, both for economical reasons and because of international phytosanitary regulations. Since a considerable part of our seedlings is sent in flask on sterile medium to non-EU destinations (the only way one can get *Paphiopedilum* species and other Appendix I species across international borders), we are not allowed to incorporate any antibiotics in our export flasks or any other compounds that temporarily suppress or mask infections.

  For what it is worth – although we made sure to follow the instructions that came with the product, we never bothered to share our experiences with others – we found that in seed cultures on medium containing PPM infections would still occasionally show up, and we have never been able to clean contaminated cultures with the help of PPM, something which with some luck can be achieved in cases of hard-leafed material as *Cattleya* and *Paphiopedilum* with sodium hypochlorite treatment. In those instances in which treated cultures of infested seedlings seemed to be clean, infection would immediately show up again after replating to PPM-free medium, and this is why we were led to believe that PPM merely suppresses infections but does not eradicate them altogether.

  I understand you are looking for information and references for the forthcoming new edition of 'Micropropagation of Orchids' and am sorry that the information above is only anecdotal."

More recently PPM was tested for its ability to control contamination in seed and seedling cultures of *Vanda sanderiana* seedlings. At 4 ml l^{-1}, PPM eliminated contamination (Bautista et al., 2002).

Altogether it seems that PPM can be useful for surface decontamination and keeping media free of contamination in orchid seed germination, seedling culture and micropropagation, but only after additional tests and experiments. PPM is available from www.ppm4plant-tc.com.

Charcoal

A clear distinction must be made between carbon black and activated charcoal. Both will turn culture media black, but this is where the similarity between them ends. Carbon black (lampblack, furnace black, channel black, and acetylene black) is a generic name for black pigments of submicron size formed by thermal degradation of hydrocarbons. These pigments are not adsorbents and are used for inks, paints, and reinforcing agents for rubber products.

Activated carbon or charcoal (Fig. 2-1) is characterized by an extremely large area : weight ratio (up to 2000 $m^2 g^{-1}$) and is used for the adsorption of substances. Both animal and vegetable charcoals are available, but the latter are preferable for culture media. They are leached during preparation to remove contaminants, but some ions still remain (Table 2-10). Pore sizes may also vary; those in decolorizing carbons are larger.

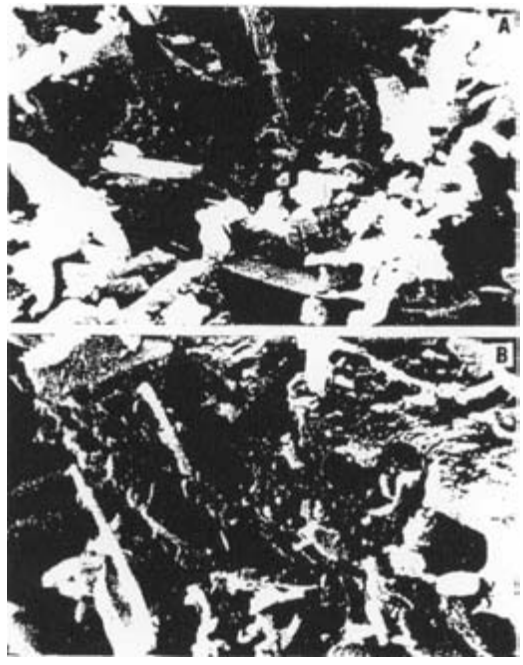


FIG. 2-1. **Scanning electron photographs of charcoal particles. A. Variation in size and shape of particles (× 10,876). B. Xylem-like structure (× 2175). (Courtesy Dr. M. A. Weatherhead, Dept. of Botany, Univ. of Hong Kong.)**

TABLE 2-10. **Mass spectrographic analysis of charcoal (Weatherhead, 1979)**

Element	Concentration[a]	Element	Concentration[a]	Element	Concentration[e]
Aluminum	90	Holmium	< 0.3	Rhuthenium	0.5
Antimony	0.5	Indium	0.2	Rubidium	0.5
Arsenic	0.1	Iodine	0.2	Samarium	< 0.9
Barium	0.2	Iridium	< 0.5	Scandium	< 0.9
Beryllium[b]	3	Iron[e]	330	Selenium	0.3
Bismuth	< 0.35	Lanthanum	0.23	Silicon	970
Boron	5	Lead	< 0.6	Silver	0.2
Bromine	0.6	Lithium	< 3	Sodium	9
Cadmium	0.6	Lutetium	< 0.3	Strontium	0.2
Calcium	46	Magnesium	900	Sulfur	60
Carbon	ND[c]	Manganese	9	Tantalum[c]	< 1.5
Cerium	0.25	Mercury	< 1	Tellurium	0.6
Cesium[d]	0.5	Molybdenum	0.7	Terbium	< 0.3
Chlorine	120	Nickel	2.2	Thallium	< 0.5
Chromium	3	Niobium	0.1	Thorium	< 0.4
Cobalt	≤ 0.1	Nitrogen	ND[c]	Thulium	< 0.3
Copper	3	Osmium	< 0.8	Tin	0.6
Dysprosium	< 0.9	Oxygen	ND[c]	Titanium[e]	< 0.37
Erbium	< 0.8	Palladium	0.3	Tungsten	< 1
Europium	< 0.5	Phosphorus	30	Uranium	< 0.4
Fluorine	ND[c]	Platinum	< 1	Vanadium	< 0.3
Gadolinium	< 1	Potassium	9	Ytterbium	< 0.9
Germanium	0.4	Praseodymium	< 0.3	Yttrium	0.15
Gold	< 0.3	Rhenium	< 0.5	Zinc	0.7
Hafnium	< 1	Rhodium	0.2	Zirconium	0.2

[a]Number of atoms of the element per 10^6 atoms of carbon.

[b]Interference from aluminum peak.

[c]Not detected.

[d]Cesium atomic line.

[e]Determined by microdensitometry.

The first attempt to darken a culture medium used for orchid seed germination was made in an effort to germinate native American *Cypripedium* species (Curtis, 1943). Lampblack (3 g l^{-1}) was used for this purpose, but germination was very poor. These species do not germinate well asymbiotically even at present and it is safe to assume that the lampblack had no effect of any kind. The addition of animal charcoal to a culture medium used for *Cymbidium* plantlets improved differentiation and plantlet growth but reduced the proliferation of protocorm-like bodies (PLBs) and the formation of aerial roots (Werkmeister, 1970*a*, 1970*b*, 1971). Darkening the culture vessels with black paper had a similar effect. Dr. Peter Werkmeister was the first to use charcoal to darken orchid culture media (for an historical account see Arditti and Krikorian, 1996). He did it to study the effects of dark media on root growth and development.

Activated vegetable charcoal (Nuchar C, 2 mg l^{-1}) improved seedling growth of the terrestrial *Paphiopedilum* (Ernst, 1974) and the epiphytic *Phalaenopsis* (Ernst, 1975, 1976) orchids. These observations led to the development of practical charcoal-containing media which gained widespread, rapid acceptance. In addition to being incorporated into media used for seedlings, charcoal is now added to many substrates employed in the tissue culture of orchids and other plants (for a few examples see Reuveni and Lillien-Kipnis, 1971; Nakamura and Itagaki, 1973; Anagnostakis, 1974; Irikura, 1975*a*, 1975*b*; Bajaj et al., 1976; Wang and Huang, 1976; Wernike and Kohlenbach, 1976; Weatherhead et al., 1978, 1979; Wann, Veazey and Kaphammer, 1997; for reviews see Yam et al., 1990; Arditti and Krikorian, 1996).

The beneficial effects of charcoal in culture media for filamentous algae and moss protonema were attributed to: (1) darkening which simulated soil conditions (Proskauer and Berman, 1970); and (2) adsorption of unidentified morphogenetically active (Klein and Bopp, 1971), toxic (Wang and Huang, 1976), or harmful (Weatherhead et al., 1979) substances.

One possible explanation of the effects of charcoal on orchid seedlings or tissue-culture-derived plantlets is that it improves aeration. Growth similar to that on charcoal-containing media was also observed when seedlings of *Paphiopedilum* and *Phalaenopsis amboinensis* were grown on Pyrex glass wool alone or in combination with Nuchar C vegetable charcoal (Ernst, 1974, 1975, 1976; Arditti, 1979; Arditti and Ernst, 1984).

A second possibility is that the charcoal adsorbs ethylene (Ernst, 1975), which can inhibit growth and differentiation. Another plausible explanation, based on careful studies of absorption characteristics and media changes during autoclaving, is that charcoal adsorbs and therefore renders harmless: (1) 5-hydroxymethylfurfural, which is produced by the dehydration of sucrose during autoclaving and is inhibitory to the growth of tobacco anthers in vitro (Weatherhead et al., 1978); and (2) inhibitory phenolics and carboxylic compounds produced by the tissues (Fridborg et al., 1978; Weatherhead et al., 1979). Charcoal can also adsorb plant hormones and vitamins and this may explain the fact that it can also be inhibitory to growth (Fridborg and Eriksson, 1975; Constantin et al., 1977; Fridborg et al., 1978; Weatherhead et al., 1978).

It is entirely possible, of course, that both of these explanations are correct, especially since Pyrex glass wool has not been shown to adsorb phenolics, hormones, and other factors (Ernst, 1974, 1975, 1976). Altogether "it would seem . . . that addition of charcoal to culture media can have a considerable effect on the composition [and aeration] of . . . media. Where the addition results in an increased response . . . it would appear pointless to include . . . components which are strongly adsorbed . . . [however, even after strong adsorption a part and/or all of these components may remain in the medium in an available form] . . . Conversely . . . adsorption of media components can lead to inhibition . . . This leads to the interesting possibility that there may be species whose tissue growth may be inhibited by phytotoxin production, and for which the addition of charcoal to negate this may lead to another type of inhibition by removal of essential nutrients" (quote from Weatherhead et al., 1979 with added comments in square brackets).

As already mentioned charcoal has the capacity to adsorb hormones and vitamins and thereby inhibit growth (Fridborg and Eriksson, 1975; Constantin et al., 1977; Fridborg et al., 1978; Weatherhead et al., 1978, 1979). For example, the thiamine level in charcoal-containing medium was 23% of that in a substrate which did not contain the darkening agent (22 mg 100 ml^{-1} vs. 96 mg 100 ml^{-1}). Niacin is absorbed even more extensively by charcoal (3.5 mg 100 ml^{-1} vs. 101 mg 100 ml^{-1}). Up to 97% of IAA and IBA can be absorbed by charcoal concentrations of up to 5% (Nissen and Sutter, 1990). On the other hand, inositol is not adsorbed by charcoal (99.6 mg 100 ml^{-1} vs. 99.8 mg 100 ml^{-1}) (Wetherhead et al., 1979).

When the pH of a medium was adjusted to 5.8 after the addition of charcoal but before autoclaving, the pH dropped to 5.4 (Sigma-neutralized charcoal), 5.9 (Sigma-acid-washed charcoal), or 5.4 (Merck No. 2186, acid-washed NuChar, and

SN-neutralized NuChar; Wann et al., 1997). In another experiment the pH of a charcoal-containing medium increased before autoclaving from 5.8 to 6.9 in the presence of 0.5% Sigma-neutralized charcoal and dropped to 6.6 after sterilization. If 0.5% Sigma-acid-washed charcoal was added the pH dropped from 5.8 to 5.7 and increased to 6.4 after autoclaving (Wann et al., 1997). In the presence of 0.5% Merck charcoal No. 2186, the pH increased from 5.8 to 6.1 and dropped to 5.7 after the medium was autoclaved (Wann et al., 1997). The pH dropped from 5.8 to 5.1 following the addition of 5% acid-washed SA NuChar and decreased further to 4.7 after the medium was autoclaved. The addition of neutralized NuChar brought about a drop to 5.3 and a post-autoclaving pH of 4.5. There was no preautoclaving drop of pH in a charcoal-free medium; the post-sterilization pH was 5.5 (Wann et al., 1997).

The addition of 5 mmol morpholinoethane sulfonic acid (MES) buffer caused the post-autoclaving pH to drop from 5.5 to 3.5 (Wann et al., 1997) and only 3% of the sucrose was hydrolyzed. In the presence of 0.5 or 0.1% activated charcoal – and when the pH dropped from 5.5 to 3.6 or 3.5 – 55 or 60% of the sucrose, respectively, is hydrolyzed. When the pH dropped to 3.8 in the presence of the 0.2% activated charcoal, sucrose hydrolysis was 14% (Wann et al., 1997).

Given these findings, charcoal should be added with caution to media that contain additives which may be adsorbed, especially if they are required by the tissues. On the other hand if explants, tissues, plantlets, and seedlings grow well on a medium that contains both charcoal and the additives it adsorbs there is no reason to make changes. In such cases it is clear that either the charcoal does not adsorb enough of any one compound to affect growth or, if it does, the substance in question is not required, or is only needed at the levels which remain in the medium. If darkening of a medium is necessary and charcoal can not be used, 2 g graphite l^{-1} (Thurston et al., 1979) can be employed instead.

Orchid tissues grow equally well on sucrose and on its components (and hydrolysis products) fructose and glucose. Therefore the effects of charcoal on hydrolysis of sucrose may be of limited, if any, importance.

Vegetable charcoal may be listed under several headings (which are not always clear) in catalogs or web sites. To ensure purchase of the appropriate charcoal, it is best to contact the suppliers and inquire.

Solvents

Distilled water must be used as a solvent in the preparation of culture media (Fig. 2-2). When this is not available deionized water may be used. Low-sodium water is the next choice.

Rain water collected in a glass or plastic container, in areas where the atmosphere is not polluted and/or rain is not acid, can also be employed. Tap and well water should be avoided, but can be used for practical (i.e., non-research) purposes after testing.

Ethanol (ethyl alcohol, 70% in distilled water) should be used as a sterilizing solvent for substances that cannot withstand autoclaving. Methanol (wood alcohol) or denatured ethanol should not be used because they are toxic.

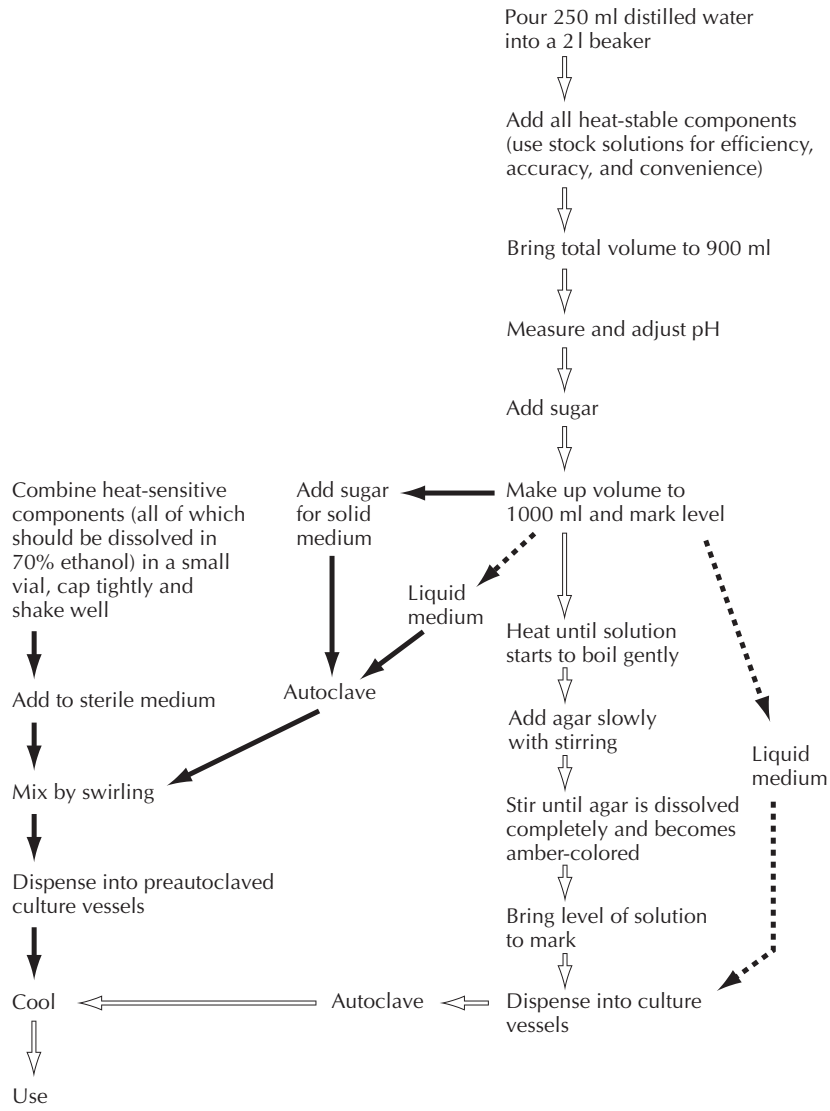
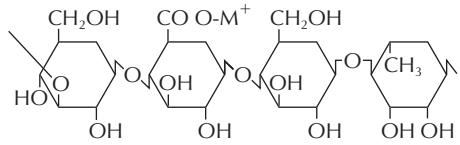


FIG. 2-2. **Preparation of culture media. Open arrows: solid media that contain only heat-stable components. Open arrowheads with dashed lines: liquid media that contain heat-stable substances. Solid arrows: solid media that contain heat-sensitive compounds. Solid arrowheads with dashed lines: liquid media that contain heat-sensitive ingredients. (Arditti, 1982*a*.)**

Solidifiers

Agar was the major solidifier used for orchid seed germination and micropropagation media for several decades. More recently, gellan gum sold under a number of brand names (Gelrite® and Phytagel™ are two of them) is being used in an increasing number of formulations (see below). Several types of agar are available from commercial sources. They differ from each other in a number of characteristics



Molecular weight $2–3 \times 10^5$ Daltons
Soluble in hot or cold water
Ash, 9.5%
Set temperature 30–50°C
Thermostable
Gelling temperature 27–31°C
pH at 1.5%, 6.5–7.0
Heat stable
Ca, 0.85%; K, 1.70%; Mg, 0.35%;
P, 0.15%; Na, 0.45%
(1 Dalton = 1.66×10^{-24} g)

FIG. 2-3. **Structure of gellan gum.**

(Table 2-11). For experimental purposes it is necessary to use reagent-grade agar (Table 2-11). Technical grade or kitchen quality agar can be used for practical micropropagation, but preliminary tests are advisable.

A widely used procedure is to add the agar slowly and with stirring to the medium at room temperature and to bring the mixture to a gentle boil. After the agar has dissolved, the medium is autoclaved before or after dispensing it into culture vessels. Another method is to disperse the agar through a cold solution and dissolve it by autoclaving.

Gellan gum (Phytagel, Gelrite) is an agar substitute consisting of glucuronic acid, rhamnose, and glucose (Fig. 2-3) produced through fermentation by *Pseudomonas elodea*. It was discovered by the Kelco Division of Merck and Co., in San Diego, California (Sanderson and Clark, 1983; O'Neil et al., 1983; Shungu et al., 1983; Kelco, 1985, 2002). The gum produces a high strength, colorless, and clear gel.

The most commonly used concentrations for plant tissue culture are 1.2–2 g l^{-1}. Phytagel and Gelrite require the presence of divalent cations for gelling. Most orchid culture media contain enough calcium and magnesium. If more dilute media are used, higher concentrations of Phytagel or Gelrite may be required.

Gellan gum (Phytagel, Gelrite) must be added to media at room temperature with rapid stirring to avoid the formation of lumps. If these gelling agents are added to hot or even warm solutions, lumps will form and the medium will not gel even after autoclaving.

pH

The term pH is indicative of the alkalinity or acidity (i.e., hydrogen ion concentration) of a medium. It is defined as the logarithm of the reciprocal of the hydrogen ion concentration, $pH = \log 1/H^+$. Moderate acidity is indicated by pH 6 to 7 ($H^+ = 10^{-5}$ to 10^{-6}); pH 5 to 6 is mildly acid, and pH below 5 is increasingly acid; pH 7 ($H^+ = 10^{-7}$) is neutral and pH 7 to 8 ($H^+ = 10^{-8}$ to 10^{-7}) is slightly alkaline. A value of pH 8 ($H^+ = 10^{-8}$) is mildly alkaline and a pH of 9 ($H^+ = 10^{-9}$) or above is increasingly alkaline. Of the important chemical elements in soil, phosphorus is available at a pH from about 4.5 to approximately 8.1; its availability is reduced between ca. pH 7.6 and 8.5 and increases after that. The availability of nitrates is best between pH 5.5 and 8 and drops after that. Magnesium is available mostly from pH 5.0–5.5 up to pH 8.5–9.0. Calcium is available from pH 5.0–5.5 to pH 8.5–9.0. The

TABLE 2-11. **Typical analyses and some characteristics of reagent-grade agars from three companies**[a]

	Difco				USB			Sigma						
								Breakdown by						
Component or characteristic	Bacto	Noble	Purified	Bitek	Bacteriological	Noble	Agar	A	E	M	High Gel	Purified	AgarGel	PhytaGel
Ash, %	4.5–6.5	2–2.6	1.75–2	6.5	3–6.5	1.6	4–6	5–6	3–4	3–6	3–4	2	4–5	
Barium, %	0.01	0.01	0.01											
Cadmium, ppm	0–0.5													
Calcium, ppm	300–3000	100–2600	2000–5000											
Chloride, %	0.43	0.18	0.13											
Chromium, ppm	0–0.1													
Cobalt	0													
Copper, ppm	0.5–1.5													
Iron, ppm	1.5–5.0													
Lead, ppm	0–0.5													
Magnesium, ppm	50–1000	0–750	400–1500											
Manganese, ppm	0–0.5													
Nitrogen, %	0.17	0.10	0.14											
Silica, %	0.19	0.26	0.09											
Sulfate, %	2.54	1.90	1.32											
Titanium	0													
Zinc, ppm	5–10													
Color of dry form	Very light beige	Off-white												
Color of solution	Very light amber	Clear	Colorless to very opalescent	Light to medium amber										
Clarity[b]	Clear, < 10 nephelometric units	Clear to very opalescent	Clear to very slightly opalescent	Slightly opalescent to opalescent										
Consistency	Granular	Powder	Powder	Free-flowing										
Gelation point (1.5%), °C	32–39	32–39	32–39	32–40	33–38	32–37.5	33–34	35–37	33–35	36	35–37	35–37	34–37	29–31
Gel pH (1.5%), (5%)					5.5–7.5	5.8–7	7–7.5	7.2–7.7	7.5–8	7–7.5	6.5–7	6.5–7	7.2–7.7	6.5–7
Gel strength (1.5%), g cm^{-2}					630–750	700	700	650–750	750–900	550–700	≥ 1000	< 800		
Loss on drying, %	16–20			≤ 20										
Melting point, °C	83–89	≥ 85	≥ 85	83–89	80–90	80–95								
Moisture, %	11–20			≤ 6	5–11									
Suggested concentration[c], %	1–2	1.5	1–2	1–2										
Absorbance, max. 430 nm						0.15								
Resistance, Ohms-min × 1000						25								
Electroendosmosis, pH 8.4 max.						0.45								

[a]*Sources*: Several releases from Difco Laboratories, P.O. Box 331058, Detroit, MI 48232-7058; US Biochemical Corp. (USB), P.O. Box 22400, Cleveland, OH 44122; and Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178. When two or more values are given for the same agar they were obtained from different documents supplied by the source(s).

[b]Light scattering qualities.

[c]These concentrations do not necessarily apply to culture media for orchids.

availability of iron is best between pH 4.0 and pH 6.5–7.0. Manganese, boron, copper, and zinc availability is best between pH 5 and pH 7.5. Molybdenum is available from pH 4 to pH 10.0.

It should be clear from the above that pH is important because it affects the availability of nutrients. It also has a direct effect on the life processes of cells. Further, if media of very low or very high pH are autoclaved the agar and other components may be hydrolyzed and/or destroyed. Hydrolysis of agar may release its major components, the sugars D- and L-galactose, which are toxic to plants in general and orchid seedlings (and probably also tissue cultures) in particular (Arditti and Ernst, 1984). For all of these reasons it is very important to adjust the pH of media carefully. In all cases it is best to set the pH to the levels recommended by the original investigators. Departures from recommended values should be avoided or undertaken only for good reasons or following experiments.

The pH of culture media should be as indicated in each method, determined experimentally, or adjusted to 4.8–6.0. Solid media may not solidify if the pH is much below 4.0 or higher than 8.0. Growth may be inhibited if the pH is lower than 4.0, higher than 8.0, or inappropriate for the plant being cultured. To measure the pH of a medium accurately it is best to use a pH meter. If one is not available, pH indicator paper may be used. When the pH of a medium is above the desired value, it is too alkaline and must be adjusted down with a few drops of acid (hydrochloric, nitric, phosphoric, or sulfuric). Should the pH be lower than required, the medium is too acid and must be adjusted up with a base (alkali) such as ammonium hydroxide, potassium hydroxide, or sodium hydroxide. Concentrated acids or bases can change the pH of a medium very rapidly and should not be used. For reasons of safety, convenience, and accuracy they should be diluted before use. A suitable concentration of acid or base for adjusting pH is 0.1N.

Recipes in this Book

In this book all recipes for media are given as published in the original papers. When a procedure lists a medium by reference citation without a recipe, the formulation given in this book is the one presented in the cited paper [for example, if a paper stated "tissues were cultured in the Murashige–Skoog medium (Murashige and Skoog, 1962)," the recipe given in this book was taken from Murashige and Skoog, 1962]. If specific modifications were made and described they are listed, and when no other details are available the remainder of the recipe is given as presented in the original paper. When the original paper provides a complete recipe this is the medium presented in the book. Recalculations and substitutions of components, including iron and chelating agent, were not made since their effects could not be predicted. Or, if changes were made, they were clearly labeled as such.

Stock Solutions

To save work and increase accuracy it is advisable to prepare stock solutions of most media components. These are concentrated solutions (10, 100, or even 1000 times)

of each compound. Stock solutions save work because only one weighing is necessary to prepare enough concentrate for 10, 100, or even 1000 liters. They increase accuracy since larger amounts are weighed and because it is easier (and faster) to measure large or small volumes of solution accurately than it is to weigh solids.

To prepare a stock solution weigh the required amount as indicated in each recipe (given in the tables), and add distilled water to the desired final volume. Label the bottle with the following information and store in a refrigerator:

1 Name of compound.
2 Formula of compound.
3 Concentration of stock solution (10×, 100×, or 1000×).
4 Amount to use per liter of culture medium.
5 Date.
6 Name of the person who made the solution (each).

Individual stock solutions should be prepared for each macroelement, vitamin, amino acid, or hormone. All microelements should be combined into one stock solution. Stock solutions containing nitrogen (as NO_3^-, NH_4^+, or urea) tend to become contaminated on standing. Therefore, these substances should be weighed every time, or if stock solutions are prepared, they must be kept frozen between uses.

Stock solutions of hormones and vitamins should be in ethanol (ethyl alcohol). If necessary, a few drops of sodium or potassium hydroxide or hydrochloric, sulfuric, nitric, or acetic acid can be added to the alcohol to increase the solubility of some substances. The use of ethanol not only prevents contamination of the stock solution, but also eliminates the need for sterilization since alcohol is a sterilant. However, under conditions of very high humidity (e.g., in Fiji, according to M. Krishnamurthi of the Fiji Sugar Corporation Experimental Station in Lautoka) solutions of 70% ethanol may become contaminated. To prevent such contamination, stock solutions in 70% ethanol should be stored in a freezer or made in 95% ethanol.

Hormones, vitamins, and amino acids may not be stable for prolonged periods. It is best, therefore, to prepare only small volumes (10–15 ml) of stock solutions. For 10 ml of stock solution, weigh carefully the required amount of substance and place it in a volumetric flask (see Appendix 1 for descriptions, and Appendix 2 for sources of glassware). Then add 5 ml of 100% (absolute) or 5.2 ml of 95% ethanol (do not use methylated spirits or any other form of denatured ethanol), and shake the flask gently. If the substance fails to dissolve, add a drop or two of dilute acid (for kinetin) and shake again (for auxin, add sodium hydroxide). Should it be necessary, one or two additional drops may be added. After the substance has dissolved completely, add another 2 ml of absolute ethanol (or 2.1 ml of 95%) and then make up the volume to 10 ml with distilled water for 70% ethanolic solution. When a 70% solution is undesirable, make up the volume to 10 ml with 95% ethanol. For 20 ml of stock solution double the amount of substance and volumes of ethanol and water. Use 2.5 times as much for 25 ml of stock solution, and multiply by 5 for 50 ml. When making larger volumes, always keep the number of acid or sodium hydroxide drops used to increase solubility to a minimum.

Stock solutions of organic substances should be stored in a freezer or refrigerator. Do not make stock solutions of inositol, sugar (sucrose or good-quality pure white refined kitchen sugar), or agar.

State of the Medium

Both solid and liquid culture media are used for orchid tissue culture, callus maintenance, plantlet regeneration, and orchid seed germination.

Sterilization

Culture media, tools, working space, and tissues must be sterilized, and work has to be carried out under aseptic conditions to ensure success. If these precautions are not taken, cultures will become contaminated and fail. A number of methods are used to ensure sterility.

Autoclaves

Spores of microorganisms in liquid (i.e., culture media) can survive elevated temperatures under normal (i.e., atmospheric) pressure. Therefore culture media must be sterilized under high temperature and pressure (usually 121°C and $1.2 \cdot 10^5$ Pa which can also be expressed as 121°C and 1 atm, or 15 psi, or $6.8 \text{ kg} \cdot 6.5 \text{ cm}^{-2}$). Such conditions are generated in autoclaves. A large number of autoclave models and sizes are available (see Appendices 1 and 2). Some require electricity, others do not. Several models are very complex and fully automatic, but there are also types that differ very little or not at all from a kitchen-type pressure cooker. Indeed, if an autoclave is not available, media, tools, culture vessels, and other items can be sterilized in a kitchen pressure cooker. Inorganic components (macroelements, microelements), sugars, agar, and some complex organic additives (coconut water, casein hydrolysate, peptone, yeast extract, banana homogenate, etc.) can be heat-sterilized. The standard conditions for sterilization can be obtained automatically in autoclaves or very easily in pressure cookers, and the entire sterilization process is very simple.

Filtration

Some media components are destroyed by elevated temperatures and cannot be heat-sterilized. Solutions containing these substances may be sterilized by passing them through very fine sterilizing filters (see Appendix 1; one example is the Millipore brand filter), which permit the passage of liquids but not particles larger than 0.22 or 0.45 μm and thereby retain all contaminants.

Some sterilizing filters can be used for a number of solvents; others are suitable only for water. Therefore it is important to read all instructions carefully. Large filtration apparatus requires vacuum pumps and other sophisticated equipment and may be expensive. For this reason filter sterilization is not recommended for small laboratories. Small disposable filter units that can be operated manually with a syringe are available from several sources (see Appendices 1 and 2). These units are well suited for small laboratories.

Sterilization in Microwave Ovens

Microwave ovens have been used to sterilize plastic tissue culture vessels (Latimer and Matsen, 1977; Sanborn et al., 1982) and Knudson C medium (Smith, 1986), as well as Murashige–Skoog, Vacin and Went, Hill's, oat, and other media (Wood and Lundergan, 1981; Marlow and Muir, 1986; Nelson, 1990; Tisserat et al., 1992). Some remained sterile for long periods, others (including the Hill's and oat media) became contaminated 5, 10, and 20 days after the sterilization.

In one of the procedures (Smith, 1986), the agar and salts (2.47 or 2.27 g of commercial preparations) and water (66.67 ml) were placed in a flask that was stoppered with a stopper sterilized by washing it in household bleach. This flask was placed in a Sharp microwave oven Model R-6210, which has an output of 600 watts. The flask was allowed to "cook" under full power until the contents started to boil. After that the flask was taken out, agitated, and "cooked" again until the contents started to boil. The medium is sterile after the second boiling and the stopper should be covered with aluminum foil which has been sprayed with a disinfectant spray or submerged in 70% alcohol for 20 min.

For the second procedure (Marlow and Muir, 1986), 70 ml of medium were placed in 250-ml flasks stoppered with cotton which was soaked in 10% (v/v) household bleach (10 ml Clorox, Purex, or Domestos made up to 100 ml with distilled water). The flasks were placed in the oven, which was turned on at full power for approximately 70 s (for a single flask) to melt the agar.

An alternative procedure is to place 500 ml of medium in a 1-l flask, melting the agar by turning the oven on for 5–6 min and dispensing 70 ml of the solution per 250-ml flask. The melted agar (regardless of the method used to melt it) should be agitated to mix the contents and placed in the oven again until the medium starts to boil for a second time. As with the previous procedure the medium is sterile after the second boiling.

One simple and effective procedure "was devised by a 12-years-old person" (Nelson, 1990), utilizing a 1000-watt microwave oven: " 'Cook' [medium] in microwave oven on high for three minutes . . . pour one-half cup liquid [medium] into each [one quart orange juice bottle] . . . insert stoppers [No. 10 drilled rubber stoppers with cotton stuffed in the hole] on an angle to let steam out . . . 'cook' in microwave oven on high until boiling (about four minutes). Reduce power level to 5 and cook for 11 minutes. Press corks down before removing [bottles from oven] with kitchen towel (bottles are hot). Then push stoppers in completely."

A comparative study (Tisserat et al., 1992) of power output, duration of exposure to microwaves, and presence in the oven of an energy sink water reservoir (ESWR, two 1-l Pyrex bottles containing 900 ml of distilled water and capped loosely with polypropylene screw caps) with a Sharp Carousel II microwave oven Model R-5E80 produced mixed results but showed that such ovens can be used to sterilize media. However, successful sterilization depended on power, volume of medium being sterilized, and the number of vessels being sterilized. "Using 700 W of power, liquid medium volumes containing 3% sucrose required the following sterilization times (ml, min): 100, 5; 250, 10; 500, 10; 1000, 15; 2000, 30 and 3000, 50. Fifteen 95×100 mm polycarbonate containers, each containing 50 ml agar medium also could be sterilized using 700 W for 15 min. Ten culture tubes 25×150 mm, containing

25 ml of agar medium could be sterilized in 15 min using 350 W or 10 min using 700 W when two 1-liter ESWR, each containing 900 ml distilled water, were included in the microwave. Syringe type filter holders [presumably plastic] with 25-mm-diameter filters, could be successfully sterilized by microwaving at 700 W for 5 min with an ESWR included."

These are clear results and useful instructions, but those who plan to sterilize culture media in microwave ovens should carry out preliminary tests. Important precautions and considerations regarding sterilization of media in microwave ovens are:

1. Completely sealed vessels such as screw cap jars or culture tubes must not be used under any circumstances since pressure will build inside them and they may explode. If such vessels must be used, the caps should be screwed on loosely or a hole must be drilled in them to prevent the build up of pressure. When a hole is drilled it must be filled with cotton to prevent subsequent contamination.
2. Metal caps must not be used.
3. Cotton stoppers (or plugs in holes) must not be allowed to come in contact with the medium since this will lead to subsequent contamination.
4. Media that contain complex additives (like oatmeal) should not be sterilized in this manner since contaminants (yeast spores for example) in these components may escape destruction and contaminate the medium later.
5. Since the effects of microwave sterilization on many components of media are not known, preliminary tests must be carried out before large-scale use. For example, microwave oven sterilization seems to reduce GA_3 (gibberellic acid or gibberellin 3) activity (Tisserat et al., 1992).
6. Aluminum foil and any other metallic objects must never be placed in a microwave oven.
7. Both containers and solutions can be very hot after microwave sterilization and should be handled with great care to prevent injury.
8. Flammable solutions should not be sterilized in a microwave oven because vapors may form, be ignited by sparks from the fan, and explode.
9. Living tissues and plants must not be sterilized in a microwave oven. The microwaves will kill or "cook" them.
10. Ovens should be checked regularly for microwave leakage.

Solvents

A simple way to sterilize heat-labile substances is to prepare their stock solutions in 70% or 95% ethanol (ethyl alcohol) in distilled water since this solvent is also an excellent sterilant. Our experience is that the addition of up to 5–6 ml of 70% ethanol per liter of medium does not have a deleterious effect on cultures. If the stock solutions are prepared properly, it is not necessary to add more than that. Methylated spirits or other forms of denatured ethanol should not be used for this purpose.

Open Flame

Burning can be used to sterilize tools and the necks of bottles while making cultures. A natural-gas burner is best because it produces a clean, non-smoking, high-

temperature flame. If one is not available, an alcohol flame (methylated spirits or denatured ethanol can be used as fuel) can be used, but it may not be hot enough. Another possibility is to dip the tools to be sterilized in alcohol (methyl, ethyl, or isopropyl) and ignite the liquid with an alcohol flame to sterilize their surfaces. A simple lamp can be prepared by filling a bottle with alcohol and inserting a wick (cotton or a piece of cloth are satisfactory). Kerosene or automotive gasoline (petrol, benzine) should not be used as fuel since they produce a lot of smoke and soot and may be explosive.

Liquids

Work areas, tools, tissues, and apparatus can be sterilized with liquid sterilants.

Hypochlorite Solutions

Preparations such as Clorox, Purex, Domestos, Milton's Snow White, and other brand-name household bleaches contain between 4.75 and 6% sodium hypochlorite and are therefore excellent sterilants. Undiluted they can be employed to wash tools, working areas, and the outsides of culture bottles. If used to sterilize seeds, capsules (orchid fruits are capsules and not pods as they are frequently and erroneously referred to), and tissues these bleaches should be diluted according to instructions in specific procedures. A wetting agent should and is often added to these dilutions. A few drops of Tween 20 are usually added by research laboratories, but a mild household liquid detergent or baby shampoo can be used for practical purposes.

To determine the correct dilution it is necessary to consider the sodium hypochlorite content of the household bleach that will be used. For example, if a procedure calls for a 50% dilution (50 ml household bleach made up to 100 ml with water) of a brand that contains 5.25% sodium hypochlorite, the diluted solution will contain 2.625% of active agent. Therefore a brand that contains only 4.7% sodium hypochlorite should be used at a lower dilution (55 ml of bleach made up to 100 ml with distilled water). On the other hand, a higher dilution must be used if a brand contains more sodium hypochlorite. For example, 44 ml of Clorox (which contains 6% sodium hypochlorite) should be diluted to 100 ml to obtain a final concentration of 2.625%.

The wetting agent (Tween 20, mild household detergent, baby shampoo) is not a sterilant. It only improves the wetting properties of the solution. Prolonged exposures to high concentrations of wetting agents and detergents can damage orchid tissues (Ernst et al., 1971*a*; Healey et al., 1971). However brief contacts with low concentrations will not have a deleterious effect. Also, neither the sterilant nor the wetting agent can come in contact with seeds when an unopened capsule is being surface-sterilized.

A saturated solution of calcium hypochlorite is used to surface-sterilize tissues and seeds. This solution is prepared by dissolving 10 g calcium hypochlorite in 140 ml water (7 g/100 ml), stirring vigorously, and allowing the solution to stand for 3–5 min. Then the solution is stirred again, allowed to stand until the precipitate

has settled, and filtered again or decanted. The clear, yellowish liquid is used as the sterilant. It should be used within 12 h.

Alcohol

Ethyl alcohol (ethanol, drinking alcohol), pure or denatured (methylated spirits), and isopropyl alcohol (isopropanol/rubbing alcohol) can be used to sterilize work areas, tools, and outside surfaces of culture vessels by swabbing. These alcohols can be used in a concentrated form or as 70% aqueous solutions (70 ml alcohol made up to 100 ml with water). If at all possible, the use of methanol should be avoided since it is toxic to people and may cause blindness.

Surface Decontamination

A number of methods are used for surface decontamination. In each case it is best to follow the specific method outlined in each procedure. Generally, sources of explants are first cleaned by a gentle washing and scrubbing to remove dirt and soil. After that they are dipped briefly in either 95 or 70% ethanol (2–3 s) and then immersed (5–20 min) in a diluted household bleach or calcium hypochlorite solution (see specific procedures for concentrations and times). The sterilant is then removed from the tissues with several sterile water washings in a sterile box or other suitable area (see Appendix 1). Sections are usually sterilized by soaking them in calcium hypochlorite for 5–20 min. The sterilant is then removed by washing with sterile distilled water.

Gas Sterilization

Under some circumstances liquid sterilants are not effective in eliminating all sources of contamination. The surface texture of an organ or seed may contain small hydrophobic crevices or indentations that shelter fungal or bacterial spores. In other cases liquid sterilants may not be able to reach and/or enter some spaces. Contaminants that are not easily eradicated by liquids can often be eliminated with the use of chlorine gas. This technique should be carried out in a fume hood, and workers must be careful not to breathe the highly toxic vapors.

Seeds and/or tissues to be treated can be placed on cheesecloth or nylon mesh (or any other porous material-paper towels may also be adequate) that has been taped to the top of a beaker or another suitable container. In this way, air can pass freely below and above the tissues during treatment.

Gas is generated by adding 3 ml of concentrated hydrochloric acid to 100 ml household bleach* (which usually contains 5.25% sodium hypochlorite) in a glass dessicator, bell jar, or other appropriate glass container. The cheesecloth- or nylon-mesh-covered beakers supporting the tissues are suspended above the solution of bleach and acid. Tissues can be kept in the closed dessicator for 5–30 min or longer

* Well-known brands such as Clorox, Purex, and Domestos; there are many others.

(30 min is suitable for taro seeds). After treatment the container should be opened carefully to allow gas to dissipate in the fume hood before attempting to remove the tissues. Seeds or tissues can then be placed in culture media using standard techniques.

Preparation of the Medium

Preparing a medium may appear complex to those who have not done it before. The step-by-step sequence described here and illustrated in Fig. 2-2 is intended to simplify the procedure.

1. Add the correct volume of each of the several macroelement stock solutions to 250 ml of distilled water. To measure volumes of 10 ml or more, use a volumetric cylinder. Smaller volumes should be measured with pipettes. In each case the smallest suitable volumetric glassware should be used. For example, 1 ml should be dispensed with a 1-ml pipette, not a 5 or 10-ml one. For 3 ml use a 5-ml pipette and not a larger one or a 1-ml pipette three times. For 7 ml use a 10-ml pipette. A 0.1-ml pipette should be used for dispensing 0.1 ml. If one is not available, a 0.5-ml or even a 1-ml pipette may be used provided they have the proper graduations (for sources of volumetric glassware see Appendix 2).
2. Dispense the proper amount of microelement stock solution.
3. If inositol is included in the medium, add it.
4. Incorporate into the medium whatever complex additives may be part of the recipe (note that some media may not require such additives).
5. Bring the total volume to approximately 900 ml.
6. Adjust the pH.
7. Weigh and add sugar (sugar may also be added before pH adjustment).
8. Pour the medium into a volumetric flask, and adjust the total volume to 1 l with distilled water. If distilled water is not available, rain water (preferably fresh and not acid) collected in a glass container may be used. Transfer the solution to an Erlenmeyer flask or bottle.
9. For solid media add agar.
10. Sterilize the medium in an autoclave or pressure cooker. The medium should be in an Erlenmeyer flask or bottle with a capacity twice the total volume of the solution being sterilized (e.g., 1 l of medium should be sterilized in a 2-l flask). Never use a volumetric flask as a container for sterilization because the heat may reduce its accuracy. A flask may be adequately covered for autoclaving by inverting a beaker over the neck.
11. Sterilize culture vessels, either before the medium is sterilized or at the same time.
12. While the medium is being sterilized, combine appropriate volumes of all hormone, vitamin, amino acid, and any other necessary stock solutions (all of which may be dissolved in 95 or 70% ethanol) in a vessel just large enough to contain the total volume (which will usually not exceed 5–6 ml). Suitable containers for this purpose are 5–10-ml Erlenmeyer flasks, 5–10-ml volumetric flasks, 5–10-ml bottles from the local pharmacy (drugstore, chemist), or small test tubes. After introducing each of the required solutions into the small container, stopper it and shake a few times to sterilize all inner surfaces. Then place the stoppered

container in the working area and sterilize its external surfaces by spraying with 70% ethanol or hypochlorite solution (described earlier).

13 After the medium has been sterilized and while it is still hot (and therefore still liquid if it contains agar), pour the contents of the container described in item 12 into the medium.

Culture Vessels

Test tubes (which are also referred to as "culture tubes"), Erlenmeyer flasks, and a variety of specially designed plastic containers (see Appendices 1 and 2) are ideal for tissue and callus cultures. However, other clear glass or plastic containers, polyethylene and polypropylene bags (Lee and Lam-Chan, 1995), and disposable film vessels (Tanaka et al., 1988; Tanaka, 1991*a*, 1991*b*) are also suitable. Containers with very wide necks which are not specially designed for tissue culture (jars, for example) should not be used because cultures in such vessels are easily contaminated unless a cap with a cotton-filled vent is screwed on tightly. To vent such caps a cotton-filled tube should be inserted into the cap and glued on with an adhesive that can withstand autoclaving. The adhesive should also be capable of bonding the tube to the cap (i.e., be able to bond two different materials) and leave no cracks. Or, a one- or two-hole stopper should be forced into an appropriate size opening on the cap. The holes in the stopper should be stuffed with non-adsorbent cotton.

Culture vessels which are specially designed for tissue culture, but are not presterilized by the manufacturer, should at least be rinsed before use. Vessels that have been presterilized by their manufacturers should be used as they come out of the packages. However it is important to remember that if these cultures are opened under non-sterile conditions their sterility will be compromised. Containers that were used previously for any purpose, including tissue culture, must be washed thoroughly with water and a good detergent and rinsed several times (at least three) with distilled water. After the rinsing these containers should be allowed to drain and dry by being placed on a rack with their openings pointing down.

When the vessels are completely dry they must be covered prior to storage. If containers such as jars, bottles, flasks, and tubes are to be used immediately after the washing they must be fitted with a cover before being filled with medium (culture vessels which are designed for tissue culture usually have their own specially designed covers).

Depending on the size of the culture vessel, the cover can be a rubber stopper (available from most laboratory supply houses) with one or two holes in them. These holes must be stuffed with non-adsorbent cotton (available from most laboratory supply houses). There is no need to insert glass tubes (curved or not) with cotton in them into the stopper holes; the cotton can be stuffed directly into the holes. Once prepared, stoppers with cotton-filled holes can be used repeatedly. Wiping them with a damp (water or 70% ethanol) towel (cloth or paper) should be enough to keep them clean. If the stoppers are washed and the cotton becomes wet it must be dried completely (an extended period in a 40°C oven should suffice) or replaced because contaminants can grow in/on the wet plugs and contaminate the cultures. There is no need to change the cotton unless it shrinks, becomes very dirty, or decomposes and is no longer snug and tight in the hole and/or cannot be dried.

Tightly fitting buns made of non-adsorbent cotton can also be used to cover containers (if the empty container can be lifted by holding the bun, it is tight enough). Both rubber stoppers and cotton buns should be covered with aluminum foil. Paper can be used if foil is not available; it should be tied or held with a rubber band below the neck. In high humidity areas moisture may condense on the cotton bun or plugs below the aluminum foil, allowing fungi to grow and contaminate cultures. In such areas paper rather than aluminum foil should be used. The use of plastic films to cover cotton buns or stoppers is not advisable because they are usually impervious to air. If tied tightly such films may prevent gas exchange and/or accumulate water condensate, which may allow growth of microorganisms that can contaminate the cultures.

Appropriate and sufficient gas exchange is very important because: (1) ethylene produced by plantlets, tissues, and/or explants can inhibit growth; and (2) oxygen and carbon dioxide may need to be replenished. Cotton plugs allow for gas exchange. So do filters that allow diffusion of gases but prevent entry of contaminants. Such filters can be self-adhesive (Milliseal™, Nihon Millipore, Ltd., Japan) or are built into lids for culture vessels (see Fig. 2-11).

Culture Conditions

The conditions under which explants, callus masses, protocorm-like bodies (PLBs), plantlets, germinating seeds, and seedlings are maintained can determine the success or failure of culture attempts and also affect growth and development.

Temperature

When adjusting the temperature for tissue culture or seed germination, it is important to follow instructions carefully. If these instructions prove to be unsuitable for a particular new cross, variety, clone, or species the appropriate temperature can be determined only by trial and error. The temperatures suggested for each procedure in this book are those recommended by the original workers and should be used, at least initially, to ensure best results or as starting points for new research.

Agitation

Liquid media must be agitated to: (1) allow for gas exchange; (2) improve contact between tissues and liquid; and (3) influence growth and development. Agitation may be gyrorotatory (wrist action), oscillatory (back and forth), or rotatory (rotating on a wheel with its axis parallel or at an angle to the ground). Shakers can be purchased (see Appendices 1 and 2) or constructed locally. Machine shops, mechanics, or simply handy persons can easily construct an adequate shaker. Oscillating and rotatory shakers are easiest to build and most suitable for the widest variety of applications. The speed of shaking is important. If shaken too fast or too slow explants, tissues, or callus may not respond as desired. In every instance it is best to use the type and speed of agitation which was employed in the original research and is

suggested in this book. If this information is not available, rotatory shakers should be set at 1–3 rpm, oscillating ones should move back and forth 60 times a minute, and gyrorotatory units should shake approximately 30–40 times per minute. These are starting speeds which should be adjusted as necessary.

Illumination

Light (see Figs 2-4 and 2-5) – its duration (photoperiods) or absence (darkness), intensity (i.e., energy levels), quality (color) and source (natural, fluorescent, incandescent, or other) – is of great importance in the micropropagation of orchids.

Presence or Absence

In most cases in vitro cultures of orchids should be illuminated for at least part of a 24-h period. However, there are also instances in which explants, cells, or protoplasts must be kept under very subdued light or in the dark, at least during the initial stages of culture. As with other aspects of micropropagation, recommendations in this book regarding light or dark regimes based on the original research should be followed in all instances. Modifications, especially when rare and/or expensive orchids are being cultured, should be introduced only following experiments with more common clones of a genus, species, or cross. During the development of a new procedure it may be advisable to place some cultures in the dark for specific periods, especially if related orchids are known to benefit from the exclusion of light.

Plantlets grown under continuous darkness do not produce chlorophyll. When moved to light such plantlets turn green. In some cases the transfer from darkness to light may have to be gradual. Plantlets growing in vitro are similar to seedlings in that they require light for normal development as well as root and/or shoot production and growth.

Growth and development of seedlings on media darkened with charcoal can be enhanced (Werkmeister, 1970*a*, 1970*b*, 1971; Ernst, 1974, 1975, 1976; for reviews see Arditti, 1979; Arditti and Ernst, 1984, 1993). The same seems to be true for plantlets in vitro. However, this fact is most probably not associated with requirements for light or darkness. It is due to the effects of the charcoal itself (see section on charcoal above).

Duration

As mentioned above, orchid cultures may be maintained under light or dark periods of duration, which can range from a few hours a day to continuous (24-h) illuminations. Appropriate photoperiods must be determined experimentally for orchids which have not been cultured before. When established procedures are employed it is best to use the photoperiods recommended by the original investigators. However, it should also be noted that the available evidence on this aspect of orchid micropropagation is far from clear and it is entirely possible that photoperiods are not an important factor in the micropropagation of orchids, so long as explants that require light are provided with sufficient illumination for at least part of the day.

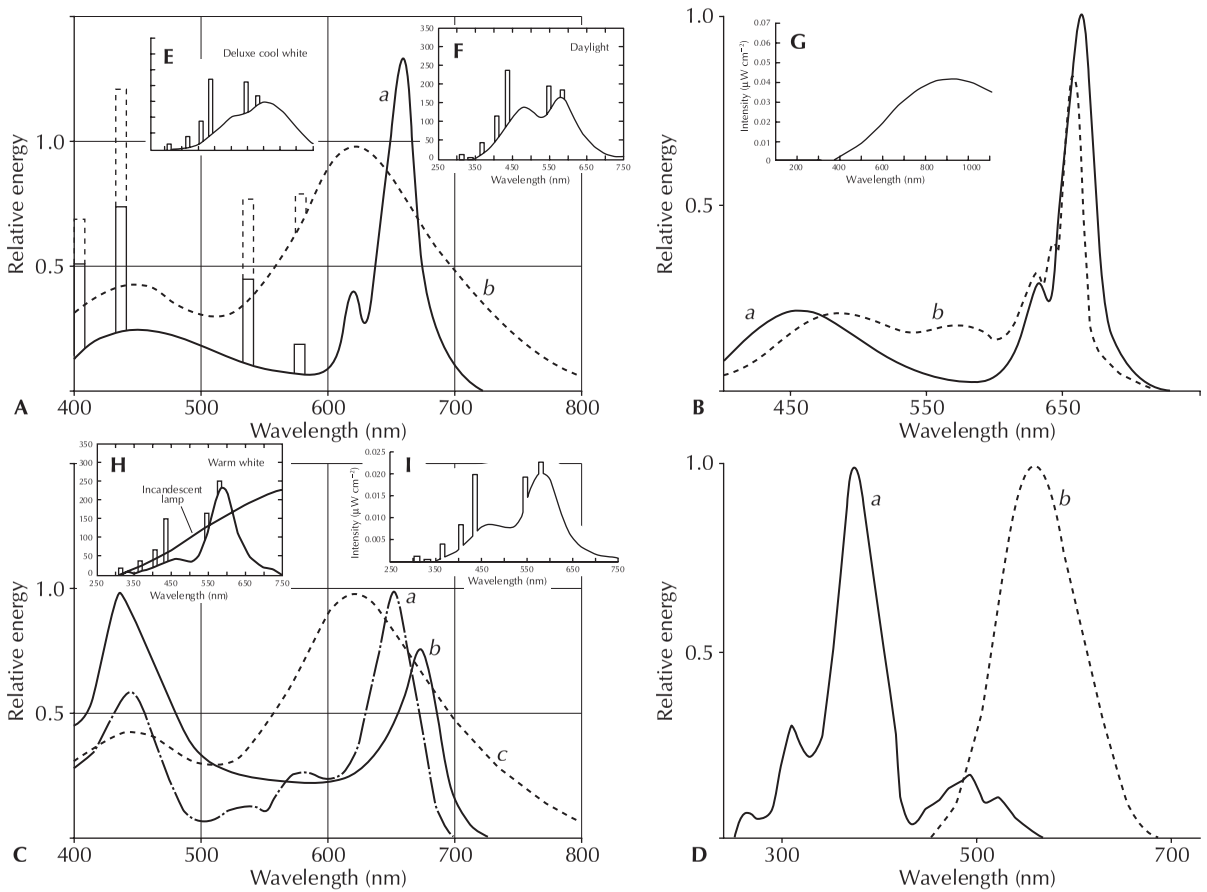


FIG. 2-4. **Light emission and action spectra. A. (*a*) Standard and (*b*) wide spectrum Gro Lux (SGL and WSGL, respectively). B. Relative spectra of (*a*) American and (*b*) European fluorescent plant grow lights. C. (*a*) Chlorophyll synthesis and (*b*) photosynthesis action spectra compared with (*c*) the WSGL emission spectrum. D. (*a*) Insect and (*b*) human vision spectra. E. Emission spectrum of a Sylvania Deluxe cool white fluorescent tube. F. Emission spectrum of a Sylvania Daylight fluorescent tube. G. Emission spectrum of a 100-W incandescent light bulb. H. Illumination produced by an incandescent bulb and a Sylvania warm white fluorescent tube. I. Emission spectrum of a Sylvania warm white fluorescent tube. (Sylvania data from Langham, 1978.)**

Intensity

"Strong" and "weak" are common terms used to describe light intensity, which is in fact visible spectrum energy produced by a source of illumination that impinges on a culture. Light intensity should be measured with appropriate instruments (two excellent discussions of light measurements were published by LI-COR Inc.; see LI-COR, 1982 and LI-COR, no date; www.licor.com). Several units and terms are used to describe light intensity and illumination:

Absorbance (sometimes called **absorptance**) is the part of the illumination that is absorbed.

Candela (cd) or **international candle** is luminous flux per unit area or luminous intensity; 1 candela = 12.57 lumens.

Candle power is the light intensity of a source in candelas.

Color temperature is the color of the light in comparison to the light color emitted by a black body heated to specific temperature expressed in Kelvin.

Einstein (E) is the energy in Avogadro's number of photons (1 E is 6×10^{23} photons s^{-1} m^{-2}; at noon on a bright summer day sunlight is approximately 1800–2200 μE). Plant scientists use units like **photosynthetic photon density** (E m^{-2}) and **photosynthetic photon flux** (E s^{-1} m^{-2}) to measure and describe light intensity. When these terms are used, E must be specified as average energy specific to the photosynthetically active radiation (PAR) wave band (360–700 nm). A problem with the Einstein is that it is not an SI unit and therefore measurements using the micromole (micromol, μmole, or μmol) are preferable. An expression used in this connection is **micromole (PAR) per second per square meter (μmol s^{-1} m^{-2} PAR)**. This is a flux of 6.022×10^{17} photons per second of photosynthetic radiation intercepted uniformly by a surface of one square meter. PAR can also be expressed as μE m^{-2} s^{-1}. Foot candles and PAR can be interconverted but conversion factors vary depending on the light source (see Tables 2-16 and 2-17).

Foot candle (ft-c), the most commonly used light intensity unit, is the illuminance resulting from a luminous flux of one lumen per square foot (1 ft-c = 1 lumen per square foot). Another definition is the amount of light emitted by an ordinary wax candle and impacting on a spherical surface measuring one square foot and located one foot from the flame. Like the lumen it is based at least in part on the relative spectral sensitivity of the human eye adapted to bright light (Fig. 2-4D; the sensitivity of insect eyes is given for comparison purposes), which differs from the spectrum requirements of chlorophyll synthesis, photosynthesis (Fig. 2-4C), and plant morphogenetic and growth responses. For this reason the foot candle is not suitable as a unit to describe light intensity for plant growth. However it can be used to describe the intensity of a known light source (Table 2-12), as for example "*X* foot candles produced by 40-W Acme Light Co. plant growth light bulbs." One foot candle is equal to 10 lux or lx (or, as stated above, 200 ft-c are equal to 2000 lx). The illumination used for orchid tissue culture is often given in foot candles probably because instruments to measure ft-c are easily available (Tables 2-12–2-15). Foot candles and PAR can be interconverted only through the use of conversion factors which are specific for each light source (Tables 2-16 and 2-17). Measurements made with photographic light meters can be converted into ft-c (Table 2-18).

TABLE 2-12. **Illumination in foot candles from two standard cool white fluorescent lamps on a white reflecting surface (Downs et al., 1966) and two Gro Lux tubes (Mpelkas, 1965) measured at several distances**[a]

	Intensity			
	Cool white lamps, ft-c			
Distance from lamps, cm	Two lamps[b,c]	Four lamps[b,c]	Four lamps[b,d]	Gro Lux lamps, μW cm^{-2}[e]
2.5	1100	1600	1800	
5	860	1400	1600	
7.6	680	1300	1400	
10.2	570	1100	1300	
12.7	500	940	1150	
15.2	420	820	1000	
17.8	360	720	900	
20.3	330	660	830	
22.9	300	600	780	
25.4	280	560	720	
27.9	260	510	660	
30.5	240	480	600	775
45.7	130	320	420	
61	100	190	260	328
91.4	159			
121.92				

[a]The candela (cd), a measure of luminous intensity, is not used in plant sciences because it is based on the sensitivity of the human eye. Lux (1 lx = 1 cd s^{-1} m^{-2} or 0.0929 ft-c) and foot candles (ft-c, not a metric unit anyway; 1 ft-c = 10.76 lx) have been used widely in plant physiology, but should not be because they are based on the sensitivity of the human eye. Lux may be used when no other measuring instruments are available. Radiant energy measurements should be accompanied by descriptions of the light source and its spectral characteristics. Conversions from lux or foot candles to irradiance (moles of photons, μmol m^{-2} s^{-1}) or energy (watts per square meter, W m^{-2}, or J s^{-1} m^{-2}) require a specific conversion factor for each light source (for a more detailed discussion of irradiance units see Salisbury, 1991). Foot candles and lux are used in this table and elsewhere in the book when these units are given in the original literature. Moles per square meter per second per nanometer (mol m^{-2} s^{-1} nm^{-1}) are used for spectral irradiance.

[b]Center to center distance between lamps = 5 cm.

[c]Lamps used for approximately 200 h before the measurements were taken.

[d]New lamps.

[e]Two rapid start 40-W Gro Lux tubes spaced 8.9 cm apart in a standard fixture with a 29.21 cm reflector.

Foot lambert is a measure of the amount of light reflected from a surface. The reflected light adds to the illumination of an area. Black surfaces reflect about 4% of the light that reaches them, whereas white areas reflect approximately 80%.

Frequency is the number of cycles or waves of electromagenetic radiation per second in Hertz (Hz).

Illuminance (E) is the luminous flux or quantity of light that falls on a unit area. The unit is the lux and the expression is E = lumens/area.

Irradiance is the radiant flux that falls on a receiving surface per unit surface area. The expression is W m^{-2}.

Lumen (lm) is the luminous flux or total visible light energy emitted by a source of illumination. A radiation of 0.00146 W is equivalent to 1 lm.

Lumen second (lms), **lumen minute** (lmm), and **lumen hour** (lmh) are the quantity and duration of light produced by a source.

Luminous efficiency (lm/W) is the ratio between luminous flux and the absorbed power.

Luminous flux (f) is the total light quantity of light emitted per second by a light source. The unit is the lux.

Lux (lx) is the intensity of illumination or the ratio of luminous flux to the area upon which it is incident. An intensity of 1 lux is produced when 1 lumen is

TABLE 2-13. **Conversion of foot candles to µW cm^{-2} for several light sources (Klein, 1973)**[a,b]

Source	Watts cm^{-2} per foot candle	
	300–800 nm	400–700 nm
Solar radiation	6.50	4.32
Incandescent lamps	4.57	
25 W (2720 K)	7.19	
40 W (2780 K)	6.00	
60 W (2820 K)	5.69	
100 W (2890 K)	5.42	
300 W (2930 K)	5.25	
500 W (3000 K)	5.14, 5.54	
1000 W (3050 K)	5.09	
Fluorescent lamps		
Cool white	2.69, 3.28	3.38
Cool white deluxe	3.18	3.68
Warm white	2.64, 2.90	3.03
Warm white deluxe	3.01	3.42
Daylight	3.61	3.71
Blacklight, BL	4.44	
Blue	6.61, 7.76	6.27
Green	1.51, 2.55	2.24
Red	8.55	9.34
Gold	1.39, 1.39	2.46
Pink	4.50	
Gro Lux (or equivalent)	8.09, 9.66	
Wide Spectrum Gro Lux (or equivalent)	4.92	
Mercury lamps		
H33-ICD	3.77	
H33-1GL/C	3.58	
H33-1GL/W	3.70	
Metalarc lamps, 400 W	5.92	
Tungsten-halogen lamps, 500 W, 3000 K	5.10	

[a]Several factors are from other sources.

[b]The candela (cd), a measure of luminous intensity, is not used in plant sciences because it is based on the sensitivity of the human eye. Lux (1 lx = 1 cd s^{-1} m^{-2} or 0.0929 ft-c) and foot candles (ft-c, not a metric unit anyway; 1 ft-c = 10.76 lx) have been used widely in plant physiology, but should not be because they are based on the sensitivity of the human eye. Lux may be used when no other measuring instruments are available. Radiant energy measurements should be accompanied by descriptions of the light source and its spectral characteristics. Conversions from lux or foot candles to irradiance (moles of photons, µmol m^{-2} s^{-1}) or energy (watts per square meter, W m^{-2}, or J s^{-1} m^{-2}) require a specific conversion factor for each light source (for a more detailed discussion of irradiance units see Salisbury, 1991). Foot candles and lux are used in this table and elsewhere in the book when these units are given in the original literature. Moles per square meter per second per nanometer (mol m^{-2} s^{-1} nm^{-1}) are used for spectral irradiance.

TABLE 2-14. **Conversion factors from lux or foot candles to watts per square meter of photosynthetically active radiation (W m^{-2} PAR), or to µmoles of photosynthetic illumination per square meter per second (µmol m^{-2} s^{-1} PAR) for several light sources (Hartman et al., 1988)**[a]

Light source	Multiplication factors for conversion to[b]			
	W m^{-2} PAR		µmol m^{-2} s^{-1} PAR	
	ft-c	lx	ft-c	lx
Average daylight	22.9	247	5.0	54
Cool white fluorescent tubes	31.5	340	7.0	74
Incandescent lamps				
Mercury lamps, high pressure	35.2 ± 5%	380 ± 5%	7.8 ± 5%	84 ± 5%
Metal halide lamps	30.2	326	6.6	71
Sodium lamps, low pressure	48.3	522	9.8	106

[a]The candela (cd), a measure of luminous intensity, is not used in plant sciences because it is based on the sensitivity of the human eye. Lux (1 lx = 1 cd s^{-1} m^{-2} or 0.0929 ft-c) and foot candles (ft-c, not a metric unit anyway; 1 ft-c = 10.76 lx) have been used widely in plant physiology, but should not be because they are based on the sensitivity of the human eye. Lux may be used when no other measuring instruments are available. Radiant energy measurements should be accompanied by descriptions of the light source and its spectral characteristics. Conversions from lux or foot candles to irradiance (moles of photons, µmol m^{-2} s^{-1}) or energy (watts per square meter, W m^{-2}, or J s^{-1} m^{-2}) require a specific conversion factor for each light source (for a more detailed discussion of irradiance units see Salisbury, 1991). Foot candles and lux are used in this table and elsewhere in the book when these units are given in the original literature. Moles per square meter per second per nanometer (mol m^{-2} s^{-1} nm^{-1}) are used for spectral irradiance.

[b]Divide reported values by the appropriate multiplication factor to convert measurements to the desired units.

Examples:

300 ft-c of light produced by a cool white tube divided by 31.5 equals 9.52 W m^{-2} PAR, and by 7.0 equals 42.857 µmol m^{-2} s^{-1} PAR

3000 lx of normal daylight divided by 247 equals 12.15 W m^{-2} PAR, and by 54 equals 55.56 µmol m^{-2} s^{-1} PAR.

TABLE 2-15. **Production of photosynthetically active radiation by several light sources (Langham, 1978)**[a]

Lamp	Input (watts)	Output in 400–700 nm wavelength (watts)	Output/input ratio
Incandescent	25	39	1.56
	40	45	1.13
	60	57	0.95
	100	69	0.69
	200	79	0.95
Fluorescent			
Cool white	46	204	4.44
Cool white	225	204	0.91
Warm white	46	199	4.30
Plant growth	46	127	2.76
Plant growth	46	146	3.17

[a]The candela (cd), a measure of luminous intensity, is not used in plant sciences because it is based on the sensitivity of the human eye. Lux (1 lx = 1 cd s^{-1} m^{-2} or 0.0929 ft-c) and foot candles (ft-c, not a metric unit anyway; 1 ft-c = 10.76 lx) have been used widely in plant physiology, but should not be because they are based on the sensitivity of the human eye. Lux may be used when no other measuring instruments are available. Radiant energy measurements should be accompanied by descriptions of the light source and its spectral characteristics. Conversions from lux or foot candles to irradiance (moles of photons, μmol m^{-2} s^{-1}) or energy (watts per square meter, W m^{-2}, or J s^{-1} m^{-2}) require a specific conversion factor for each light source (for a more detailed discussion of irradiance units see Salisbury, 1991). Foot candles and lux are used in this table and elsewhere in the book when these units are given in the original literature. Moles per square meter per second per nanometer (mol m^{-2} s^{-1} nm^{-1}) are used for spectral irradiance.

TABLE 2-16. **Interconversion between photosynthetically active radiation (PAR) and foot candles**[a]

A Conversion factors			**B** Examples			
			Sunlight		Cool white fluorescent	
Light source	PAR (μmol m^{-2} s^{-1}) to foot candles	Foot candles to PAR (μmol m^{-2} s^{-1})	PAR	Foot candles	PAR	Foot candles
Sunlight	5.01	0.200	10	50.1	10	68.7
Cool white			100	501	100	687
fluorescent	6.87	0.146	200	1002	200	1374
High pressure			300	1503	300	2061
sodium lamps	7.62	0.131	600	3006	600	4122
High pressure metal			1000	5010	1000	6870
halide lamps	6.60	0.152	2000[b]	10020[b]	2000	13740
Low presure						
sodium lamps	9.85	0.102				

[a]Source: Apogee Instruments Inc. (www.apogee-inst.com/conv_.htm), makers of a meter which can measure both PAR and foot candles (Dual Radiation MeterDRM-FQ). Parts A and B of the table are independent of each other.

[b]Full sun at noon on a clear summer day. Multiply PAR (or photosynthetic photon flux, PPF) by the conversion factor to obtain foot candles. For example, full sunlight 2000 μmol m^{-2} s^{-1} × 5.01 = 10020 ft-c. Multiply foot candles by the conversion factor to obtain PAR (or PPF). For example, 10020 ft-c × 0.2 = 2000 μmol m^{-2} s^{-1}.

TABLE 2-17. **Light conversion factors**[a]

	Conversion factors[b]			
			Photometric	
Light source	Radiometric, W m^{-2}	Photosyntheically active radiation, μE m^{-2} s^{-1}	Foot candles	Lux
Cool white fluorescent lamp, 215 W	1	4.6	34.2	367
		1	7.44	80.0
			1	10.8
High pressure sodium lamps, 400 W	1	5	33.5	360
		1	6.7	72.3
			1	10.8
Metal halide lamps, 400 W	1	4.6	29.6	319
		1	6.5	69.5
			1	10.8
Mercury lamps, 400 W	1	4.7	30.8	332
		1	6.5	70
			1	10.8

[a]Source: Provided by Dr. Ching-yeh Hu, Biology Department, William Patterson University, Wayne, NJ 07470, chu@frontier.wilpaterson.edu to Kitchen Culture Kits, Inc, www.kitchenculturekit.com.

[b]To convert, multiply by the appropriate conversion factor.

TABLE 2-18. **Light intensity estimations in foot candles using a camera or a light meter**[a]

Shutter speed, sec	ASA/ISO 25						ASA/ISO 100								
	f stop						*f* stop								
	2.8	4	5.6	8	11	16	1.4	2	2.8	4	5.6	8	11	16	22
1/4							0.5	1	2	4	8	16	32	64	125
1/8							1	2	4	8	16	32	64	125	250
1/15							2	4	8	16	32	64	125	250	500
1/30							4	8	16	32	64	125	250	500	1000
1/60	200	370	750	1500	2800	5000	8	16	32	64	125	250	500	1000	2000
1/125							16	32	64	125	250	500	1000	2000	4000
1/250							32	64	125	250	500	1000	2000	4000	8000
1/500							64	125	250	500	1000	2000	4000	8000	16000
1/1000							125	250	500	1000	2000	4000	8000	16000	32000
1/2000							250	500	1000	2000	4000	8000	16000	32000	64000

[a]Sources: ftp://ftp.nmt.edu/pub/orchids/lighting1.2, Eiich L. Koch, Jack Blumenthal (blumenthal@penny.net), Joachim Saul, and Bob Hamilton. The foot candle is a measure of light intensity in terms of the sensitivity of the human eye. It does not measure energy levels. A formula which can be used to convert light meter measurements to foot candles is 20 (A^2)/(shutter speed in seconds)(film speed in ASA/ISO). Another formula is 20 × A^2/shutter speed × film ASA. To use this table the film speed indicator should be set to ASA/ISO 25 or 100, and after that the light meter or camera should be pointed toward the light source. The shutter speed should be set after that. The indicated value is then read. Foot candles are obtained from the table after that. Example: Set film speed to ASA/ISO 100 and shutter speed to 1/125 of a second. Point meter or camera to light source. If the indicated *f* stop is 5.6 the light intensity is 250 ft-c. Conversions: 1 lx = 0.029 ft-c; 1 ft-c = 0.76 lx; 1 lx = 1 lm^{-2}; 1 ft-c = 1 lm ft^{-2}; 1 ph = 1 lm cm^{-2}; 1 lmh = 60 lmm.

distributed uniformly over an area of 1 square meter. Ten lux equal one foot candle (i.e., to convert foot candles to lux it is necessary to multiply by 10; for example 200 ft-c equal 2000 lx). The light intensities used for some orchid tissue culture procedures are given in lux, especially in more recent papers. The lux is sometimes referred to as a metric unit because it is the amount of light emitted by an ordinary wax candle and impacting on a spherical surface measuring one square meter at a distance of one meter from the candle.

Lux second (lxs) is the quantity of illumination or a product of intensity and duration.

PAR *see* Photosynthetically active radiation.

Phot (ph) is the specific luminous radiation of a surface or the ratio of the radiated luminous flux to a specific surface area. It equals 10,000 lx.

Photosynthetically active radiation (PAR) is illumination in the wavelengths used in photosynthesis. Light sources differ in the production of PAR (Table 2-15) and several conversion factors must be used (Tables 2-12–2-15).

Radiance is the radiant flux emitted by a unit area of source.

Radiant flux is amount of light emanating from a source per unit time. The unit is the watt (W). The expression is J s^{-1}.

Watt (W) is a radiometric unit of energy per unit of time and area irrespective of wavelength. Measurements of W in the PAR area are known as W PAR. Light intensity can be expressed as W, mW, or μW cm^{-2}. Reported illumination intensities used for orchid tissue culture vary considerably, but it is not certain at present whether this is due to requirements by the plants, preferences by individual investigators, or simply availability of light sources. Normal daylight or that coming through a window, cool or warm white fluorescent tubes, plant growth lights (like Gro Lux), incandescent bulbs only, various mercury or sodium lamps, and combinations of these all seem to be suitable (also see below).

Quality

This term refers to the spectra of light sources (Figs 2-4 and 2-5) and implies color. Since plants require specific parts of the spectrum for normal growth and development, the quality of light used to illuminate cultures is important. In general, incandescent lamps produce more red and less blue wavelengths (Figs 2-4G, H and 2-5C) than fluorescent tubes (Figs 2-4A–F, H, I and 2-5C, D). Other light sources have their own specific spectra. Lamps designed especially for plant growth provide light of somewhat better balance (Fig. 2-4E, F, H, I), but may cost more in terms of initial price and energy input.

In a study with *Tradescantia fluminensis* (Biran and Kofranek, 1976) the best yields per electrical energy input unit were obtained under illumination with cool white lamps (Fig. 2-4E). In comparison with these lamps, the relative yield under daylight lamps (Fig. 2-4F) was 88%; Deluxe cool white (Fig. 2-4E), 73%; Plant Light (probably similar to Fig. 2-4A–C), 72%; and pink and blue lamps, 36%. Similar results were obtained with calculations based on the photosynthesis action spectrum of an average leaf (Biran and Kofranek, 1976).

A formula which can be used to predict the photosynthetic efficiency of lamps (Biran and Kofranek, 1976) is:

$$PP = \left[\sum_{i=1}^{i=30} P_i \cdot E_i \right] (\text{lm/W}) \qquad \text{(XIII)}$$

where:

E_i = μW or photons 10 nm^{-1} lm^{-1} which can be calculated from spectral energy distribution curves provided by lamp manufacturers;
lm/W (L/W in the original paper) = lumen output per total electrical input in watts;
P_i = relative PAR value per unit of incident energy flux (McCree, 1971, 1972*a*, 1972*b*), or photon flux (Balegh and Biddulph, 1970) for thirty 10-nm portions of the visible spectrum between 400 and 700 nm;
PP = predicted PAR value per electrical input in watts.

This equation can also be used to evaluate PAR at plant level (which is a certain distance below the lamps). In such cases lm/W should be the light measurement at plant level and an assumption is made that illumination levels are directly proportional to the total output of lumens (Biran and Kofranek, 1976).

Light Sources

A number of light sources (Figs 2-6–2-8) are available in addition to the ones already mentioned (Figs 2-4 and 2-5; Tables 2-12–2-18). Fluorescent lamps are manufactured in a bewildering array of sizes, shapes, intensities, and emission spectra (Figs 2-4–2-7). They consist of a partially evacuated glass tube with an anode at one end and a cathode at the other. When the light is turned on a small amount of mercury vapor inside the tube becomes ionized and emits ultraviolet light, which causes a phosphor that coats the inside of the tube to fluoresce and give off light. The nature of the phosphor determines the emission spectrum of the lamp.

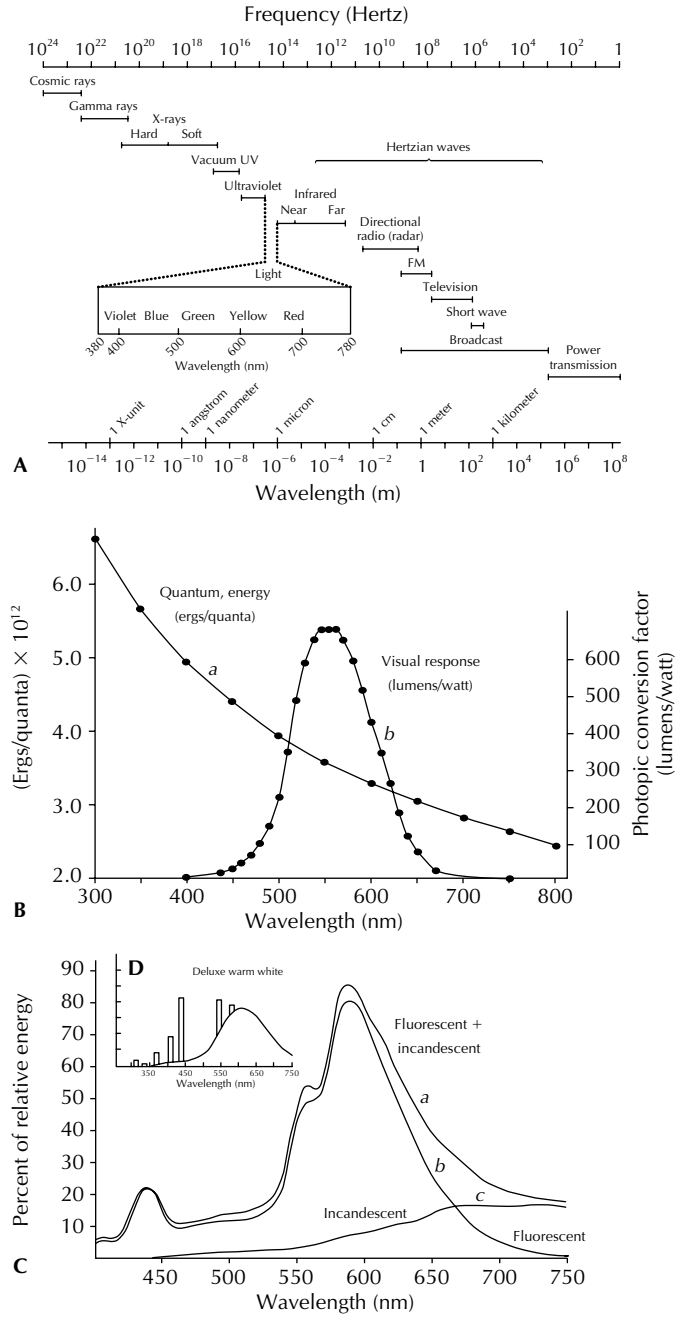


FIG. 2-5. **Light spectra and energy distribution. A. The electromagnetic (radiant energy) spectrum. B. Quantum energy (*a*), wavelength, and the human visual response (*b*). The sensitivity of the human eye differs from the action spectrum of photosynthesis (see Fig. 2-4C). This is why units used to measure light intensity in terms of vision are not suitable for photosynthesis-related determinations. C. Spectra of several light sources and combinations: (*a*) fluorescent and incandescent, (*b*) fluorescent, and (*c*) incandescent. D. Emission spectrum of a Sylvania Deluxe warm white fluorescent tube.**

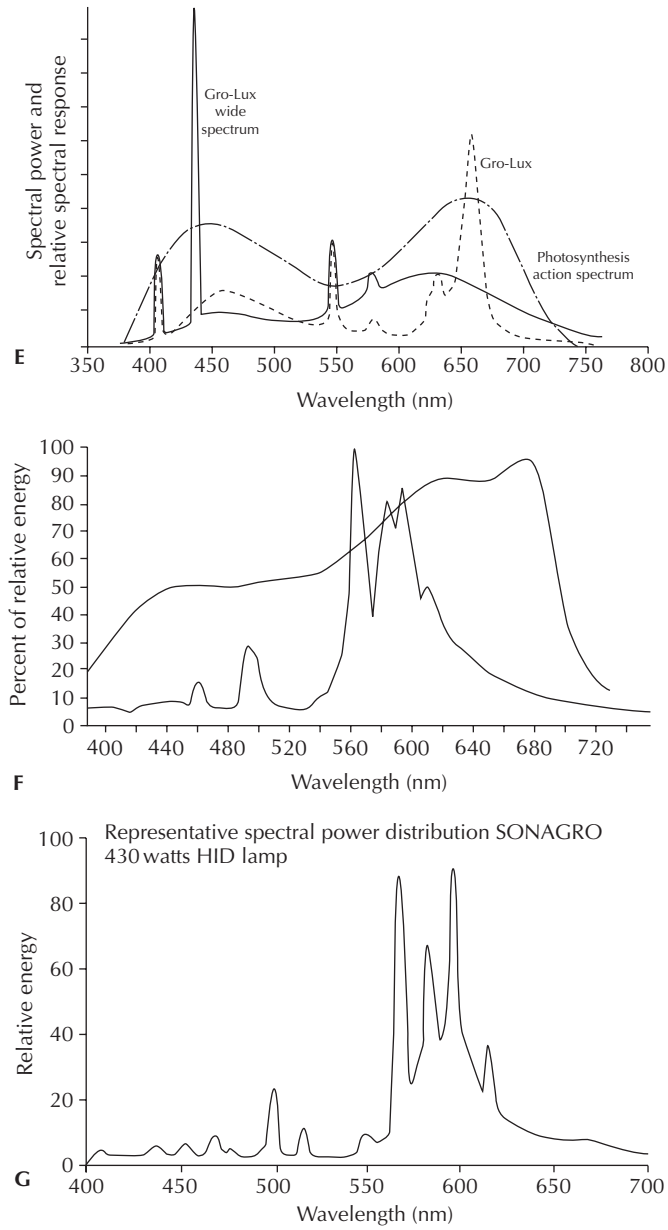


FIG. 2-5. (***Continued***) **E. Photosynthesis action spectrum and emission spectrum of Gro Lux tubes. F. Hortilux spectral distribution and plant sensitivity curve. G. SON AGRO 430-W high intensity discharge (HID) tube.**

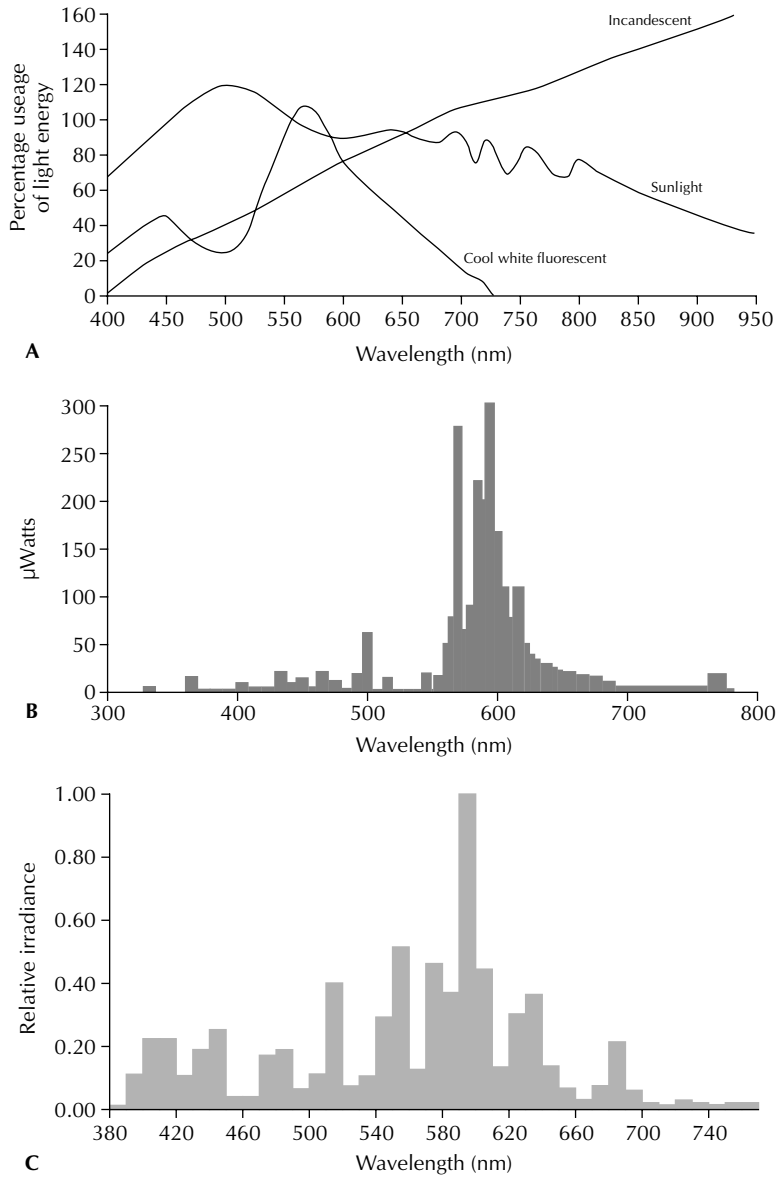


FIG. 2-6. **Light emission by several sources, and wavelength utilization by plants. A. Blue and red are the most important part of the spectrum for photosynthesis. Flowering, germination, stem elongation, and aspects of growth and development are affected by red and far red light. Blue light affects phototropism and other physiological functions (source: www.biocontrols.com). B. Master Son-T PIA Agro 400 W (source: www.philips.com). C. Sun Master Warm Deluxe metal halide lamp (source: www.hydroponics.com).**

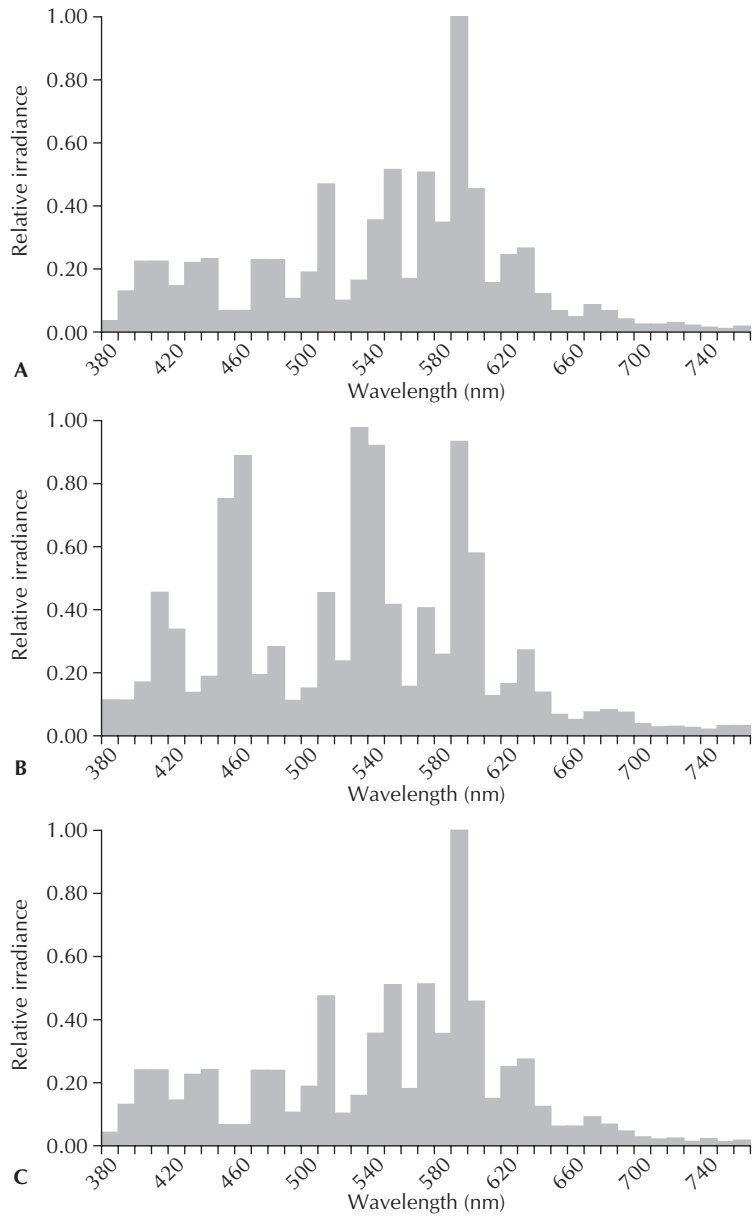


FIG. 2-7. **Light emission by metal halide bulbs. A. SunMaster Neutral Deluxe metal halide lamp. B. SunMaster Cool Deluxe metal halide lamp. C. SunMaster conversion lamp, high pressure sodium to metal halide. (Source: www.hydroponics.com.)**

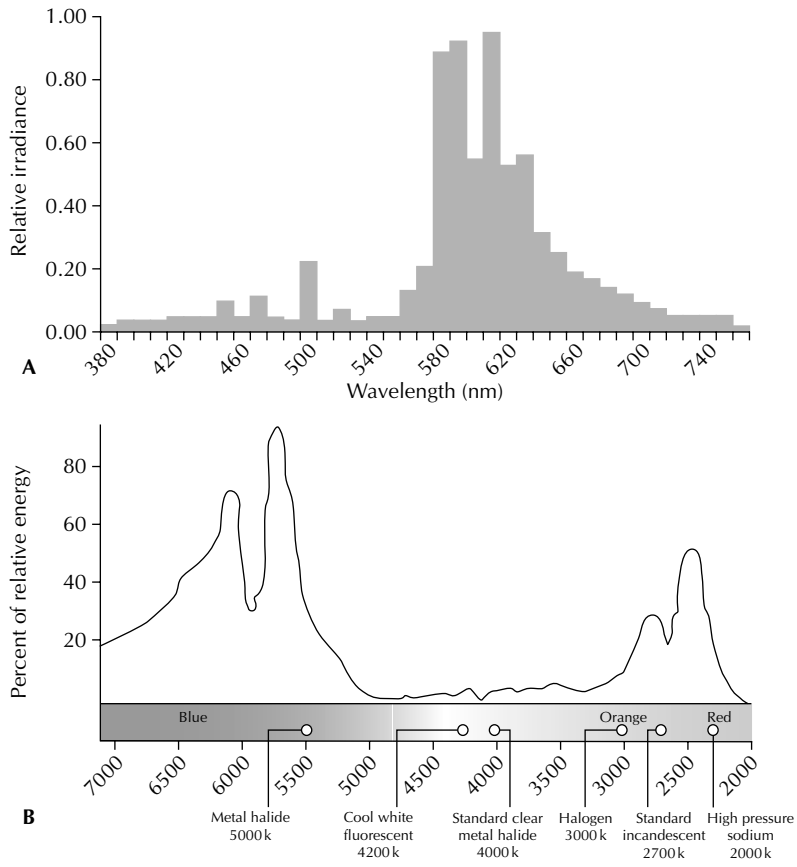


FIG. 2-8. **Light emission and temperature. A. SunMaster High Pressure Sodium Deluxe lamp (source: www.hydroponics.com). B. Light absorption by chlorophyll (*y*-axis and shaded areas), light temperature in Kelvin (*x*-axis), and several light sources (source: www.kingswoodorchids.com).**

The diameter of fluorescent tubes can be 1 inch or about 2.5 cm [designated as T8 or Slimline, and produced in lengths of 24, 36, and 48 inches (approximately 60, 90 and 120 cm)] or 1.5 inches or ca. 3.8 cm [the more common T12 designation; these tubes can be 18, 24, 36, 48, 72, and 96 inches (about 46, 60, 90, 180, and 245 cm) long, round or U-shaped, and signed for several voltages]. Depending on quality, fluorescent tubes produce adequate illumination for approximately 12 months only, even if they appear to be functioning properly.

As can be expected the least expensive fluorescent tubes are the ones that are produced and sold in the largest quantities. These are the cool and warm white tubes. Their emission spectra are not optimal for plant growth, but if used in combination with incandescent lamps these tubes produce excellent results (Biran and Kofranek, 1976). Plant growth fluorescent tubes produced by a number of manufacturers have emission spectra that are adjusted to promote growth (Figs 2-4–2-7), but they can be expensive.

Incandescent (i.e., the common) household light bulbs consist of a tungsten filament that glows in a vacuum when the electricity is turned on. These bulbs produce a considerable amount of heat and very little light in the shorter wavelength (blue) end of the spectrum. Most of their output is in the red (longer) wavelengths (Fig. 2-4G). If used they should be combined with cool white fluorescent lamps (Biran and Kofranek, 1976). The tungsten in incandescent lamps evaporates, coats the inside of the bulb, and reduces light output.

Halogen bulbs are modified incandescent lamps in which small amounts of bromine or iodine (both halogens) are present and combine with the vaporized tungsten to form tungsten bromide (WBr_2) or tungsten iodide (WI_3). These iodide or bromide molecules move to the tungsten filament where they split. The tungsten remains on the filament, whereas the iodine or bromine returns to the atmosphere of the bulb. Halogen lamps are very hot because these reactions require a temperature of 200°C. The emission spectrum of halogen bulbs is heavy in the red end.

High intensity discharge (HID) lamps are big, bright, produce a tremendous amount of heat, and range from 70 to 2000 W. They contain a vapor and work with an arc. The vapor can be mercury (mostly bluish-white light and very little red), sodium (yellow), or metal halide (similar to sunlight; Figs 2-6–2-7).

The choice of lamps for a culture room may depend on many factors, not the least of which is cost and coverage (Fig. 2-9). Incandescent lamps are the least expensive, but are not very suitable due to their spectrum and heat production. Halogen lamps have the same disadvantages. HID and metal halide lamps are very expensive. Standard or plant growth fluorescent lamps, alone or in combination with incandescent bulbs, are easily available, economical, simple to maintain, and suitable in terms of their emission spectra and intensity (Figs 2-4–2-7; Tables 2-12–2-15). These are the reasons why fluorescent lights are used extensively in culture rooms throughout the world.

Recommendations. A suitable and inexpensive light source for orchid tissue culture and micropropagation can consist of two cool white tubes mounted on a standard fluorescent light fixture. The addition of two 25–50-W incandescent bulbs between the fluorescent tubes will improve the light spectrum to which the plants will be subjected. A combination of one cool white and one warm white tube may produce somewhat better illumination, especially if they are combined with incandescent bulbs. Other combinations are also possible. The area illuminated by these lights should be roughly equal to (or slightly larger than) that of the fixture itself, which is usually mounted 45–50 cm above the plants.

If only two cool white tubes are used the light intensity provided at plant level by such a fixture should be between 110 and 130 ft-c (ca. 3.81 W m^{-2} or 17.14 μmol m^{-2} s^{-1} PAR) or 1100–1300 lx. Four tubes may provide 250–320 ft-c (2500–3200 lx, or ca. 9.52 W m^{-2} PAR or 42.86 μmol m^{-2} s^{-1} PAR), whereas new ones can be expected to produce 350–420 ft-c (3500–4200 lx, or ca. 12.70 W m^{-2} PAR or 57.14 μmol m^{-1} s^{-1} PAR). The addition of incandescent bulbs will increase the illumination levels and broaden the spectrum. The emission spectra of plant growth tubes contain wavelengths appropriate for plant growth and should be considered, especially if their prices are reasonable. Combinations of plant growth and other fluorescent tubes and/or incandescent bulb can also produce good results.

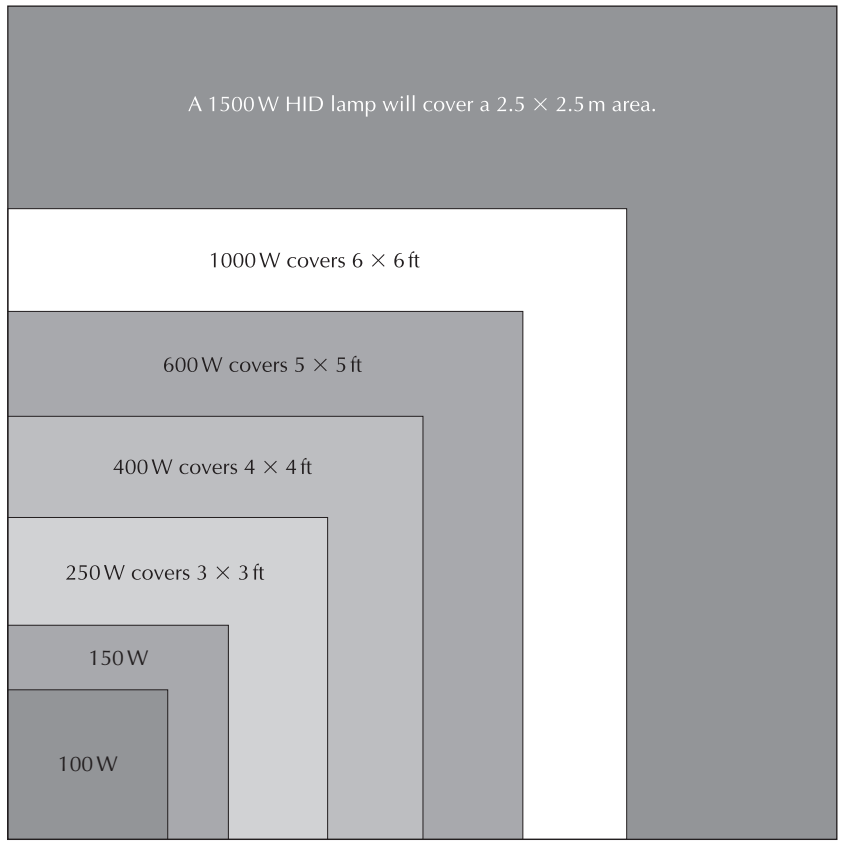


FIG. 2-9. **Areas of illumination. HID, high intensity discharge. (Source: www.kingswoodorchids.com.)**

Studies with red and blue light-emitting diodes (LED; Fig. 2-10) have shown that red light promoted leaf growth in *Cymbidium* plantlets, but decreased chlorophyll content. This was reversed by blue light. Growth under a combination and red and blue LED was comparable to that of plants illuminated by fluorescent lamps (Tanaka et al., 1998). Given the current cost of LEDs there does not seem to be a good reason to use them except under special circumstances.

Carbon Dioxide

Despite its relatively low concentration in the atmosphere (Table 2-19), carbon dioxide (CO_2) plays a very important role in plant life. It is the source of carbon for photosynthesis, but the low atmospheric levels can be a limiting factor. Therefore higher levels of CO_2 can bring about increased growth by orchid plantlets and seedlings (Figs 2-11 and 2-12; Hew et al., 1995; Lootens and Heursel, 1998; Mitra et al., 1998; Tanaka et al., 1998; Gouk et al., 1999).

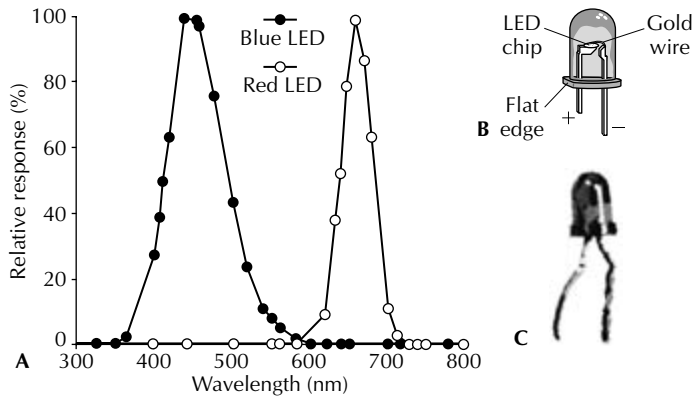
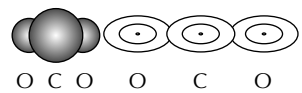


FIG. 2-10. **Light-emitting diodes: spectra (A), diagram (B), and photograph (C).**

TABLE 2-19. **Some facts about carbon dioxide**

Parameter	Value, description, or illustration
Name	Carbon dioxide
Synonyms	Carbonic acid gas, carbonic anhydride
Chemical formula	CO_2, O=C=O
State	Inorganic colorless and odorless gas
Structure	 O C O O C O
Absorption	Absorbed by alkaline solutions
Atmosphere, concentration in	370 ppm or 0.37 ml l^{-1}
Boiling point	There are two values in the literature, –56.6°C and –78.5°C
Content in air, %	0.03–0.037
Content in air, ppm	300–370
Content $liter^{-1}$ of air	0.3–0.37 ml
Density at 21.1°C and 1 atm	1.977 mg m^{-3}
Flammability	None
Freezing point	There are two values in the literature, –76°C and –78.5°C
Humans, effects on	Over 1.5%: headaches, hyperventilation, visual disturbance, tremors, loss of consciousness, death 3–6%: dyspnea, headaches, perspiration 6–10%: dyspnea, headaches, perspiration, tremors, unconsciousness, visual disturbance Over 10%: unconsiousness
Melting point	–78.5°C
Mole volume	22.4 liters
$Molecules\ mole^{-1}$	6×10^{23}
Molecular weight	44.01 = [(C = 12.01) + 2(O = 16)]
Solubility in alcohol	Slight
Solubility in water at 20°C	87.8% by volume
Specific gravity	1.53
Vapor density	1.53 (air being 1.0)
$Volume\ mole^{-1}$	22.4 liters at standard temperature and pressure
$Weight\ mole^{-1}$	44.01 g

Several experiments (Hew et al., 1995; Lootens and Heurserl, 1998; Mitra et al., 1998; Tanaka et al., 1998; Gouk et al., 1999) have shown that subjecting orchid plantlets to higher levels of CO_2 brought about increased growth and dry weight of *Cymidium*, *Mokara*, and *Phalaenopsis*. Growth of *Mokara* Yellow was enhanced greatly

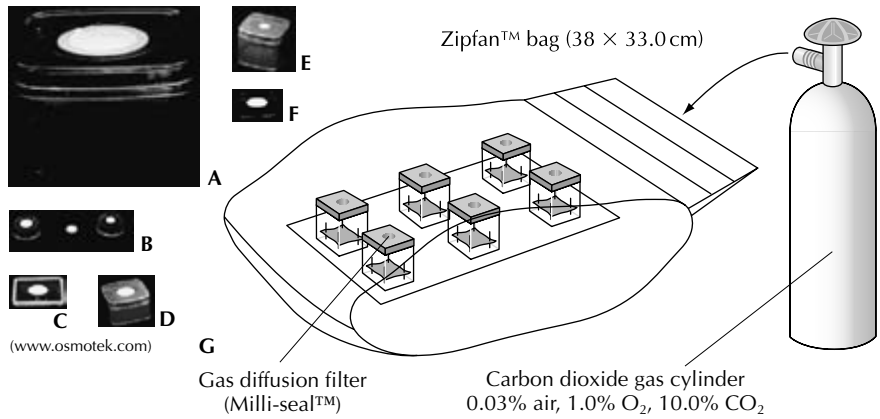


FIG. 2-11. **Carbon dioxide enrichment. A–F. Vented lids that allow for gas exchange, including the diffusion of carbon dioxide. G. A carbon dioxide enrichment system developed by Professor Choy sin Hew (National University of Singapore) and Dr. John W. H. Yong (Nanyang Technological University, Singapore). (Hew and Yong, 1997.)**

when the plants were exposed to 1% CO_2 for 3 months. These plantlets also had higher levels of soluble sugars like glucose and sucrose (the latter also implies increased amounts of fructose), starch, and an extensive thylakoid system (Gouk et al., 1999). *Phalaenopsis* hybrids exposed to 950 ppm CO_2 for 1 week exhibited uptake of the gas which was 82% higher than that of plants exposed to 380 ppm CO_2. This increased uptake can bring about better growth.

There are no simple or inexpensive systems for CO_2 enrichment of the atmosphere that surrounds culture vessels. This is probably the reason why CO_2 enrichment is not a common practice.

A system developed by Professor Choy sin Hew of the National University of Singapore and Dr. John W. H. Yong of Nanyang Technological University in Singapore (Fig. 2-11; Yong et al., 2002) could be used in cases where improved growth is desirable or necessary. Culture vessels with vented caps are available from www.osmotek.com, www.sigmaaldrich.com, www.duchefa.com, and www.unicornbags.com. However such vessels are not strictly necessary. Culture vessels (tubes, flasks) which are covered with cotton buns are also suitable. Also, it is possible that CO_2 levels in the bags can be increased by simply placing a small piece of dry ice in each bag. This possibility has not been tested experimentally. Therefore those who may wish to try it should experiment with it first and accept the fact they are doing it at their own risk and cannot hold anyone else responsible in the event of failure and losses.

Placing Plant Material in Culture

General requirements of tissues, organs, and explants at the start of culture vary. Attention must be paid to these requirements to ensure success.

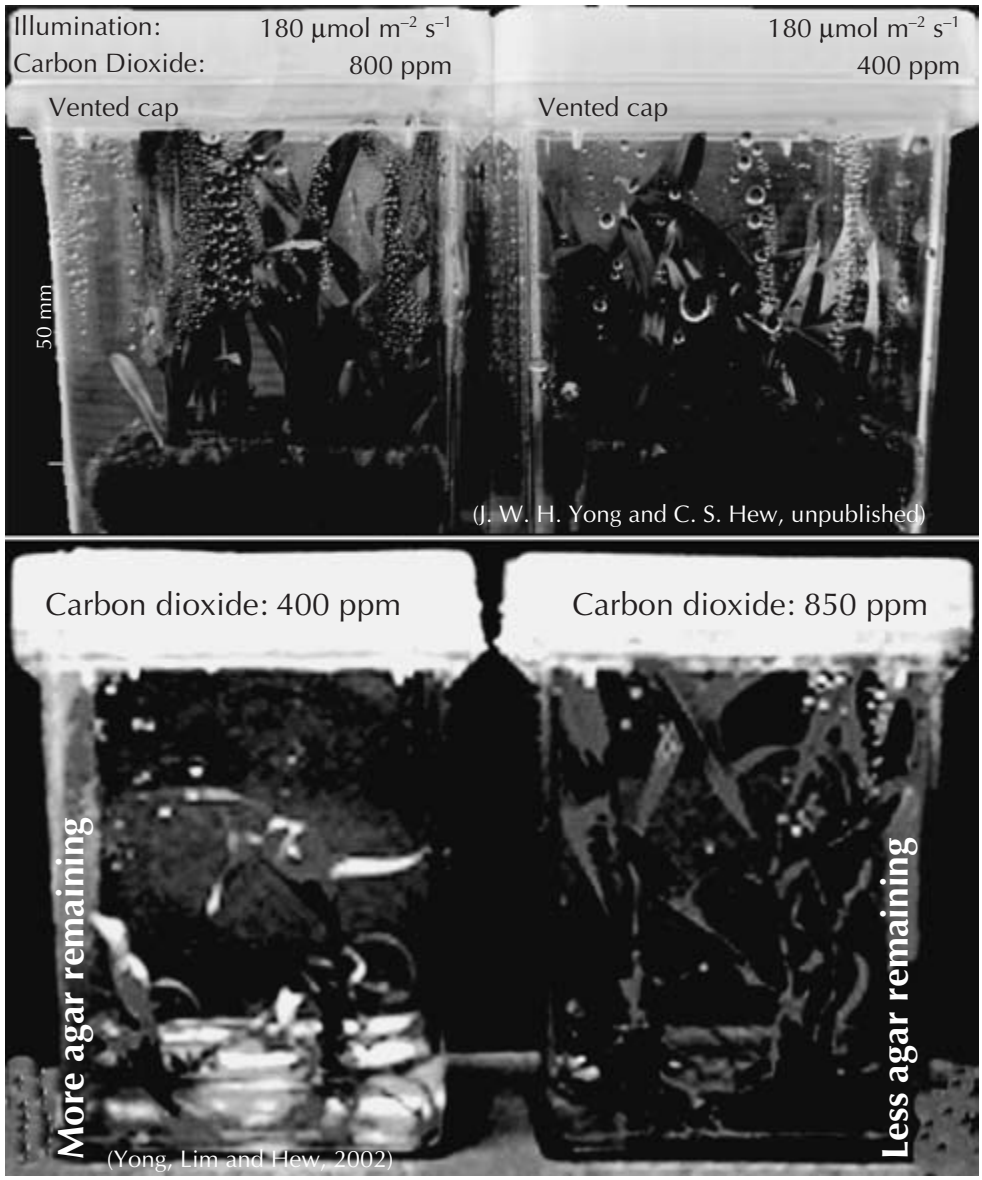


FIG. 2-12. **Effects of increased carbon dioxide levels (800 and 850 ppm) on the growth of *Cymbidium* plantlets (Yong et al., 2002; Dr. J. W. H. Yong and Prof. C. S. Hew, unpublished).**

Seeds

When seeds are placed on a solid medium it is important to establish good contact between them and the medium, and to distribute them evenly. They should not be totally buried in the agar in order to prevent death from improper gas exchange. All inoculations should be carried out in a sterile work area, except as noted otherwise. Explants should be treated similarly (see below).

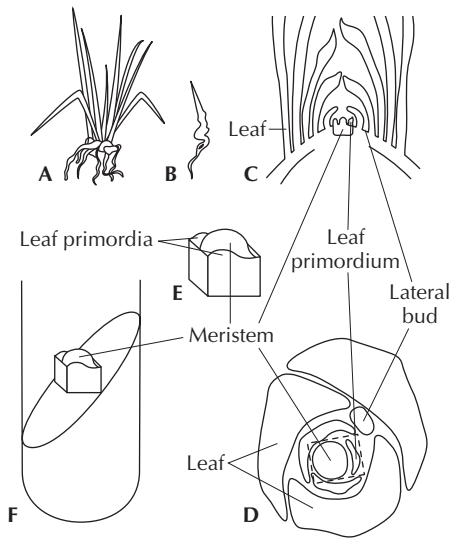


FIG. 2-13. **Excision and culture of a meristem. A. Mature plant; the meristem is located at the tip surrounded by leaf or petiole bases that must be removed to expose it. B. Excised shoot tip. C. Longitudinal section of excised shoot tip showing mature leaves, leaf primordial, lateral buds, and the area (*dotted lines*) to be excised for culture. D. Top view of shoot tip after all large leaf bases have been removed showing meristem, several developing leaves, and lateral buds. E. Excised shoot tip with two leaf primordia. F. Shoot tip on agar. (Modified from Arditti and Strauss, 1979.)**

Tissue Explants

The selection of specific tissues (Figs 2-13 and 2-14) as primary explants depends on the ultimate goal of tissue culture (mass, rapid, clonal propagation or micropropagation, production of callus for in vitro selection, or a source of protoplasts or cells for cultures). The response of a tissue or explant to in vitro conditions may vary widely between families, genera, species, hybrids, clones, and genotypes, and even within the same genotype grown under different environmental conditions. There may also be endogenous physiological rhythms in plants that undergo periodic (annual, seasonal, diurnal) fluctuations which can play critical roles in the establishment of successful cultures. Endogenous cycles may play a particularly critical role in the establishment of protoplast cultures from various tissues of plants.

Shoot tips or meristems (Fig. 2-13B–E) can be utilized as primary explants for the establishment of callus or for mass clonal propagation. Techniques for excision vary slightly with the growth form of the shoot. Generally, meristems are located at the tips of shoots or buds protected by sheathing petioles, leaves, or scales. The shoot tips are sterile and the protective structures maintain their sterility. These structures also protect the shoots from surface sterilants. The shoot tips of *Paphiopedilum* do not have such protection and can be damaged during surface sterilization. This is one reason for the limited success in the culture of shoot tips from mature plants of this genus. In-vitro-grown seedlings are easier to culture because they do not require surface sterilization.

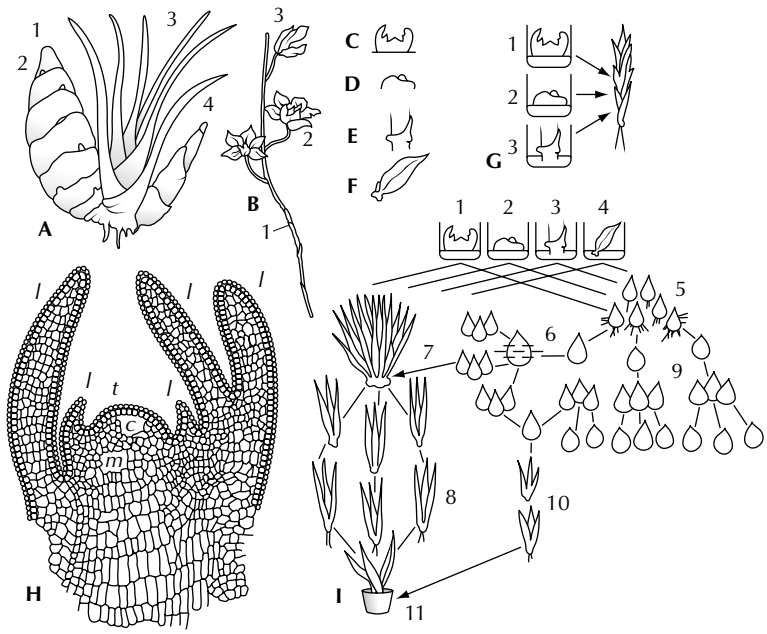


FIG. 2-14. **Sources, utilization, and development of explants. A. (1) Old, (2) young, and (3) new growth, and (4) pseudobulbs and axillary bud. B. Inflorescence with (1) axillary bud, (2) open flowers, and (3) bud in the process of opening. C. Apical meristem. D. Bud. E. Bud on a flower stalk. F. Young leaf. G. Shoots can be formed from (1) shoot tips, (2) buds, and (3) flower-stalk buds. H. Shoot tip (*c,* corpus; *l,* leaf primordial; *m,* meristem; *t,* tunica). I. Development of (1) shoot tips, (2) buds, (3) flower-stalk buds, and (4) young leaves, into (5) protocorm-like bodies (PLBs). The PLBs can be (6) cut and subcultured, or (7) eventually form, (8) multiple, or (9) proliferate naturally, or (10) single shoots that (11) develop into plantlets. (Czerevczenko and Kushnir, 1986.)**

The process of meristem or shoot-tip excision from stems, buds, or other organs requires several important, sensitive, and critical steps. First, the entire plant or growth (corm, tuber, stems, leaves, roots) is removed from the soil or potting mix or a part of a plant is excised.

The next step is to wash and scrub the plant (or the excised parts) with a soft bristle (or a well-used and discarded) toothbrush or another suitable brush, a mild household detergent or baby shampoo, and ample running tap water. Long exposures to high concentrations of some detergents or surfactants (even mild ones) can damage plant tissues (Ernst et al., 1971*a*; Healey et al., 1971), but a brief exposure during washing followed by a thorough rinse will not have deleterious effects in the great majority of cases.

Dissection is initiated after the wash and rinse by careful removal of mature leaves, scales, dead tissues, debris, roots, and other parts using a sharp scalpel (see Fig. A1-4, Appendix 1) or razor blade, taking care not to damage the young internal tissues and/or the part to be excised and cultured. When excising a shoot tip or meristem, the lower portion of the organ (usually a stem) should be removed so that the exposed cut edge is perpendicular to the axis. The top portion can then be mounted by placing the flat cut edge on a Styrofoam block or a cork and affixing it with long

TABLE 2-20. **Dilutions of household bleaches containing different concentrations of sodium hypochlorite**

Percentage of sodium hypochlorite (NaOCl) in household bleach	Volume of household bleach which should be diluted to 100 ml with distilled water to obtain a desired concentration, ml[a]						
	2.0%	2.2%	2.4%	2.5%	2.6%	2.8%	3.0%
4	50	55	60	63	65	70	75
4.25	47	51	57	59	61	66	71
4.5	44	49	53	56	58	62	67
4.75	42	46	51	53	55	59	63
5	40	44	48	50	52	56	60
5.25	38	42	46	48	50	53	57
5.5	36	40	44	46	47	51	55
5.75	35	38	42	44	45	49	52
6	33	37	40	42	43	47	50

[a]Volumes of bleach to use are rounded to the nearest whole figure. There are many brands of household bleach which contain sodium hypochlorite. The concentration of sodium hypochlorite in any given brand can change. Therefore it is advisable to check the label. Pour the bleach into a container (preferably a volumetric flask), adjust to 100 ml with distilled water and add a few drops of surfactant (Tween 20, mild household detergent, or baby shampoo). The diluted bleach must be used within 6–8 h or less.

pins inserted at an angle. Mounting in this fashion stabilizes the organ and allows for: (1) easy surface sterilization by inverting the mounted organ into a sterilizing solution; and (2) microexcision of the part to be cultured (shoot tip or any other tissue explant). Commonly, about 2.4–2.6% of sodium hypochlorite is a suitable solution for surface sterilization. The amount of household bleach to use to obtain such a concentration will depend on the levels of sodium hydroxide in the brand being used (Table 2-20).

Instructions for surface sterilization are given in every method discussed in Chapter 3.

Shoot tips are located at the top of the shoot system and may or may not be covered by mature leaves. Their dissection requires careful removal of leaf primordia under a dissecting microscope and excision of the meristem on a cube of subjacent tissue. The younger leaf primordia surrounding the shoot tip are smaller than older ones and more closely associated with the meristem. These primordia are more difficult to excise without damaging the apical dome (Fig. 2-13C–F). Often, damage to the meristem itself can be avoided by excising primordia under (or with the aid of) a dissecting microscope.

When a dissecting microscope is used, the body and stage must first be wiped with towel (cloth or paper) moistened with water that contains a few drops of detergent. After that the microscope must be wiped with a towel that has been dipped in distilled water. And, just before the dissection, the tissues must be wiped carefully several times with a towel wetted with 70% ethanol (74 ml of 95% ethanol brought up to 100 ml with distilled water) to eliminate contaminants. Once most of the young primordia have been removed, the shoot tip or meristem can be excised on a small cube (Fig. 2-13E) of subjacent tissue (usually not larger than 1 cm^3, and not smaller than 0.5 cm^3) and lifted on a scalpel or a loop to an agar slant (Fig. 2-13F).

Lateral buds can also be removed during the dissection process prior to excision of the terminal meristem or shoot tip. In most orchids, lateral buds are clearly visible in the axils of leaves and appear as glistening raised domes (usually 0.5–1 cm in diameter), similar in appearance to the apical meristem (Fig. 2-13E, F). As each

leaf is dissected, lateral buds along with subjacent tissue can be excised and transferred to agar slants. The size of lateral buds is dependent on genotype, location, and growth conditions, but explants should be in the same size range as those taken from shoot-tip meristems, as described above.

Root sections, tips, or primordia can also be excised and cultured. Reports of callus production and plantlet formation from roots are not as common as those from shoot tips, but the available evidence indicates that these tissues can be useful for micropropagation. The presence of mycorrhiza may make surface sterilization and decontamination of the root difficult or even impossible. The problem can be avoided by using only aerial roots that have not been in contact with soil, potting mix, benches, bark surface, or any other object that could lead to penetration of a fungus.

Seedling tissues, shoot tips, leaves, or roots and their sections or parts can also be used as explants. However, since the nature (or quality) of seedlings is not known there is nothing to be gained from clonal propagation of any one seedling. However, when very few seedlings are produced by what seems to be a very desirable cross, micropropagation may be the only means of increasing their number. Explants from seedlings growing in vitro can also be very useful when the intent is to test a medium or a procedure while eliminating the possible effects of surface sterilization.

Any young or mature tissue from leaf blades, petioles, scale-like leaves, roots, rhizomes, corms, tubers, stems, flowers, or fruits may be suitable as primary explants for the production of either callus or adventitious shoot buds. Dissection of these tissues is usually very simple and involves cutting and removing explants of appropriate size after surface sterilization. Detailed descriptions of excision methods for such tissues are given as part of specific procedures in Chapter 3.

The placement, density, and size of explants as well as the nature of the dissection (e.g., longitudinal vs. cross sections through an organ) may contribute to successful proliferation, growth, and/or differentiation of primary explants. Therefore it is best to follow published reports precisely. If there is no previous work, or when the information is not given in the original publication, it is best to change these parameters independently of each other and only one at a time at first. This approach may increase the likelihood of hitting on the right combination.

Internal Contaminants

There are reports in the literature about microorganisms that reside in plant tissues and contaminate cultures after explants are placed on appropriate media. The literature also contains suggestions regarding the handling of such contamination. In nearly half a century of culturing orchid seeds and explants in our laboratory we have never encountered contamination by internal microorganisms in orchids (except of course mycorrhizal fungi). We also never saw such contamination in other laboratories. The only exception is contamination by organisms found under the scales which cover the flower stalk buds in *Phalaenopsis*. When these scales are removed before surface sterilization there is no such contamination. Anecdotal reports (mostly oral) about contamination by internal microorganisms in orchids do exist, but in the absence of publication in major (and peer-reviewed) journals they remain no more than unconfirmed anecdotes. This being the case not much can or should be written about

presumed contamination of this nature. Anecdotal information, even if advanced or contrived by self-styled experts, has no place in a book like this one. Should contamination by internal microorganisms occur, anticontaminants and PPM may prove useful in combating it.

Enzymes for Protoplast Isolation

A number of mixtures, each consisting of several enzymes, are used for protoplast isolation. Many of the original procedures were carried out with enzymes obtained from Japanese companies. Some or all of these enzymes are still being used, but preparations that have become available more recently are also being utilized. Since success may often depend on using each method exactly as it was formulated originally it is not only advisable but actually imperative to use exactly the same enzymes and preparations as in the original reports. They should be purchased from the sources listed in the original publications.

An enzyme solution used by a foremost orchid micropropagation expert in Japan, Professor Syoichi Ichihasi of the Department of Biology, Aichi University of Education, consists of 1.0% cellulase Onozuka RS (Yakult Pharmaceutical Ind. Co., Ltd., www.yakult.co.jp/ypi/english/index.html), 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., www.katayamakagaku.co.jp/menu-top.htm), 0.5% Driselase (Kyowa Hakko Kogyo Co., Ltd., www.kyowa.co.jp/eng/index.htm), 160 mg $Ca(NO_3)_2 \cdot 4H_2O$ 100 ml^{-1}, 10 mg KH_2PO_4 100 ml^{-1}, 58.5 mg 2-(*N*-morpholino)-ethane sulfonic acid (MES) 100 ml^{-1}, and 0.3 mol sorbitol l^{-1}, pH 5.6. Professor Ichihashi's method for the isolation of protoplasts is now published with the proceedings of the World Orchid Conference held in Kuala Lumpur, Malaysia in early 2002 (Ichihashi and Shigemura, 2005).

Work Area

Mixing heat-sterilized solutions with components in 95 or 70% ethanol stock solutions, pouring medium into culture vessels, and excising explants and placing them in culture must be carried out under aseptic conditions. Such conditions can be obtained in several ways.

Laminar-flow Hoods

The best and most efficient means of assuring sterility in the working area is to use a laminar-flow hood (see Appendix 1). Air coming into these hoods is driven through filters that remove all particles. The sterile air is blown gently across the working area toward the operator, and this generally prevents contamination of cultures. Tools and work surfaces must, of course, be sterilized, even when used in such a hood, and care must be taken to prevent the introduction of contaminants from unsterile surfaces into vessels.

An important point to keep in mind is that only the air coming into the hood is sterile. The surfaces inside the hood are not sterile and must be sterilized before the

hood is used. This can be done by spraying the inside of the hood with 70% ethanol prior to use. In addition, the inside of the hood should be irradiated with a germicidal ultraviolet (UV) lamp prior to use or while it is not being used, with the front opening curtained off with a plastic curtain. This curtain can prevent the entry of dust into the hood and is also necessary for the protection of workers because UV light can cause severe damage to eyes (people should never look at the UV lamp even through the curtain).

The work space inside these hoods is large enough for comfortable, fast, and efficient movements. There is also space inside for tools, a gas or alcohol burner for flaming tools, aluminum foil and glassware necks, culture vessels, magnifying glasses, and microscopes. The prices of smaller hoods are now low enough to justify their purchase by most laboratories where seed germination, seedling culture, and micropropagation are or may become routine activities.

Sterile Rooms

These are usually small rooms fitted with hard-surface benches that are kept clean by swabbing with alcohol or hypochlorite solution and irradiation with sterilizing UV lamps (which must be allowed to stay on for at least 30 min to ensure sterility but have to be turned off when the operator enters the room). All culture vessels and tools are placed in these rooms, sterilized by washing or spraying with alcohol or hypochlorite, and irradiated with UV light.

When everything is sterile, an operator enters, having washed his or her hands carefully (short clean nails are important if no gloves are worn). Alternatively, the operator can wear surgical gloves, which are kept sterile by periodic swabbing with alcohol or hypochlorite. All hair must be under a shower cap. Tools must be kept sterile by flaming and/or dipping them in alcohol or hypochlorite before and after every use. The necks of culture vessels must be flamed after removing the cotton buns, following the introduction of tissues, and after replacing the buns.

Sterile rooms were popular at one time, but they tend to be expensive and not very comfortable, efficient, or desirable working areas. At present they have been largely replaced by sterile hoods. They are mentioned here mainly for historical reasons and because some may still be in use. The construction of new sterile rooms and the use of existing ones are not advisable.

Sterile Boxes

An enclosure made of plastic, glass, stainless steel, or wood painted with hard polyurethane or plastic, or a cardboard box lined with aluminum foil (see Appendix 1) can prove to be a fairly satisfactory aseptic working area for tissue culture. The inside of the box is kept sterile by washing or spraying it with alcohol or hypochlorite. Irradiating it with sterilizing UV lamps is very desirable but not strictly necessary. Tools, an alcohol burner, culture vessels containing medium, and containers with sterilizing solutions are placed in the box and sterilized by spraying or swabbing them with alcohol or hypochlorite. Approximately 20 min after the swabbing

or spraying, the operator (preferably wearing gloves) can insert his or her hands into the box through long plastic bags attached to the front openings and start to work. All other procedures are as in a sterile room.

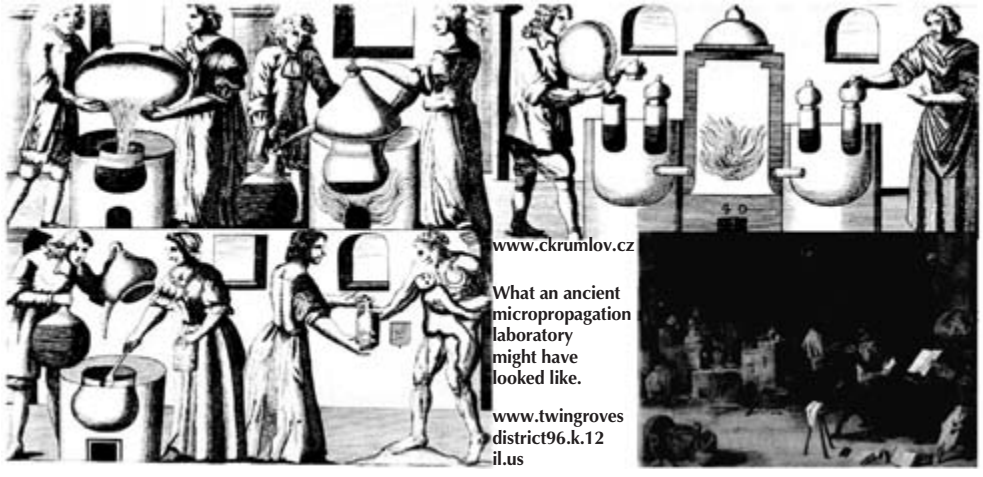
Sterile boxes are suitable for a small laboratory or one that is just initiating a tissue culture program. Also, sterile boxes can be useful for those who are just starting or trying to decide if micropropagation is something they may want to do. With a minimum of training, dexterity, and experience, most operators can use such a box successfully. At present, sterile boxes are no longer used extensively because small sterile hoods are inexpensive and much more convenient, safe, efficient, and productive. Sterile boxes are mentioned here for historical reasons and because some are seen occasionally. On the whole their use is no longer recommended.

Clean Laboratory Bench

In some locations (clean laboratories, areas of low atmospheric humidity), experienced operators can simply use a clean laboratory bench as an appropriate work area, but this is not generally advisable.

Washing Glassware

It is imperative that all glassware (culture vessels, volumetric flasks or cylinders, beakers, test tubes, etc.) be chemically clean. This is especially true for containers that have been used previously for other purposes (to hold ketchup, liquor, soft drinks, or medicines, for example) or even tissue culture and for new vessels that were not manufactured for use as they come out of their boxes. Disposable presterilized culture vessels are widely available and relatively inexpensive at present. They do not require washing prior to initial use.



www.ckrumlov.cz

What an ancient micropropagation laboratory might have looked like.

www.twingroves district96.k.12 il.us

CHAPTER THREE

Methods for Specific Genera

Acampe

Although not of major commercial importance *Acampe* has attracted some attention as a subject for studies of tissue culture and the isolation of protoplasts.

Isolation of Protoplasts from *Acampe praemorsa*

For the most part, research on the micropropagation of orchids, and especially protoplast culture, has centered on species and hybrids of commercial importance. The approach at the Tropical Botanic Gardens and Research Institute at Trivandrum, India, is to initiate "a major research programme . . . to [screen] some wild species and known hybrids of orchids for protoplast isolation . . ." (Seeni and Abraham, 1986). One of the species screened was *Acampe praemorsa*.

Plant Material. Root tips of mature plants and mesophyll from young leaves (second or third leaves from the top) are suitable protoplast sources. Thin slices of these explants "were suspended in . . . enzyme mixture. . . ."

Surface Sterilization. There is no mention of surface sterilization in the original paper. Methods used for surface sterilization of leaves and roots in other methods should prove suitable.

Culture Vessels. Details are not given in the original paper (Seeni and Abraham, 1986), except for one mention of what are presumably Petri dishes. However, it is reasonable to assume that apparatus and culture vessels used in other protoplast isolation procedures would be suitable (see *Aranda* entry for example).

Isolation and Culture Conditions. Tissue should be suspended in the enzyme mixture at room temperature (ca. 22°C) in the dark. Suitable conditions for the culture of protoplasts are 26°C in the dark in Petri dishes sealed with parafilm.

Isolation Reagent, Washing Solution, and Culture Medium. The enzyme consists of 0.5% (w/v) Macerozyme R-10, Onozuka cellulase R-10, 2% (see *Aranda* entry, Isolation and Culture of Mesophyll Protoplasts from leaves of *Aranda* Noorah Alsagoff, for sources of enzymes), 5-m*M* (976.2 mg l^{-1}) 4-MES buffer (see Appendix 2, for sources), 1-m*M* (111 mg l^{-1}) $CaCl_2$ (or 147 mg l^{-1} $CaCl_2 \cdot 2H_2O$), and 0.3-*M* (54.65 g l^{-1}) sorbitol. The pH should be adjusted to 5.5.

The washing solution is 1-*M* (342.30 g l^{-1}) sucrose, whereas 0.3-*M* (54.65 g l^{-1}) sorbitol should be used to suspend the washed protoplasts. The culture medium for protoplasts includes components of two standard solutions as well as other additives (Table Acampe-1).

Procedure. Suspend thin sections of tissue, taken from 600 mg (fresh weight) of tissue, in 5 ml of enzyme mixture at room temperature (ca. 22°C) in the dark with no shaking at all or with gentle agitation at 2-h intervals for 6 h. Remove undigested tissues and large debris by filtering the mixture through a nylon mesh with 100-μm pores. Then mix the filtrate with an equal volume of the 1.0-*M* sucrose solution, and centrifuge the filtrate at 120 g for 3 min. After the centrifugation the protoplasts will be in the supernatant, which must be decanted carefully. Seeni and Abraham (1986) state that "protoplasts were washed once," which can be taken to mean that the supernatant was mixed with 0.3-*M* sorbitol and recentrifuged to form a pellet. This pellet should then be suspended in 2 ml of the 0.3-*M* sorbitol solution. The number of protoplasts can be determined with a hemocytometer.

Before culturing the protoplasts it is necessary to wash them by resuspending the pellet in the culture medium (Table Acampe-1) and recentrifuging them. The new pellet should be resuspended in the culture medium at a concentration of approximately 10^6 protoplasts per milliliter in Petri dishes sealed with parafilm.

Developmental Sequence. The protoplast yield from leaves of *A. praemorsa* was 1.2×10^4 g per tissue or 10,000 per gram of tissue. Roots yielded only 0.2×10^4 protoplasts per gram of tissue. The enzyme mixture turned brown after the *A. praemorsa* tissues were placed in it, "possibly due to oxidation of phenolic compounds," and this may be one reason for the low yields. It may also be a reason why the original paper does not report whether the protoplasts divided in culture.

General Comments. The orchid protoplast research program at the Tropical Botanic Gardens and Research Institute at Trivandrum is laudable, and their results with *A. praemorsa* and other orchids suggests that it will be successful.

TABLE ACAMPE-1. **Modified Vacin and Went medium (Vacin and Went, 1949) for protoplast culture of *Acampe praemorsa* (Seeni and Abraham, 1986)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (a concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Polyol				
8	*myo*-inositol[e]	100	No stock	No stock	Weigh
	Auxin				
9	Naphthaleneacetic acid (NAA)		100 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
10	Benzyladenine	0.2	20 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1}	1	
	Sugar alcohol				
14	Mannitol	36.4 g	No stock	No stock	Weigh
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve and it is not possible to prepare a stock solution. To facilitate the preparation of medium place 200 mg of the salt in 500 ml water and stir and/or heat until it dissolves. Add the other components of the medium after that.

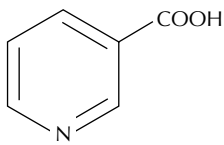
[d]Like other iron salts, this one is hard to dissolve. It is, therefore, preferable to chelate the iron. To prepare a stock solution of chelated iron dissolve 3.73 g chelating agent, Na_2EDTA, and 2.78 g of ferrous sulfate, $FeSO_4 \cdot 7H_2O$, in 1 l water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Also known as inositol, *i*-inositol, or *meso*-inositol.

[f]If auxins and cytokinins do not dissolve, a few drops of KOH or HCl, respectively, can be used to solubilize them.

[g]Keep refrigerated between uses.

[h]Add items 1, 3–8, and 14 to the 500-ml solution of calcium phosphate (item 2). Bring volume to 900 ml with distilled water (item 16); set pH as required (not given in this case, but 5.8 should be suitable); add sugar (item 15) and adjust volume to 1000 ml with distilled water (item 16). Autoclave solution, add hormones and vitamins (items 9–13) under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.



Niacin (also known as nicotinic acid) is one of the most commonly used vitamins in orchid micropropagation. Pharmaceutical or nutritional supplement preparation should be avoided, or used only after prior testing because the tablets contrain binders or other unknown additives

Plantlet Regeneration from Leaf Tips of *Acampe rigida*

In Hong Kong *Acampe rigida* grows on rocks in sunny location. It is of sparse distribution, but populations tend to be large. A method for plantlet regeneration from leaf tips was developed as part of research associated with a Ph.D. dissertation (Yam, 1989; Yam and Weatherhead, 1991*a*).

Plant Material. In the original research, leaf tips 1–1.5 cm in length were taken from 2-cm-long leaves on 6-month-old seedlings. It is important to use a sharp and sterile scalpel blade. Tips should be cut in a sliding action, and pressure on the tissues should be avoided.

The seeds were germinated and the seedlings were grown on Knudson C medium supplemented with 20% (v/v) coconut water from mature nuts. They were maintained under conditions of 25 ± 2°C and 16-h photoperiods of 2000 lx provided by four 40-W Gro Lux fluorescent tubes placed 60 cm above the cultures.

Surface Sterilization. Since these tips are taken from aseptic seedlings, there is no need to surface-sterilize them.

Culture Vessels. Wide mouth, gamma-irradiated plastic flasks, 75-ml capacity (Johns Mallinkrodt, Johns Division, Mallinkrodt Australia, Pty., Ltd.) were used in the original research. Other containers like Erlenmeyer flasks, various bottles, and test tubes are also suitable.

Culture Conditions. In the original research the flasks were wrapped in aluminum foil and placed on shelves next to seedling cultures until plantlets were formed (ca. 2–3 months). Thus the cultures were maintained in the dark, and other facilities that provide darkness would also be suitable. After plantlets were formed, they were transferred first to 500 lx (one 40-W Gro Lux tube ca. 60 cm above the flask) for 1 month, then to 1000 lx (two 40-W Gro Lux tubes 60 cm above the cultures) for another month, and finally to 2000 lx.

Culture Media. If explants are placed on a modified Heller's medium (Table Acampe-2), the number of leaf tips that form plantlets is larger. However, fewer plantlets are produced per leaf tip. On the Ichihashi–Yamashita medium (Table Acampe-3) the number of plantlets per leaf tip is larger, but fewer explants survive.

Procedure. Cut and place leaf tips flat on the medium with their upper epidermis facing upward. Examine the cultures at regular intervals, and when plantlets are formed, move them to the light.

Developmental Sequence. PLBs form after approximately 1 month of culture. Plantlets develop on these bodies after 1–2 months (a total of 2–3 months in culture).

General Comments. This method can be used with other orchids and may be employed to increase the number of plantlets in cases where only a few seedlings of a specific

TABLE ACAMPE-2. **Heller's medium (Heller, 1953) as modified for the culture of leaf tips from seedlings of *Acampe rigida* (Yam, 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (a concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium chloride, $CaCl_2 \cdot 2H_2O$	75	7.5 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25.7 g l^{-1}	10	
3	Monophosphate, $NaH_2PO_4 \cdot H_2O$	125	12.5 g l^{-1}	10	
4	Potassium chloride, KCl	750	75 g l^{-1}	10	
5	Sodium nitrate, $NaNO_3$[b]	600	60 g l^{-1}	10	
6	**Micronutrients**[c]				
(a)	Aluminum chloride, $AlCl_3$	0.03	3 mg l^{-1}	10	One solution
(b)	Boric acid, H_3BO_3	1	100 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.03	3 mg l^{-1}		
(d)	Ferric chloride, $FeCl_3 \cdot 6H_2O$	1	100 mg l^{-1}		
(e)	Manganese sulfate, $MnSO_4 \cdot H_2O$	0.1	10 mg l^{-1}		
(f)	Nickel chloride, $NiCl_2 \cdot 6H_2O$	0.03	3 mg l^{-1}		
(g)	Potassium iodide, KI	0.01	1 mg l^{-1}		
(h)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1	100 mg l^{-1}		
	Auxin				
7	Naphthaleneacetic acid (NAA)[d,e]	0.2	20 mg 100 ml^{-1} 95% ethanol	1	
	Cytokinin				
8	Benzyladenine (BA)[e,f]	1	100 mg 100 ml^{-1} 95% ethanol	1	
	Vitamin				
9	Thiamine·HCl (vitamin B_1)[e]	1	100 mg 100 ml^{-1} 95% ethanol	1	
	Sugar				
10	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar, Difco Bacto[g]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add all the microelements to the same 1 l of distilled water, and stir and/or heat until all components are dissolved. It may be advisable to replace the $FeCl_3$ with chelated iron. To prepare a chelated-iron solution add 3.73 g Na_2EDTA (chelating agent) and 2.78 g $FeSO_4 \cdot 7H_2O$ to the same 1 l of distilled water, and stir and/or heat until both are dissolved. Add 10 ml of this solution to 1 l of culture medium.

[d]If the auxin fails to dissolve, add a few drops of dilute KOH.

[e]Keep refrigerated or frozen between uses.

[f]If the cytokinin fails to dissolve add a few drops of dilute HCl.

[g]Add items 1–6 to 900 ml distilled water (item 11), set pH to 5.6, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring solution to a gentle boil, and add agar (item 12) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense solution into a 2-l flask and autoclave. Add auxin (item 7), cytokinin (item 8), and vitamin (item 9) to hot solution under sterile conditions, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

species or cross are available. A change in the procedure that may increase its efficiency would be to culture the leaf tips on Heller's medium (Table Acampe-2) until PLBs form and then to transfer them to the Ichihashi–Yamashita solution (Table Acampe-3).

Acampe is derived from the Greek *akampes* (ἀκαμπής), which means "rigid" (Schultes and Pease, 1963).

TABLE ACAMPE-3. **Ichihashi–Yamashita medium (Ichihashi and Yamashita, 1977) as modified for the culture of leaf tips from seedlings of *Acampe rigida* (Yam, 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (a concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium phosphate, $NH_4H_2PO_4$[b]	391	3.91 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	828	8.28 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	172	1.72 g l^{-1}	10	
4	Potassium nitrate, KNO_3	747	7.47 g l^{-1}	10	
5	**Microelements**[c]				
(a)	Boric acid, H_3BO_3	1	100 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.03	3 mg l^{-1}		
(c)	Ferric EDTA, Fe_2EDTA	25	2.5 g l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	0.1	10 mg l^{-1}		
(e)	Nickel chloride, $NiCl_2 \cdot 6H_2O$	0.03	3 mg l^{-1}		
(f)	Potassium iodide, KI	0.01	1 mg l^{-1}		
(g)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(h)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1	100 mg l^{-1}		
	Auxin				
6	Naphthaleneacetic acid (NAA)[d,e]	0.2	20 mg 100 ml^{-1} 95% ethanol	1	
	Cytokinin				
7	Benzyladenine (BA)[e,f]	1	100 mg 100 ml^{-1} 95% ethanol	1	
	Vitamin				
8	Thiamine·HCl (vitamin B_1)[e]	1	100 mg 100 ml^{-1} 95% ethanol	1	
	Sugar				
9	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[g]	To 1000 ml			
	Solidifier				
11	Agar, Difco Bacto[g]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add all the microelements to the same 1 l of distilled water, and stir and/or heat until all components are dissolved. The iron can be omitted and replaced with a chelated-iron solution prepared by adding 3.73 g Na_2EDTA (chelating agent) and 2.78 g $FeSO_4 \cdot 7H_2O$ to the same 1 l of distilled water and stirring and/or heating until both are dissolved. Add 10 ml of this solution to 1 l of culture medium.

[d]If the auxin fails to dissolve, add a few drops of dilute KOH.

[e]Keep refrigerated or frozen between uses.

[f]If the cytokinin fails to dissolve, add a few drops of dilute HCl.

[g]Add items 1–5 to 900 ml distilled water (item 10), set pH to 5.6, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. The agar can also be added to the cold solution which is then brought to a boil and stirred. When the agar is completely dissolved, dispense the solution into a 2-l flask and autoclave. Add auxin (item 6), cytokinin (item 7), and vitamin (item 8) to hot solution under sterile conditions, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

Direct Shoot Regeneration from Foliar Explants of *Acampe praemorsa*

Acampe is an orchid genus consisting of 15 epiphytic species. Of the five species found in India, *Acampe praemorsa* has attracted attention with its yellow and red flowers. A micropropagation procedure for this species was developed at the Post-Graduate Department of Botany, Utkal University, Bhubaneswar, Orissa, India (Nayak et al., 1997*a*).

Plant Material. Fully expanded third and fourth leaves from in vitro grown plants are most suitable for this procedure. It is not clear from the original source whether the plants from which leaves were taken were seedlings or plantlets produced through micropropagation.

Surface Sterilization. None is needed because the explant sources grow in vitro.

Culture Vessels. Screw-capped glass test tubes, 20 mm in diameter (Fig. Acampe-1D) and 150-ml Erlenmeyer flasks with 30 ml of nutrient solution were used in the original research. Other containers are also suitable.

Culture Conditions. The original cultures were maintained at 25 ± 1°C, 55–60% relative humidity, and 16-h photoperiods of 35–50 μE m^{-2} s^{-1} irradiance provided by Phillips (India) cool white fluorescent tubes.

Culture Media. Modified MS medium is used to induce shoot formation (Table Acampe-4). The shoots elongate on another modification of MS (Table Acampe-5). Rooting occurs on a third modification (Table Acampe-6).

Procedure. The leaves must be inserted vertically (Fig. Acampe-1A–C) into the first medium (Table Acampe-2). Shoots should be transferred to the second medium (Table Acampe-5) for elongation and the third medium (Table Acampe-6) for rooting.

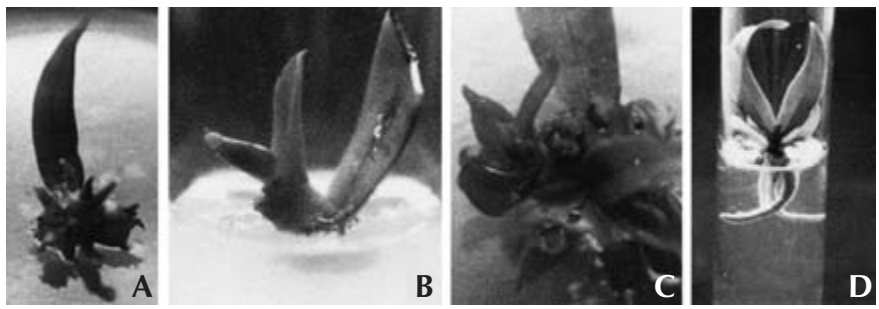


FIG. ACAMPE-1. **Micropropagation of *Acampe*. A. Entire leaf is inserted vertically in the medium. Multiple buds develop on its base after 10 weeks of culture on MS medium containing 1.0 mg TDZ l^{-1}. B. Only a single shoot developed from the base of a leaf (also inserted vertically) after 12 weeks on MS which contains 1 mg NAA l^{-1}. C. Multiple shoot formation after 12 weeks on MS supplemented with 1.0 mg TDZ l^{-1}. D. A rooted shoot on MS with 2 mg IBA l^{-1}. (Nayak et al., 1997*a*.)**

TABLE ACAMPE-4. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Acampe praemorsa* leaf explants (Nayak et al., 1997*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	1-Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
	Cytokinin				
11	Thidiazuron (TDZ)[g]	1.0	10 mg in 10 ml^{-1} 0.1N NaOH in 95% ethanol	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar[h]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1N KOH.

[g]Pure TDZ is expensive (Sigma sells 25 mg for $54.15). It is the active principle in Dropp (50 wettable powder), a cotton defoliant, which is much less expensive. For practical use Dropp can be used at the rate of 2 mg l^{-1}. The solution may be slightly cloudy, but this does not seem to have a deleterious effect on tissue culture media. The problems with using Dropp may be legal (since in some areas it is only approved for use on cotton) and availability (it may be sold in larger amounts than a laboratory can use in a long time). One suggestion on PLANT-TC@TC.UMN.EDU regarding the latter is to find a cotton grower and ask nicely for a small sample. This discussion group offered no suggestions on how to deal with legalities. The only possible (and wise) suggestion here is not to engage in illegal activities.

[h]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.8, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to cold water which is then brought to a boil and stirred. When the agar is completely dissolved distribute the solution into culture vessels, autoclave, and allow the medium to cool before use. As a rule amino acids, hormones, and vitamins should not be autoclaved. However in this case all components were autoclaved by the original researchers.

TABLE ACAMPE-5. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified to bring about elongation of *Acampe praemorsa* shoots produced by leaf explants (Nayak et al., 1997a)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	1-Naphthaleneacetic acid (NAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[f]	1	
	Cytokinin				
11	Benzyladenine (BA)[g]	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar[h]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]A combination of 1 mg NAA l^{-1} and 10 mg BA l^{-1} can also be used to induce shoots on the leaf explants but it is not as effective as NAA and thidiazuron (Table Acampe-2).

[h]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.8, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved distribute the solution into culture vessels, autoclave and allow the medium to cool before use. As a rule amino acids, hormones and vitamins should not be autoclaved. However in this case all components were autoclaved by the original researchers.

TABLE ACAMPE-6. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for rooting *Acampe praemorsa* shoots produced by leaf explants (Nayak et al., 1997*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indole-3-butyric acid (IBA)	1.0	100 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[h]	To 1000 ml			
	Solidifier				
16	Phytagel[h]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1N KOH.

[g]A combination of 1 mg NAA l^{-1} and 10 mg BA l^{-1} can also be used to induce shoots on the leaf explants but it is not as effective as NAA and thidiazuron (Table Acampe-2).

[h]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.8, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Add the Phytagel (item 16) to the cold medium slowly while stirring vigorously. When the Phytagel is completely dissolved distribute the solution into culture vessels, autoclave, and allow the medium to cool before use. Phytagel should not be added to warm or hot water because it will form clumps which will not dissolve or disperse later or during autoclaving. As a rule, amino acids, hormones, and vitamins should not be autoclaved. However, in this case all components were autoclaved by the original researchers.

Developmental Sequence. In the original research, bases of leaves cultured on MS medium containing TDZ expanded and became swollen within 5–7 weeks in culture. Buds formed after 8–9 weeks. They appeared as small green protuberances. This process continued for 12 weeks. There was no callus or PLB formation. When shoots were formed on the TDZ-containing medium, they failed to elongate. Elongation did take place 12–15 days after the shoots were transferred to a medium containing NAA and BA. Roots were formed on a medium containing IBA.

General Comments. Shoot regeneration occurred only on the lower portions of leaves (Fig. Acampe-1D). If leaves were cut in half horizontally, shoots did not form on the upper halves. The highest frequency of shoot regeneration was recorded on fully expanded third and fourth leaves. There was no shoot formation on first, second (both expanding), and fifth and sixth (older) leaves. The context of the original paper suggests that leaves may have been taken from seedlings. If so, selection for desirable cultivars is not possible. A method for seed germination and seedling culture of *A. praemorsa* was also developed in India (Kanjilal et al., 2001).

In Vitro Propagation of *Acampe praemorsa* from Leaf Explants

A method for micropropagation of *Acampe praemorsa* using young leaves was developed at Karnatak University in India (Pyati and Murthy, 1999).

Plant Material. Basal or tip segments, 0.5–1.0 cm long, from young leaves up to 2 cm in length taken from 16–20-week-old axenic seedlings should be used (similar leaves from greenhouse-grown plants do not grow).

Surface Sterilization. There is no need to surface-sterilize the explants because they are taken from axenically grown plants.

Culture Vessels. The original paper does not mention culture vessels. However, photographs in it suggest that test tubes containing 30 ml of medium are suitable.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 12-h photoperiods of intensity similar to that used in the previous procedure. No information is presented in the original paper regarding the source(s) of illumination.

Culture Media. MS medium containing 1 mg BA l^{-1} should be be used to induce formation of PLBs (Table Acampe-7), which occurs on 65% of the explants after 4 weeks of culture. Callus is formed on 45% of explants after 4 weeks on MS supplemented with 1 mg NAA l^{-1} (Table Acampe-8). For callus formation followed by PLB production the explants should be cultured on MS with 1 mg BA and 1 mg NAA l^{-1} (Table Acampe-9). To induce shoot and root formation, the PLBs should be transferred to fresh MS containing 1 mg BA l^{-1} (Table Acampe-7).

TABLE ACAMPE-7. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for induction of protocorm-like bodies from leaf explants of *Acampe praemorsa* (Pyati and Murthy, 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzylaminopurine (benzyladenine, BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.6, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), BA (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. This method of preparing the medium assumes that the amino acid, cytokinin, and vitamins should not be autoclaved. The original paper does not make it clear whether they were autoclaved or not. If a test shows that the medium is effective after these components are autoclaved, items 1–13 should be added to 900 ml of distilled water (item 15). Following that the steps are the same as above until the agar is dissolved. After that the medium should be dispensed into culture vessels and autoclaved.

TABLE ACAMPE-8. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for induction of callus from leaf explants of *Acampe praemorsa* (Pyati and Murthy, 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	1-Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N NaOH.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.6, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), NAA (item 10), and vitamins (items 11–13 to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. This method of preparing the medium assumes that the amino acid, auxin, and vitamins should not be autoclaved. The original paper does not make it clear whether they were autoclaved or not. If a test shows that the medium is effective after these components are autoclaved, items 1–13 should be added to 900 ml of distilled water (item 15). Following that the steps are the same as above until the agar is dissolved. After that the medium should be dispensed into culture vessels and autoclaved.

TABLE ACAMPE-9. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for induction of callus followed by protocorm-like bodies from leaf explants of *Acampe praemorsa* (Pyati and Murthy, 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	1-Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (benzyladenine, BA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), NAA (item 10), BA (item 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. This method of preparing the medium assumes that the amino acid, cytokinin, and vitamins should not be autoclaved. The original paper does not make it clear whether they were autoclaved or not. If a test shows that the medium is effective after these components are autoclaved, items 1–14 should be added to 900 ml of distilled water (item 16). Following that the steps are the same as above until the agar is dissolved. After that the medium should be dispensed into culture vessels and autoclaved.

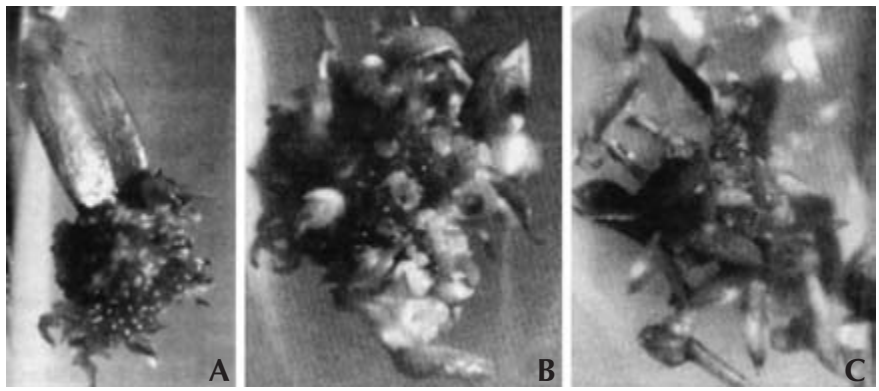
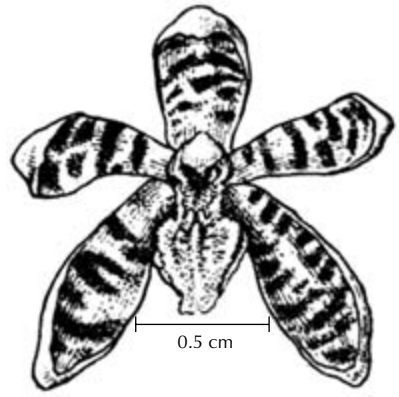


FIG. ACAMPE-2. **Leaf culture of *Acampe praemorsa*. A. PLBs on explant after 8 weeks of culture on MS medium containing 1 mg BA l^{-1}. B. Shoot on PLB after 12 weeks of culture (medium not indicated in caption for illustration in the original paper). C. Plantlets which formed after 12 weeks of culture (medium not indicated in original caption). (Pyati and Murthy, 1999.)**

Procedure. Explants taken from young plants should be sectioned and placed on the medium (Fig. Acampe-1). Once formed, PLBs should be moved to fresh medium for shoot and plantlet formation (Fig. Acampe-2).

Developmental Sequence. Depending on the medium, the explants may form PLBs (Table Acampe-7), callus (Table Acampe-8), or callus followed by PLBs (Table Acampe-9). The PLBs form plantlets when moved to fresh medium.

General Comments. A clear advantage of procedures that use leaf explants is that the donor plant is not endangered. However, propagating seedlings of unknown quality may be useful only in cases when there are few viable seeds in a capsule.



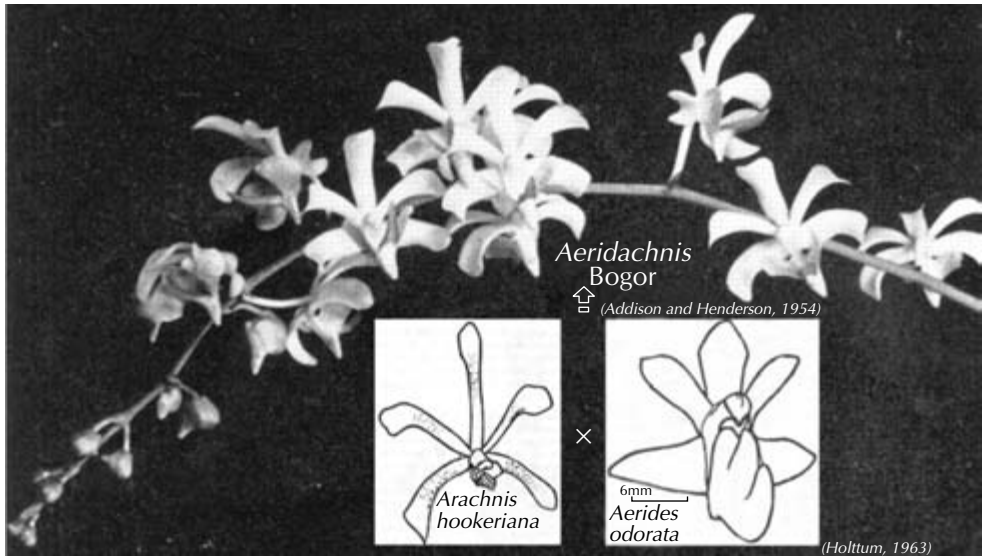
***Acampe praemorsa* flower (Abraham and Vatsala, 1981)**

Aeridachnis

A hybrid genus, *Aeridachnis* or *Aëridachnis*, is a cross between *Aerides* and *Arachnis*. The first hybrid, *Aeridachnis* Bogor, was reported in 1954.

Culture of Apical Buds of *Aeridachnis* Bogor

Apical and axillary buds of *Aeridachnis* Bogor 'Apple Blossom' (*Arachnis hookeriana* × *Aerides odorata*), *Aeridachnis* Alexandra (*Aeridachnis* Bogor × *Arachnis flos-aëris* var. *insignis*), and *Aeridachnis* Elizabeth Howe (*Arachnis* Ishbel × *Aerides lawreanceae*) were cultured at the Singapore Botanic Gardens by the method used for *Arachnis* (Lim-Ho, 1981). The explants are first cultured in a liquid modification of the Vacin and Went medium (see Table Arach-4). Two solid modifications (see Tables Arach-4 and Arach-5) are used for proliferation and differentiation. A third solid modification is employed for plantlet formation (see Table Arach-6). Explant growth is slow.



Aerides

An epiphytic genus, *Aerides*, consists of approximately 20 species which are found in South East Asia.

Protoplast Culture of *Aerides ringens*

The procedure developed for *Acampe praemorsa* was used to obtain 1.0×10^4 and 1.6×10^4 protoplasts per gram of tissue from leaves and roots, respectively, of *Aerides ringens* (Seeni and Abraham, 1986).

Micropropagation of *Aerides maculosum* through the Culture of Leaf Explants

A south Indian orchid valued for its inflorescences, *Aerides maculosum* has been over-exploited and its population is dwindling. Attempts to germinate it symbiotically have not been very successful. Only 0.3% of the seeds germinate. Therefore, a micropropagation method was developed with a view towards conservation and commercialization (Murthy and Pyati, 2001; Murthy et al., 2001).

Plant Material. Young leaves up to 2 cm long are taken from axenic seedlings and sectioned into 0.5–1.0-cm segments. Both leaf-tip and leaf-base sections of young leaves can be used as explants. Leaves from mature plants cannot be used.

Surface sterilization. There is no need to surface-sterilize the explants because they are taken from axenically grown seedlings.

Culture Vessels. Culture vessels are not described in the original paper, but photographs (Fig. Aer-1) suggest that test tubes, 20 mm in diameter, containing 30 ml of culture medium are suitable.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 12-h photoperiods of 40 μmol PAR m^{-2} s^{-1}. The light sources are not mentioned, but almost any combination of two 40-W fluorescent tubes (cool white or Gro Lux, for example) and two 50–100-W incandescent bulbs (if cool white tubes are used) per fixture placed 50 cm above the cultures should be suitable.

Culture Media. MS medium containing 2.0 mg BA l^{-1} (Table Aer-1) should be used to induce PLB formation. On transfer to basal MS (Table Aer-2) the PLBs produce plants.

Procedure. The explants (leaf sections) must be inserted vertically (Fig. Aer-1A) with their cut ends in the medium (Table Aer-1). Once they are formed, PLBs (Fig. Aer-1B) should be transferred to the second medium (Table Aer-2) for plantlet formation

TABLE AER-1. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for induction of protocorm-like bodies from leaf explants of *Aerides maculosum* (Murthy and Pyati, 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzylaminopurine (benzyladenine, BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.6, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), BA (item 10) and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. This method of preparing the medium assumes that the amino acid, cytokinin, and vitamins should not be autoclaved. The original paper does not make it clear whether they were autoclaved or not. If a test will show that the medium is effective after these components have been autoclaved, items 1–13 should be added to 900 ml of distilled water (item 15). Following that the steps are the same as above until the agar is dissolved. Following this the medium should be dispensed into culture vessels and autoclaved.

TABLE AER-2. **Basal Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as used for shoot and root formation on protocorm-like bodies generated from leaf explants of *Aerides maculosum* (Murthy and Pyati, 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol[f]	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[g]	To 1000 ml			
	Solidifier				
15	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]This substance is also known as inositol and *meso*-inositol. Actually there is no definitive proof that this polyol is required by explants. It is being added routinely to media as part of the original formulation. Inositol can be found among nutrition supplements and vitamins in many pharmacies and food stores. However care should be taken in using inositol from these sources because the preparations may contain other substances.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 14), adjust pH to 5.6, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8) and vitamins (items 10–12) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. This method of preparing the medium assumes that the amino acid and vitamins should not be autoclaved. The original paper does not make it clear whether they were autoclaved or not. If a test will show that the medium is effective after these components have autoclaved, items 1–12 should be added to 900 ml of distilled water (item 14). After that the steps are the same as above until the agar (item 15) is dissolved. Following this the medium should be dispensed into culture vessels and autoclaved.

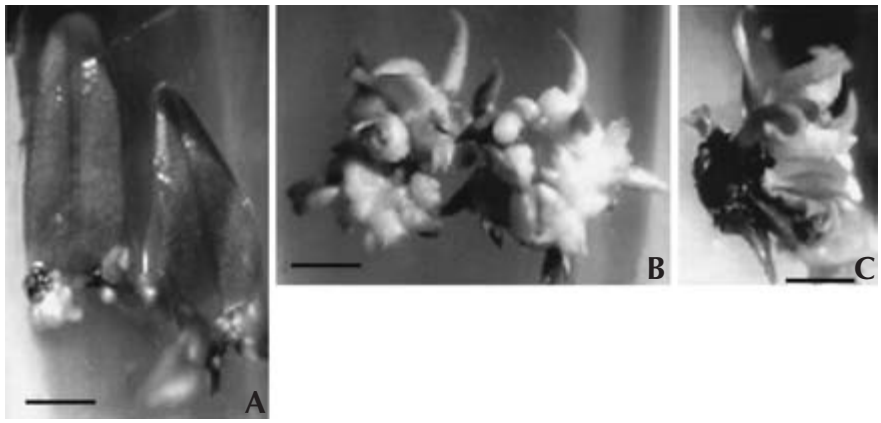


FIG. AER-1. **Culture of *Aerides maculosum* leaf explants. A. Leaf-tip explants inserted vertically in medium and developing PLBs on their bases. B. Increase in the number of PLBs. C. Development of shoots and roots. (Murthy and Pyati, 2001.)**

(Fig. Aer-1C). In the original research plantlets that formed on the second medium (Table Aer-2) were transferred to vermiculite for further development. After that they should be potted. A potting mix consisting of brick pieces, charcoal, and chopped dried coconut husks (in the ratio 1 : 3 : 1; probably v/v/v) was used in the original research. Survival in the last potting mix was 84%. A different potting mix may also be suitable.

Developmental Sequence. Bases of explants start to expand and swell within 2 weeks of being inserted in the first medium (Table Aer-1). PLBs start to differentiate after 4 weeks and continue to do so until the 8th week (Fig. Aer-1A, B). There is no callus formation. This occurs only on the bases of explants. Shoots and roots differentiate (Fig. Aer-1C) following transfer to the second medium (Table. Aer-2). Mature plants develop in the vermiculite and the potting medium.

General Comments. When an orchid is propagated through the culture of explants from seedlings it is not possible to select for desirable characteristics because the quality of the donor plants is not known. This usually reduces the value of the propagation procedure. However, in this case, selection is of secondary importance because the seeds of *A. maculosum* do not germinate well and the current procedure makes possible multiplication for conservation and commercialization purposes.

Micropropagation of *Aerides multiflorum* through Leaf Segments

As has been pointed out several times, use of shoot tips for micropropagation "requires the sacrifice of [an] entire new growth or the only growing point and has a limited utility in monopodial taxa where it endangers the survival of the mother plant" (Vij and Pathak, 1990). To overcome this problem, Professor Suraj P. Vij, founding editor of the *Journal of the Orchids Society of India* and Professor of Botany at

Panjab University, and his associate Dr. Promila Pathak developed micropropagation procedures, using foliar explants for several orchid species, including *Aerides multiflorum* (Vij and Pathak, 1990).

Plant Material. Young leaves, up to 2 cm in length, from 16–40-week-old axenic seedlings should be used. Leaf explants from mature plants die after 10–20 weeks in culture.

Surface Sterilization. No surface sterilization is needed because the leaves are taken from axenically grown plants.

Culture Vessels. Culture vessels are not described in the original paper. Test tubes, 20 mm in diameter, containing 30 ml of culture medium are appropriate.

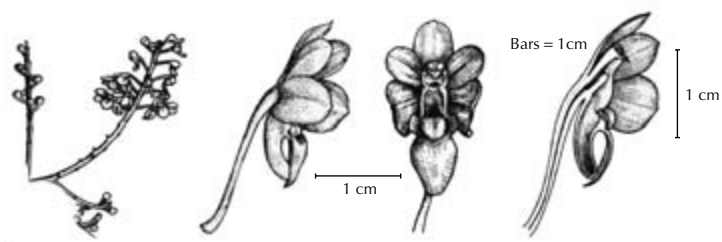
Culture Conditions. In the original experiments cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx.

Culture Media. One medium is suitable for induction of PLBs on leaf-tip explants (Table Aer-3). Another formulation should be used for basal explants (Table Aer-4).

Procedure. After removal from plants the leaves are sectioned under sterile conditions. The sections should be inserted (presumably vertically) in the culture medium and allowed to remain there until plantlet formation or PLBs and/or callus can be subcultured. Well-developed PLBs or small plantlets will probably grow well on Knudson C (see Tables Cym-2 and Cym-3), Tsuchiya (see Table Cym-4), Vacin and Went (see Table Cym-5) or basal MS (Table Aer-2) media.

Developmental Sequence. Explants will produce callus and after that PLBs on MS medium containing activated charcoal, IAA, and yeast extract (Table Aer-3). Only PLBs are produced on a medium which contains activated charcoal, IBA, kinetin, and yeast extract (Table Aer-4). Leaf and shoot primordia develop after 6 and 12 weeks in culture of PLBs from apical explants and 10 and 20 weeks on basal section PLBs.

General Comments. As with other procedures that use explants from seedlings, this method does not allow for the selection of desirable forms. However, it can be used to propagate an orchid whose seeds may not germinate well, an endangered species, or plants that are in demand commercially.



***Aerides ringens* inflorescence (Abraham and Vatsala, 1981)**

TABLE AER-3. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of apical sections of *Aerides multiflorum* leaf explants (Vij and Pathak, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indoleacetic acid (IAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
14	Yeast extract	1.0 g	No stock	No stock	Weigh
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	9.0 g	No stock	No stock	Weigh
	Darkening agent				
18	Activated charcoal[h]	2.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve add a few drops on 0.1 N NaOH.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. When the agar is completely dissolved, add darkening agent (item 18) slowly with vigorous stirring; after it has been dispersed completely, dispense solution into culture vessels and autoclave. The agar can also be added to the cold water which is brought to a boil and stirred. The darkening agent should be added as above when the agar is completely dissolved. After that pour the solution into a 2-l flask and autoclave. As a rule the amino acid (item 8), auxin (item 10), and vitamins (items 11–13) should be added to the hot solution under sterile conditions with sterilized pipettes and mixed well before the medium is distributed into preautoclaved culture vessels. However in this case the original paper implies that all components of the medium were autoclaved.

[h]Only vegetable charcoal should be used. Bone charcoal is not suitable.

TABLE AER-4. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of basal explants of *Aerides multiflorum* leaves (Vij and Pathak, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indolebutyric acid (IBA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Kinetin	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
15	Yeast extract	1.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	9.0 g	No stock	No stock	Weigh
	Darkening agent				
19	Activated charcoal[h]	2.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve add a few drops on 0.1 N NaOH.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.5, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. When the agar is completely dissolved, add darkening agent (item 19) slowly with vigorous stirring; after it has been dispersed completely, dispense solution into culture vessels and autoclave. The agar can also be added to the cold water which is brought to a boil and stirred. The darkening agent should be added with vigorous stirring when the agar is completely dissolved. After that pour the solution into a 2-l flask and autoclave. As a rule the amino acid (item 8), auxin (item 10), cytokinin (item 11), and vitamins (items 12–14) should be added to the hot solution under sterile conditions with sterilized pipettes and mixed well before the medium is distributed into preautoclaved culture vessels. However in this case the original paper implies that all components of the medium were autoclaved.

[h]Only vegetable charcoal should be used. Bone charcoal is not suitable.

Micropropagation of *Aerides multiflorum* through Root Explants

Roots are among the last orchid organs to be cultured and to be used as explants for micropropagation. One reason for this is their tendency to be recalcitrant. Another is the presence of mycorrhizal fungi, which complicates matters. One of the more extensive and successful programs of orchid root explant culture was carried out by Professor S. P. Vij and his associates Anil Sood, Promila Pathak, Sanjeev Arora, Kusam Mahant, Parminder Kaur, and Vishal Sharma at the Botany Department, Panjab University, Chandigarh, India (Vij, 1993).

Plant Material. Young and actively growing roots from 16–30-week-old axenic seedlings should be used. Roots from mature plants die after 8–10 weeks in culture.

Surface Sterilization. No surface sterilization is needed because the roots are taken from axenically grown plants.

Culture Vessels. Culture vessels are not described in the original paper. Standard culture tubes containing 30 ml of culture medium should be appropriate.

Culture Conditions. In the original experiments cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx.

Culture Media. A medium containing yeast extract, activated charcoal, an auxin, and a cytokinin induced the formation of PLBs and subsequently leaves and roots (Table Aer-5).

Procedure. After removal from plants the roots are placed on the medium (Table Aer-5). Well-developed PLBs or small plantlets should grow well on the Knudson C (see Table Cym-3), Tsuchiya (see Table Cym-4), Vacin and Went (see Table Cym-5) or basal MS (Table Aer-2) media.

Developmental Sequence. Roots start regeneration 1 week after being placed in culture. The first leaf and root are formed after 5 and 12 weeks of culture, respectively. If only IAA is present in the medium the time required for leaf and root formation is doubled.

General Comments. Since the roots are taken from seedlings it is not possible to use this method for propagation of outstanding cultivars. However this method can be used to propagate orchids in cases where only a few seedlings become available. It may also be possible to adapt it to mature plants. If so care should be taken to use root tips free of mycorrhiza.

TABLE AER-5. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of root explants of *Aerides multiflorum* leaf explants (Vij, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	62.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indoleacetic acid (IAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Kinerin (6-furfuryl aminopurine)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
15	Yeast extract	1.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	9.0 g	No stock	No stock	Weigh
	Darkening agent				
19	Activated charcoal[h]	2.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve add a few drops on 0.1 N NaOH.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.5, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. When the agar is completely dissolved, add darkening agent (item 19) slowly with vigorous stirring. After it has been dispersed completely, dispense solution into culture vessels and autoclave. The agar can also be added to the cold water which is brought to a boil and stirred. If this is done the darkening agent should be added as above when the agar is completely dissolved.

[h]Only vegetable charcoal should be used. Bone charcoal is not suitable.

Anacamptis

Anacamptis pyramidalis seems to be the first European terrestrial orchid propagated by meristem culture (Morel, 1970).

Anacamptis pyramidalis

Plant Material. Few details are given, but it appears that meristems are excised like those of *Cymbidium* or *Cattleya*. They are then put in culture (Morel, 1974).

Surface Sterilization. Since no details are given, the assumption is, again, that it is accomplished as with *Cymbidium* or *Cattleya* (Morel, 1974).

Culture Vessels. Use 16-mm-diameter test tubes and 50- or 125-ml Erlenmeyer flasks, containing 3–5, 15, and 25 ml medium respectively.

Culture Conditions. No details are given regarding photoperiods, light intensity, or temperature. Therefore it seems that the same conditions as for *Cymbidium* or *Cattleya* should be employed (Morel, 1974). Or use 12-h photoperiods and 100–200 ft-c provided by Sylvania Gro Lux lamps at 22°C.

Culture Media. "The meristem of *Anacamptis pyramidalis* which was cultured on . . . Murashige–Skoog medium" (Murashige and Skoog, 1962) is the statement given (Morel, 1970). However, only the minerals of this medium are listed along with the suggestions that auxin (IAA, NAA, or IBA), 0.5–1 mg l^{-1}, and coconut water must be included in the solution. The medium listed for use with *A. pyramidalis* is based on these suggestions (Table Anac-1). However, it is possible that the versions of the MS medium used for *Epidendrum* leaf tips and Knop's solution employed for *Dendrobium* stem nodes could also be used.

Procedure. Place explants in culture and treat like *Cymbidium* or *Cattleya*.

Development Sequence. "Das Meristem von *Anacamptis pyramidalis*, das auf einem Nährboden von Murashige und Skoog gezüchtet wird, bildet ebenfalls protokormartige Gewebe, die man unendlich vermehren kann; ihr Wachstumprozess ist allerdings sehr langsam" [Meristems of *A. pyramidalis* cultured on Murashige–Skoog medium form protocorm-like bodies, which can be used for unlimited propagation; however, their growth is very slow] (Morel, 1970).

General Comments. According to one report (F. R. Gomm, Nature Conservancy Council, Merlewood Research Station, Grange-over-Sands, UK, personal communication, 1974), only 2–3 plants of native *Cypripedium calceolus* are left in England. An effort is being made to save the species by seed and, if possible, tissue culture propagation. Hence, propagation using tissue culture may well be applied in conservation efforts. The development of such a method of *A. pyramidalis* points to the fact that this is possible and should serve as encouragement for others to devise procedures for other species.

TABLE ANAC-1. **Modified Murashige–Skoog (MS) medium for the culture of *Anacamptis pyramidalis* meristem (Morel, 1970)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements[b]				
1	Ammonium nitrate, NH_4NO_3[b]	1.65 g	82.5 g l^{-1}	20	Or weigh
2	Potassium nitrate, KNO_3[b]	1.9 g	85 g l^{-1}	20	Or weigh
3	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
5	Potassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c,d]				
(a)	Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution[c,d]
(b)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution[d]
(b)	Managanese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(c)	Zinc chloride, $ZnCl_2$	3.93	393 mg l^{-1}		
(d)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(f)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(g)	Cobalt chloride, $CoCl_2 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
	Auxin[e,f]				
8	Indoleacetic acid (IAA) or naphthalenelacetic acid (NAA)	1	100 mg 50 ml^{-1} acidified 95% ethanol[f]	0.5	
	Cytokinin				
9	Kinetin	2.60	100 mg 50 ml^{-1} basic 95% ethanol[f,g]	1.3	
	Amino acid				
10	Glycine	20	2 g 100 ml^{-1} 70% ethanol[h]	1	
	Vitamin[i]				
11	Thiamine (vitamin B_1)	0.1	100 mg 100 ml^{-1} 95% ethanol	0.1	
	Complex additive[j,k]				
12	Coconut water from immature (green) nuts	100–250 ml	No stock	No stock	If available
	Sugar[j,k]				
13	Sucrose	30 g	No stock	No stock	
	Solvent				
14	Water, distilled[j,k]	To 1000 ml			
	Solidifier[k]				
15	Agar	10–15 g	No stock	No stock	

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, they must be kept frozen between uses.

[c]Items 6a and 6b are added to the same 1 l. Add 10 ml per liter of culture medium.

[d]Add all microelements to the same 1 l, and keep at 60°C (a waterbath might be suitable) for 24 h in the dark. It is possible to combine solutions 6 and 7: add items 6 and 7 to the same 1 l, and keep at 60°C for 24 h. In such cases the amount of Na_2EDTA should be doubled. Use 10 ml per liter culture solution in either case.

[e]2,4-Dichlorophenoxyacetic acid (2,4-D) may also be used at the rate of 1 mg l^{-1} of culture medium (0.5 ml of a 50 mg per 25 ml 95% ethanol stock). Keep refrigerated.

[f]If the auxin or cytokinin does not dissolve, add a few drops of dilute HCl or KOH solution, respectively to solubilize it. Keep refrigerated.

[g]Benzyladenine (BA) may also be used at the rate of 1 mg l^{-1} of culture medium (0.5 ml of 50 mg per 25 ml 95% ethanol stock solution). Keep refrigerated.

[h]Keep frozen between uses to prevent contamination.

[i]Keep refrigerated between uses.

[j]To keep the solution liquid, mix items 1–12 with 500 ml distilled water (item 14). Then adjust pH to 5.2–5.5, add sugar (item 13), and bring volume to 1000 ml with more distilled water (item 14). Sterilize the medium through 0.45-µm or 0.22-µm millipore filters (Millipore Filter Corp., Bedford, MA 01730) or a Morton UF fritted-glass filter (Corning Glass Co., Corning, NY 14830). An alternative method is to mix items 1–7 with 500 ml distilled water (item 14), adjust pH to 5.2–5.5, add sugar (item 13), adjust volume to 750 ml, and autoclave (Solution A). Then add items 8–11 to 200 ml green coconut water (item 12), adjust pH to 5.2–5.5, bring volume to 250 ml with more coconut water (item 12), and filter-sterilize the solution (Solution B). Mix Solutions A and B in a sterile box after each has been sterilized. If no coconut water (item 12) is to be used, mix items 1–7 with 500 ml distilled water (item 14), adjust pH to 5.2–5.5, and then add sugar (item 13). Bring volume to 1000 ml (or 997.1 ml for those who are extra fussy) with distilled water (item 14), and autoclave the solution. Add items 8–11 to this solution under sterile conditions (with sterilized pipettes or syringes) when it has cooled to about 60°C. Dispense sterile medium into sterilized (i.e., autoclaved) culture vessels (test tubes, bottles, Erlenmeyer flasks, etc.).

[k]Add agar (item 15) only if solid medium is desired. Sterilization can be accomplished in several ways. One is to mix items 1–12 (or 1–11 if no coconut water is used) with 100 ml distilled water, adjust pH to 5.2–5.5, add sugar (item 13), bring volume to 250 ml, and filter-sterilize. Add agar to 750 ml distilled water, dissolve by bringing the solution to a gentle boil, and autoclave. Mix the two solutions while the agar is still liquid, and dispense into sterilized containers. A second method is to mix items 1–7 with 500 ml distilled water, adjust pH to 5.2–5.5, add sugar (item 13), bring volume to 750 ml with more distilled water, dissolve the agar as above, and autoclave. Then add items 8–11 to 200 ml coconut water, adjust pH to 5.2–5.5, bring volume to 250 ml with more coconut water, and sterilize the solution by filtration. Mix the two solutions, and dispense as above. The third method, if no coconut water is used, is as follows: mix items 1–7 with 500 ml distilled water, adjust pH to 5.2–5.5, add sugar (item 13), adjust volume to 1000 ml (or 997.1 ml for the extra fussy), dissolve agar as above, and autoclave the solution. Add items 8–11 under sterile conditions following autoclaving, and dispense solution as above.

Micropropagation of Crimean *Anacamptis pyramidalis*

A method for the micropropagation of *Anacamptis pyramidalis* (Fig. Anac-1) was developed in the Crimea and reported in an abstract which is a jumble of information (Popkova, 2000) that is nearly impossible to unravel. "Explants . . . were parts of stems, seed-buds [sic], buds, roots and closed seeds bull [sic] with seeds." No information is given as to which of these explants were used for *A. pyramidalis*. Flower buds were surface-sterilized with 70% alcohol (presumably ethanol). There was "also gradual sterilization [with] 70% alcohol + 15% peroxide [presumably hydrogen peroxide] or 70% alcohol + 10% chloramine for roots, part[s] of stem[s] [and] seed bull [sic, whatever that is]." MS, Knudson C, Burgeff, and Prasad and Mitra media were used for "seeds and tissues." Since no details are given about the medium it seems reasonable to suggest that the medium used for meristems of this species (Table Anac-1) should be the first one to try. Cultures were maintained under "24–250" [sic, probably meaning 25°C] and 16-h photoperiods of 1500–3500 lx, but the abstract also states: "in the thermostat of cultivated were in darkness where the temperature was 24–250 [sic, probably meaning 25°] C" (the editors of *Plant Physiology and Biochemistry* clearly needed to do a much better job of editing this abstract).

Anacamptis pyramidalis formed protocorms of "protocorm-like structures" (but the abstract does not state if these were of seed or explant origin). On reaching a diameter of 1–4 mm they were transferred to Knudson C medium supplemented with "meso[myo]inositol, peptone, vitamins and kinetin" (amounts not listed).

This abstract is neither clear nor useful, but is presented here for the sole purpose of providing coverage of micropropagation methods that is as nearly complete as possible.

FIG. ANAC-1. ***Anacamptis pyramidalis*** **from Italy (A–C) and Russia (E–G). A, G. Inflorescence. B, E. Flower. C. Tubers. D. Leaves. (Sources: A–D, Scrugli, 1990; E–G, Vahrameeva, 1991.)**

Angraecum

Angraecum is a large and varied genus with many interesting species from Madagascar. Few if any have been cultured in vitro.

Angraecum eburneum

See discussion in following paragraph.

Isolation of Protoplasts from Green Sepals of *Angraecum giryamae*

Isolated protoplasts of orchids could be used to produce interesting new hybrids through fusion and advanced plant bioengineering research. A method for the isolation of protoplasts from green sepals of *Angraecum giryamae* was developed at Cornell University (see Price and Earle, 1984; note that the species is misspelled there as *Angraecum gyrimae*). This taxon is treated as a distinct species as parent of hybrids and for horticultural purposes in the Sander's List of Orchid Hybrids, but is considered "by some authorities . . . [as being] within [*Angraecum*] *eburneum*]. . . ."

Plant Material. Green sepals from flowers of *A. giryamae* were used in the original research as sources of protoplasts. Small strips of sepal tissue (50–150 mg) are incubated in 2 ml of the enzyme mixture.

Surface Sterilization. Tissues are agitated in 20% Clorox (20 ml Clorox adjusted to 100 ml with distilled water) for 15 min, rinsed three times with sterile distilled water, and dried by gently pressing them between sterile paper towels.

Culture Vessels. Incubation and protoplast isolation are carried out in 35-mm-diameter Petri dishes. Centrifugation requires centrifuge tubes. Mixing of protoplasts for counting is carried out in test tubes. Plastic Petri dishes, 60-ml capacity, are used for fusion. In addition to the culture vessels this procedure requires Buchner funnels, centrifuge tubes, sterilizing filters and holders, a Fuchs–Rosenthal hemocytometer (see Appendices 1 and 2), and 80-μm nylon-mesh filters (Textco, 420 Saw Mill River Rd., Elmsford, NY 10523). Other equipment needed is an epifluorescence microscope (a brand made by Zeiss was used in the original research, but other brands can also be used) with a filter that can be set to a wavelength of 450–490 μm.

Culture Conditions. Incubation of tissue for protoplast release is carried out at room temperature (23 ± 2°C) on a gyrorotatory shaker at 30–50 rpm. It is not clear from the original paper whether this is done in the dark or under illumination. Protoplast cultures are incubated in the dark. Normal laboratory illumination and 22–24°C are suitable conditions for fusion.

Culture Media. Several media are used for protoplast release, isolation, culture, and fusion. For the isolation of protoplasts the tissues are incubated in an enzyme

solution consisting of 2% Cellulysin (Cal Biochem, P.O. Box 12087, La Jolla, CA 92112), 91.1 g sorbitol, and 1.5 g l^{-1} calcium chloride ($CaCl_2 \cdot 2H_2O$). The enzyme mixture is diluted 1 : 1 with a solution of 91.1 g sorbitol, 1.5 g calcium chloride ($CaCl_2 \cdot 2H_2O$), and 5 mmol (approximately 976 mg) MES (pH 6.0). This medium must be sterilized by filtration; use filters from Millipore Filter Corporation (see Appendices 1 and 2). The medium may not be autoclaved.

Protoplasts are cultured in a specially enriched medium (Table Angcm-1) or a minimal solution (Table Angcm-2). To check viability, protoplasts are stained with a solution of 100 μg fluorescein diacetate (available from Aldrich Chemical Co. and Sigma Chemical Co.; see Appendices 1 and 2).

Procedure. Following sterilization brush the undersides of sepals "100 times in each direction with small amounts of sterile carborundum" (Fisher 320 grit available from the Fisher Scientific, 2761 Walnut Ave., P.O. Box 9800, Tustin, CA 92681). Use a dry watercolor brush 1.25-mm wide sterilized by being soaked in 95% ethanol. Remove excess carborundum by washing the petals three to four times with sterile distilled water.

Incubate the sepals in the enzyme solution in 35-mm plastic Petri dishes for 1 day. At the end of the incubation period dilute the enzyme mixture 1 : 1 with the sorbitol–$CaCl_2$–MES solution. The mixture is filtered into a 15-ml centrifuge tube through two 80-μm nylon-mesh filters. (In the original research the nylon filters were held in place by a two-piece plastic Buchner funnel.) Sediment protoplasts by centrifuging the tubes at $100 \times g$ for 5 min. Resuspend the pellet in 5 ml sorbitol–$CaCl_2$–MES solution, and centrifuge again. After the second centrifugation, resuspend the protoplasts in 1 ml of either the enriched medium (Table Angcm-1), or the minimal solution (Table Angcm-2), and transfer the medium to sterile 35-mm-diameter plastic Petri dishes for incubation in the dark.

To count protoplasts place a specific volume of the suspension in a Fuchs–Rosenthal hemocytometer and count at a 400× magnification. Viability can be checked by staining the protoplasts with fluorescein diacetate, allowing them to stand for 15–30 min, and counting those that show a yellow-green fluorescence.

Fusion experiments were not carried out with *A. giryamae*, but a good starting point would be the method used for *Dendrobium*.

Developmental Sequence. Protoplast yield per gram of fresh weight was "very few." There are no reports of callus formation and plantlet regeneration from *A. giryamae* protoplasts.

General Comments. This procedure is clearly an early one and the beginning of a research process that will undoubtedly lead to effective procedures for: (1) the isolation and fusion of orchid protoplasts; and (2) the regeneration of plantlets from the protoplasts and/or fusion products.

TABLE ANGCM-1. **Enriched B5 medium (Gamborg et al., 1968) for the culture of *Angraecum giryamae* protoplasts (Price and Earle, 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	134	13.4 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	735	73.5 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	2500	250 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150	15 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.0	300 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	10	1 g l^{-1}		
(e)	Potassium iodide, KI	0.75	75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2	200 g l^{-1}		
	Amino acids				
8	L-Arginine	210	No stock	No stock	Weigh
9	L-Asparagine	300	No stock	No stock	Weigh
	Polyol				
10	*myo*-Inositol	100	No stock	No stock	Weigh
	Auxins				
11	2,4-Dichlorophenoxyacetic acid (2,4-D)	1	100 mg 100 ml^{-1} water[e,f]	1	
12	2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)	0.5	50 mg 100 ml^{-1} water[e,f]	1	
	Vitamins and related substances[e,g]				
13	*p*-Aminobenzoic acid				
14	Ascorbic acid (vitamin C)	2	200 mg		
14	Biotin	0.01	1 mg		
16	Calcium pantothenate	1	100 mg	1	One solution
17	Choline chloride	0.2	20 mg		
18	Folic acid	0.4	40 mg		
19	Niacin (nicotinic acid)	1	100 mg		
20	Pyridoxine (vitamin B_6)	1	100 mg		
21	Riboflavin	0.2	20 mg		
22	Thiamine (vitamin B_1)	10	1000 mg		
23	Vitamin A	0.01	1 mg		
24	Vitamin B_{12}	0.02	2 mg		
25	Vitamin D_3	0.01	1 mg		
	Complex additive				
26	Casamino acids (vitamin-free)	250	No stock	No stock	Weigh
	Sugars				
27	Glucose	81 g	No stock	No stock	Weigh
28	Ribose	500	No stock	No stock	Weigh
29	Xylose	500	No stock	No stock	Weigh
	Solvent				
30	Water, distilled[h]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add the microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxins do not dissolve, add a few drops of KOH or HCl.

[g]Add all vitamins to the same 100 ml of distilled water.

[h]Add items 1–7, 10, and 26 to 750 ml of distilled water (item 30). Adjust pH to 5.8, add sugars (items 27–29), and adjust volume to 900 ml with distilled water (item 30). Pour solution into a 2-l flask, and autoclave. Dissolve amino acids (items 8 and 9) in 100 ml 95% ethanol, and to this solution add auxins (items 11 and 12) and vitamins (items 13–25, all in 1 ml). Filter-sterilize mixture (Millipore Filter Corp., Bedford, MA 01730), add the mixture to autoclaved medium after it has cooled, mix well, and distribute medium to sterile containers as needed.

TABLE ANGCM-2. **Protoplast medium (Dudits et al., 1976*a*, 1976*b*) modified for the culture of *Angraecum giryamae* protoplasts (Price and Earle, 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	250	25 g l^{-1}	10	
2	Ammonium sulfate, $(NH_4)_2SO_4$[b]	134	13.4 g l^{-1}	10	
3	Calcium chloride, $CaCl_2 \cdot 2H_2O$	450	45 g l^{-1}	10	
4	Calcium phosphate, $CaH_4(PO_4)2 \cdot H_2O$	100	No stock	No stock	Weigh
5	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	2500	250 g l^{-1}	10	
7	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150	15 g l^{-1}	10	
8	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
9	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.0	300 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	10	1 g l^{-1}		
(e)	Potassium iodide, KI	0.75	75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2	200 g l^{-1}		
	Polyol				
10	*myo*-inositol	100	No stock	No stock	Weigh
	Amine				
11	N-Z amine type A	250	No stock	No stock	Weigh
	Auxin				
12	Naphthaleneacetic acid (NAA)	185 µg	18.6 mg 100 ml^{-1} water[e,f]	1	
	Cytokinin				
13	Zeatin	22 µg	2.2 mg 100 ml^{-1} water[e,f]	1	
	Vitamins[e,g]				
14	Niacin (nicotinic acid)	1	100 mg 100 ml^{-1} 95% ethanol		
15	Pyridoxine (vitamin B_6)	1	100 mg 100 ml^{-1} 95% ethanol	1	One solution
16	Thiamine (vitamin B_1)	10	1000 mg 100 ml^{-1} 95% ethanol		
	Complex additive				
17	Coconut water	50 ml	No stock	No stock	Measure
	Sugars				
18	Glucose	68.4 g	No stock	No stock	Weigh
19	D-Ribose	250	No stock	No stock	Weigh
	Solvent				
20	Water, distilled[h]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add the microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops KOH or HCl, respectively.

[g]Add the vitamins to the same 100 ml of distilled water.

[h]Add items 1–11 and 17 to 800 ml of distilled water (item 20). Adjust pH to 5.5, add sugars (items 18 and 19), and adjust volume to 900 ml with distilled water (item 20). Pour solution into a 2-l flask and autoclave. Add items 12–16 to 100 ml distilled water, and sterilize through filters (Millipore Filter Corp., Bedford, MA 01730). Pour this solution into autoclaved medium after it has cooled, mix well, and distribute as needed.

Anoectochilus

Orchids are usually grown for their flowers. However "a small group . . . popularly called Jewel orchids is appreciated [and cultivated] for their foliage." Among these is the genus *Anoectochilus* "whose leaves appear as though woven from shimmering silken threads – a blending of cinnamon, purple, olive and moss green" (Gangaprasad et al., 2000). This genus consists of approximately 25 species distributed throughout Asia, Australia, and Polynesia. Unfortunately "seed capsules are seldom produced by the plants or under cultivation." Therefore, propagation through seeds is difficult and not common. Attempts to culture embryos were not successful. Even if it were successful, embryo culture cannot be used if capsules do not set. Conventional vegetative propagation is slow. Demand for jewel orchids is high and extensive collection has reduced native populations. They have been used as house plants, which means that selected cultivars have to be propagated clonally. A micropropagation method was developed to overcome these problems (Gangaprasad et al., 2000).

Anoectochilus elatus

Many protoplasts (550,000–3,100,000) were obtained from leaf mesophyll of this species (Gopalakrishnan and Seeni, 1987).

Anoectochilus formosanus (*Anoectochilus roxburghii*)

A tissue culture procedure for clonal propagation of seedlings was developed in Taiwan (Chow et al., 1982).

Plant Material. Seedlings produced through seed germination in vitro and maintained under aseptic conditions are used as sources of lateral bud explants and cuttings and for layering.

Surface Sterilization. Seeds are surface-sterilized in accordance with standard procedures (Arditti et al., 1982). There is no need to surface-sterilize the seedlings.

Culture Vessels. Erlenmeyer flasks and culture tubes are suitable.

Culture Conditions. The cultures should be maintained at 25 ± 2°C.

Culture Media. Seeds are germinated on a medium consisting of 3 g Hyponex, 2 g Bacto-peptone, and 30 g sucrose per liter; pH is adjusted to 5.2. MS medium containing (per liter) 0.5 mg NAA and 2 mg kinetin is used for lateral bud culture (Table Anct-1). The same medium containing 0.5 mg of either NAA, or 2,4-D and 3 mg kinetin per liter is used for cuttings in vitro (Table Anct-2). For in vitro layering 0.1 mg NAA and 2 mg kinetin are added per liter of MS medium (Table Anct-3).

TABLE ANCT-1. **Modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as used for the culture of lateral buds of *Anoectochilus formosanus* (*Anoectochilus roxburghii*) (Chow et al., 1982)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Potassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
11	Kinetin	2	200 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyriodoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add the microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference is probably without much of an effect.

[e]Keep frozen between uses.

[f]If the auxin and cytokinin do not dissolve, add a few drops of KOH or HCl, respectively, to solubilize them.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 16), adjust pH as required, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. The agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add items 8 and 10–14 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

TABLE ANCT-2. **Modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for rooting of *Anoectochilus formosanus* in vitro (Chow et al., 1982)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	Chelated iron[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)[f] or 2,4-dichlorophenoxyacetic acid (2,4-D)	0.5	50 mg 100 ml^{-1} 95% ethanol	1	
	Cytokinin[f]				
11	Kinetin	3	300 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add the microelements to the same 1 l of distilled water, and stir and/heat until all components are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference is probably without much of an effect.

[e]Keep frozen between uses.

[f]If the auxin and cytokinin do not dissolve, add a few drops of KOH or HCl, respectively, to solubilize them.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 16), adjust pH as required, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add items 8 and 10–14 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

TABLE ANCT-3. **Modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for in vitro layering of *Anoectochilus formosanus* (Chow et al., 1982)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
11	Kinetin	2	200 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyriodoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add the microelements to the same 1 l of distilled water, and stir and/heat until all components are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference is probably without much of an effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin do not dissolve, add a few drops of KOH or HCl, respectively, to solubilize them.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 16), adjust pH as required, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add items 8 and 10–14 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

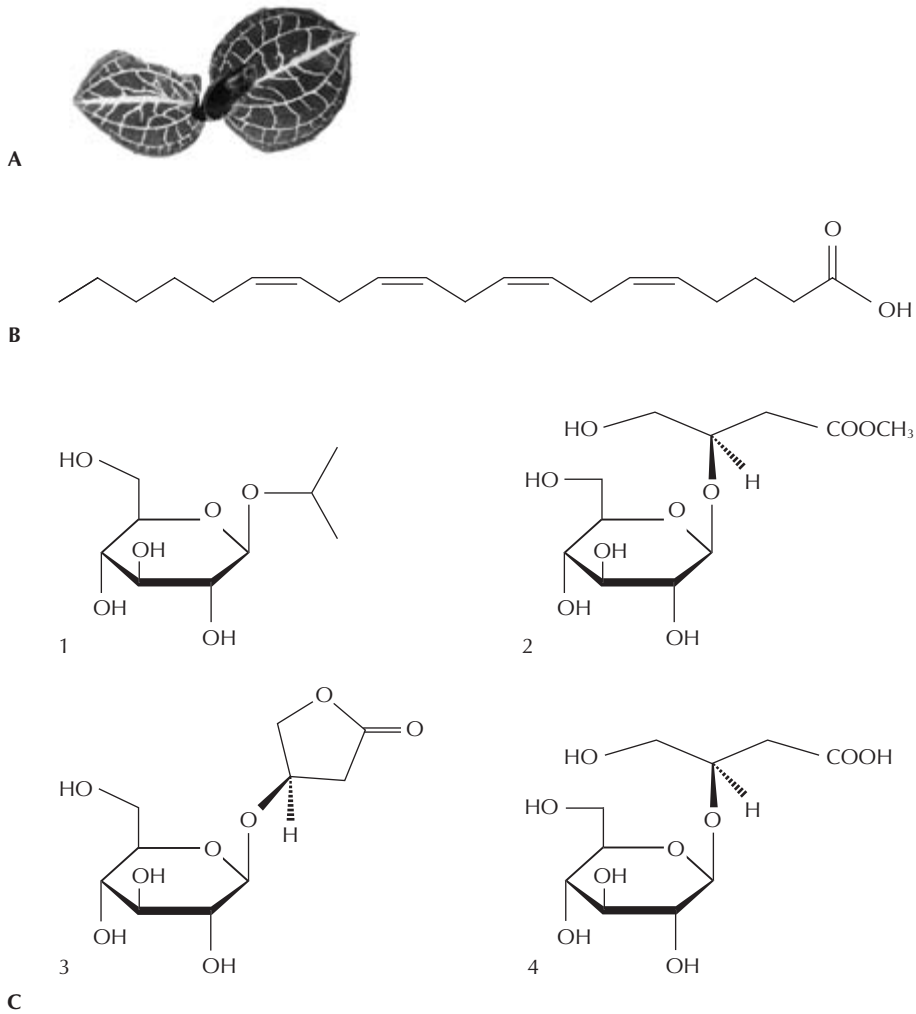


FIG. ANCT-1. **A. *Anoectochilus formosanus*. B. Substances from *A. formosanus*: arachidonic acid. C. Substances from *A. formosanus*: (1) 1-*O*-isopropyl-β-D-glucopyranoside; (2) methyl 3-*O*-β-D-glucopyranosyl-(3R)-4-dihydroxybutanoate; (3) 3-*O*-β-D-glucopyranosyl-(3R)-hydroxybutanolide; (4) 3-*O*-β-D-glucopyranosyl-(3R)-4-diydroxybutanoic acid. (Sources: B, internet; C, Du et al., 1998.)**

Procedure. Germinate seeds as usual. When the seedlings are large enough they can be layered and/or used as sources of lateral buds and/or cuttings.

Developmental Sequence. Plantlets form as a result of layering, from excised lateral buds, and from the cuttings.

General Comments. A difficulty encountered during the preparation of this entry is the fact that the original paper is not in English. The procedure itself seems workable, but it has been tried only with seedlings. This leaves unanswered the question

of whether this method can be used with explants from adult plants. Propagating seedlings may be necessary when seeds are not available and/or only a few seeds have germinated. However, from a horticultural standpoint it is more desirable to propagate outstanding mature plants.

As with most orchids, the nomenclature relating to this species is neither clear nor fully agreed upon. The original paper employed *Anoectochilus formosanus* Hayata in the title and that is why this name is being used here. *Anoectochilus roxburghii* has also been applied to this species.

Tissue culture of *Anoectochilus formosanus*

An orchid found in forests below 1500 m altitude in Taiwan, *Anoectochilus formosanus*, is used in Chinese herbal medicine to treat hypertension, tuberculosis, impotence, and underdeveloped children. These uses have lead to overcollection. Wild type *A. formosanus* contains substances that affect the metabolism of arachidonic acid, which is involved in cardiovascular system functions. A tissue culture method for *A. formosanus* was developed in Taiwan to determine whether "metabolic pathways of a plant tissue culture may differ from [those] of the original plant" (Huang et al., 1991).

Plant Material. Stem segments with nodes were taken from seedlings "originally collected in the field and . . . supplied by [the] Taiwan Agricultural Research Institute."

Surface Sterilization. "The herb" (probably meaning whole seedlings) was surface-sterilized in 70% alcohol (presumably ethanol) for 15 s and then washed in tap water for 20 min. After that the seedlings were sterilized a second time by sonication for a few (number not given) minutes in 1% sodium hypochlorite (probably a dilution of household bleach). The hypochlorite solution was decanted following the sonication and the plants were washed 4–5 times with sterile distilled water. Sonication may well be superfluous especially for plants that are immersed in a 1% sodium hypochlorite solution. However, tests should be carried out before eliminating it and sterilizing the seedlings only by immersion in 70% alcohol and 1% sodium hypochlorite. Also, since the number of minutes in 1% sodium hypochlorite is not given, the appropriate period may have to be determined through experimentation.

Culture Vessels. The original paper does not describe culture vessels. However the context of the paper suggests that they were Erlenmeyer flasks, 250-ml capacity or larger.

Culture Conditions. During the original research the cultures were maintained at 25°C under 16-h photoperiods of 27 μmol photons m^{-2} s^{-1}(400–700 nm) at plant height provided by Sylvania cool white fluorescent tubes.

Culture Media. Stem segments with nodes should be layered on half-strength MS medium containing 0.3 mg NAA l^{-1} and 3 mg kinetin l^{-1} (Table Anct-4). After the segments develop 3–4 leaves it is necessary to transfer them to MS medium

TABLE ANCT-4. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as used for layering of *Anoectochilus formosanus* stems (Huang et al., 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.3	30 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine	3.0	300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The term "half strength" pertain only to the macroelements.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.2, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE ANCT-5. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as used for leaf-bearing stem segments of *Anoectochilus formosanus* (Huang et al., 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Kinetin	0.3	30 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.2, add sugar (item 15), and raise the volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

supplemented with 1 mg NAA and 0.3 mg kinetin l^{-1} (Table Anct-5) to induce root formation.

Procedure. After leaves and roots of the seedlings are removed, stem sections containing nodes (length and distance from nodes to cuts not given) should be placed on the first culture medium (Table Anct-4). When the explants develop 3–4 leaves they should be transferred to the second solution (Table Anct-5). Plantlets with leaves and well-developed root systems can be potted in a mixture of "moss peat, vermiculite and crushed tree fern" (ratio not given).

Developmental Sequence. Explants form leaves on the first medium (Table Anct-4), produce roots, and develop into plantlets on the second medium (Table Anct-5) and grow into plants in the potting mix.

General Comments. In the original research plants were propagated for use in pharmacological research and studies of butanoic acid glucosides (Du et al., 1998). This culture procedure can also be used for horticultural purposes because the limitations imposed by the use of seedlings as sources of explants do not apply to *A. formosanus*. It is grown for its decorative leaves and desirable variegations may be evident even in seedlings.

Micropropagation of *Anoectochilus regalis*

Plant Material. Nodal segments of shoots, 1 cm in length, were taken from plants collected in the Agasthyamalai area in the southwestern Ghats region of India and cultivated in the orchidarium of the Tropical Botanic Garden and Research Institute, Paloda, Trivandrum.

Surface Sterilization. Stem-tip cuttings, 4–5 cm long, should be washed well with water before their leaves are removed. After that the cuttings should be sectioned into 1-cm-long nodal segments. Sterilization of these segments involves several steps: a 15-min wash with running water and a soaking (period not specified) in 1% (v/v) mild detergent (Labolene, Glaxo India, Bombay was used in the original research; baby shampoo can also be used) before "passage through ethanol (70%) for 30 seconds and diluted Steriliq [Combii Organic Chem., (P) Ltd., New Delhi] containing 2% hypochlorite [a dilution of household bleach containing 2% sodium hypochlorite will probably be a suitable replacement] for 10 min." After that the explants should be washed once in sterile distilled water, immersed in 0.1% (w/v) mercuric chloride (this is a dangerous chemical which must be used with great care) for 6–7 min and rinsed three times with sterile distilled water. The last step is to cut off browned and damaged cut ends of the segments. This sterilization sequence seems overly elaborate and could perhaps be simplified based on experience in laboratories that decide to use the procedure.

Culture Vessels. Culture tubes, 25 × 150 mm containing 15 ml of medium were used in the original research. Other containers can also be used.

Culture Conditions. During the original research, the cultures were maintained at 25 ± 2°C under 12-h photoperiods of 25–50 µmol photons m^{-2} s^{-1} provided by fluorescent tubes (type not listed in the original paper). The lights employed for the *A. formosanus* procedure can be used.

Culture Media. Explants are cultured on a medium initially developed for woody plants supplemented with 2.2 µmol 6-benzylaminopurine (Table Anct-6). Shoots that form on the initial substrate are rooted on different modifications of the same medium containing 2.7 µmol of NAA (Table Anct-7).

Procedure. Explants are cultured on the first medium (Table Anct-6). Shoots which form on this medium should be transferred to the second solution (Table Anct-7) for rooting. Rooted plantlets must be washed with lukewarm water to remove all agar before they can be potted in community pots in a potting mixture composed of charcoal, broken tiles, and river sand (in the ratio 1 : 1 : 1, v/v/v). In the original research these pots were irrigated well and placed in a mist chamber where the relative humidity was 70–90%. After 6 months, established plants can be planted in humus-rich soil at 22–34°C and 80–90% relative humidity.

Developmental Sequence. Explants form shoots on the first medium (Table Anct-6), produce shoots on the second medium (Table Anct-7), and grow into hardened plants in the community pots.

General Comments. Plants produced by this method were reintroduced into their natural habitat and became established with 70% efficiency.

Micropropagation of *Anoectochilus sikkimensis*

The method developed for *Anoectochilus regalis* can also be used for this species (Gangaprasad et al., 2000).

Anoectochilus from the Greek ἀνοικός, *anoektos* (open) and χειλος, *cheilos* (lip), "referring to the lip which is adnate to the column but which, through a sharp bend in the isthmus, has its blades spreading to give it the appearance of openness." (Schultes and Pease, 1963)

TABLE ANCT-6. **Woody plants medium (Llyod and McCown, 1980; McCown and Sellmer, 1987) as used for the culture of *Anoectochilus sikkimensis* shoot cuttings (Gangaprasad et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400.0	40.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2$	96.0	9.6 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	556.0	55.6 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
5	Potassium sulfate, KSO_4	990.0	99.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	62.0 mg l^{-1}		
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.25	25.0 mg l^{-1}		
(c)	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	6-Benzylaminopurine	0.32	320 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.2	20 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	5.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

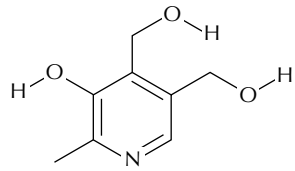
[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1N HCl.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.2, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10) and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.



Pyridoxine/vitamin B_6

TABLE ANCT-7. **Woody plants medium (Llyod and McCown, 1980; McCown and Sellmer, 1987) as used for the culture of *Anoectochilus sikkimensis* shoots (Gangaprasad et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400.0	40.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2$	96.0	9.6 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	556.0	55.6 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
5	Potassium sulfate, KSO_4	990.0	99.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	62.0 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.25	25.0 mg l^{-1}		
(c)	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid	0.502	50.2 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.2	20 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	5.0 g	No stock	No stock	Weigh
	Darkening agent				
17	Charcoal	2.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1N KOH.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.2, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, add darkening agent (item 17) slowly with vigorous stirring; after it has been completely dispersed pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10) and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

Arachnis

Species and hybrids of *Arachnis* are of importance as cut flowers for exporting countries like Singapore and Thailand. Hobby growers also grow members of this genus. Many hybrids have been produced since World War II with the best known being *Arachnis* Maggie Oei (*Arachnis hookeriana* × *Arachnis flos aëris*). John Laycock, a well-known lawyer and political leader, made the cross, R. E. Holttum at the Singapore Botanic Gardens germinated the seeds, and the hybrid was registered in 1940.*

Clonal Propagation of *Arachnis* in Vitro

The first method for tissue culture of *Arachnis* was developed at the Chulalongkorn University Botany Department in Thailand by the husband-and-wife team of Thavorn and Montakan (Mon) Vajrabhaya (Vajrabhaya and Vajrabhaya, 1976*a*).

Plant Material. According to the original research, terminal and lateral buds of *A. hookeriana* are cultured. Only the four uppermost lateral buds are used. They are excised following removal of leaves.

Surface Sterilization. The newly excised buds should be surface-sterilized by immersing them in 5% Clorox (5 ml Clorox diluted with distilled water to 100 ml) for 5–8 min. After the leaves, sheaths, and other coverings are removed, the explant is placed in 1% Clorox (1 ml Clorox diluted to 100 ml) for 3 min. It is advisable to wash the sterilized explants three times with sterile distilled water.

Culture Vessels. Culture tubes are used for solid cultures, and 250-ml capacity Erlenmeyer flasks are employed for liquid media.

Culture Conditions. Cultures are maintained at 26–30°C under 14-h photoperiods and a light intensity of 2500 lx provided by fluorescent tubes. Liquid cultures should be placed on a reciprocating shaker at 50 rpm.

Culture Media. Buds are first placed on a solid modified MS medium (Table Arach-1). After 1 month the buds are transferred to a liquid medium (Table Arach-2).

Procedure. Excise the buds and place them on the solid medium (Table Arach-1). Move them daily from one position on the surface of the agar to another by gently tapping the sides of the culture vessel. When the buds are moved to the liquid medium (Table Arach-2) you may have to remove developing leaf primordia. Callus masses that form within 1–3 months should be subcultured. Regenerate plantlets by placing callus sections on solid sugar-containing medium (Table Arach-1).

* Laycock named the hybrid after his friend Ms. Maggie Oei, who cannot be traced at present. According to some longtime residents in Singapore she may have been employed at the time as a bar hostess or a dance hall dancer.

TABLE ARACH-1. **Modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as used for the culture of *Arachnis* buds (Vajrabhaya and Vajrabhaya, 1976*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (a concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	10	1 g l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	25	2.5 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	10	1 g l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
9	L-Tyrosine	100	100 mg 100 ml^{-1} 95% ethanol[f]	1	
	Polyol				
10	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
11	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.5	100 mg 100 ml^{-1} 95% ethanol[g,h]	0.5	
	Vitamins				
12	Niacin (nicotinic acid)	5	500 mg 100 ml^{-1} 95% ethanol[h]	1	
13	Pyridoxine (vitamin B_6)	0.5	100 mg 100 ml^{-1} 95% ethanol[h]	0.5	
14	Thiamine (vitamin B_1)	0.1	20 mg 100 ml^{-1} 95% ethanol[h]	0.5	
15	Folic acid	0.5	100 mg 100 ml^{-1} 95% ethanol[h]	0.5	
16	Biotin	0.05	10 mg 100 ml^{-1} 95% ethanol[h]	0.5 mg	
	Complex additive				
17	Coconut water	100 ml	No stock	No stock	
	Sugar				
18	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[i]	To 1000 ml			
	Solidifier				
20	Agar, Difco Bacto[i]	6 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the microelements to the same 1 l of distilled water, and stir and/heat until all components are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect. There are no cobalt or iodine salts in the Vajrabhaya–Vajrabhjaya medium, but these elements are present in the original formulation.

[e]Keep frozen between uses.

[f]This is not a stock solution but a means of sterilizing the tyrosine which must be dissolved or suspended in alcohol.

[g]If the auxin does not dissolve, a few drops of KOH can be used to solubilize it.

[h]Keep refrigerated between uses.

[i]Add items 1–7, 10, and 17 to 750 ml distilled water (item 19). Adjust pH to 5.2, add sugar (item 18), and adjust volume to 1000 ml with distilled water (item 19). Bring solution to a gentle boil, and add agar (item 20) slowly while stirring. Agar can also be added to the solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acids, vitamins, and hormones (items 8–16) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

TABLE ARACH-2. **A medium for the culture and proliferation of *Arachnis* buds (Vajrabhaya and Vajrabhaya, 1976*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (a concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	500	50 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Potassium chloride, KCl	250	25 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	125	12.5 g l^{-1}	10	
6	Monosodium phosphate, $NaH_2PO_4 \cdot 2H_2O$	125	12.5 g l^{-1}	10	
7	**Chelated iron**				
(a)	Chelating agent, Na_2EDTA	22.4	2.24 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	16.7	1.67 g l^{-1}		
8	**Microelements**[c]				
(a)	Boric acid, H_3BO_3	1	100 mg l^{-1}	10	One solution
(b)	Cobaltous chloride, $CoCl_2 \cdot 6H_2O$	0.02	2 mg l^{-1}		
(c)	Copper chloride, $CuCl_2 \cdot 2H_2O$	0.01	1 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	2	200 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.02	2 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.1	10 mg l^{-1}		
	Amino acid				
9	Glycine	7.5	750 mg 100 ml^{-1} 95% ethanol[d]	1	
	Vitamins				
10	Calcium pantothenate	0.25	25 mg 100 ml^{-1} 95% ethanol[d]	1	
11	Niacin	1.25	125 mg 100 ml^{-1} 95% ethanol[d]	1	
12	Pyridoxine hydrochloride (vitamin B_6)	0.25	25 mg 100 ml^{-1} 95% ethanol[d]	1	
13	Thiamine hydrochloride (vitamin B_1)	0.25	25 mg 100 ml^{-1} 95% ethanol[d]	1	
	Complex additive				
14	Coconut water	100 ml	No stock	No stock	
	Sugar				
15	Sucrose[e]	0 or 20 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[f]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the microelements to the same 1 l of distilled water, and stir and/heat until all components are dissolved. Dispense the volume indicated per liter of culture medium.

[d]Store in a refrigerator of freezer between uses.

[e]No sugar is added to the liquid medium used for culture of 1-month-old explants moved from the initial medium (Table Arach-1). When this medium is used to regenerate plantlets, it must contain 20 g sucrose per liter.

[f]Add items 1–7 and 14 to 700 ml distilled water (item 16), and adjust pH to 5.2. Add sugar (item 15) only to medium used to regenerate plantlets from callus but not to liquid medium used to subculture explants from first medium. Adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring to medium used for plantlet regeneration from callus (not the sugar-free medium used to subculture explants from the first medium). Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into culture vessels and autoclave. Add amino acid (item 9) and vitamins (items 10–13) under sterile conditions with sterilized pipettes to the medium while its is still hot and liquid.

Developmental Sequence. Callus masses form within 1–3 months. When subcultured onto suitable media these tissues regenerate plantlets. Callus induction occurs on a high-salt solution containing medium to high levels of sucrose, 10% coconut water, and 0.5 ppm 2,4-D. Growth of callus is best on a glucose- and auxin-free low-salt medium that contains 10% coconut water. Regeneration takes place on a solution that has a medium level of sucrose, 10% coconut water, and no auxin.

General Comments. This is a very useful and successful method (68% of the explants form callus) that has not attracted wide attention only because it was published in the Thai language. The outline given here was supplied by Vajrabhaya and Vajrabhaya. Monopodial orchids are somewhat more difficult to culture than sympodial ones. T. Vajrabhaya has written in a letter to JA that "monopodial orchids generally grow better in coconut water [containing media] *without adding sucrose*. For callus growth low-salt formulae plus coconut water are much better than the high salt (with high ammonium ion levels) media."

Tissue Culture Propagation of *Arachnis* in Vitro

A tissue culture laboratory was established at the Singapore Botanic Gardens in the early 1970s. Within the first few years this laboratory was successful in culturing several orchid hybrids. In 1978 the laboratory initiated a tissue culture service for local growers. By 1981 the laboratory was successful in culturing six orchid species and more than 100 hybrids (Lim-Ho, 1981).

Plant Material. Apical and axillary buds from the upper 10 cm of shoots of *Arachnis hookeriana* and *Arachnis* Capama 'Merah' (*Arachnis* Maggie Oei × *Arachnis breviscapa*) were cultured at the Singapore Botanic Gardens. Other species and hybrids of this genus can probably also be cultured. Cubes 0.2 mm on each side are excised from the apices of buds. Care should be taken to excise only meristematic cells and not the surrounding tissue.

Surface Sterilization. Leaves are removed to expose the apical and axillary buds. The shoot is then washed with detergent and water before being submerged in 10–12% Clorox (10–12 ml Clorox diluted to 100 ml with distilled water) for 10–15 min. The original paper does not mention subsequent rinses with sterile distilled water, but they are advisable.

Culture Vessels. Erlenmeyer flasks of 100-ml capacity and containing 20 ml of medium were used in the original research, but other containers are also suitable.

Culture Conditions. All cultures are maintained under continuous illumination of 2000 lx at 21–25°C. Liquid cultures are shaken. No information is given about shakers or rpm, except the indication that "the mode of shaking is not significant, however, too violent shaking has been observed to cause browning-off of protocorm[-like bodies]."

TABLE ARACH-3. **Modified Vacin and Went medium (Vacin and Went, 1949) for initial culture of shoot tips from apical and axillary buds of *Arachnis* (Lim-Ho, 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (a concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.84	2.78 g l^{-1}	10 ml	One solution
(b)	Chelating agent, Na_2EDTA	37.24	3.72 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
9	Coconut water	150 ml	No stock	No stock	Measure
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml of water, and stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]The original formulation contains an iron source that is hard to dissolve. Chelated iron is preferable.

[e]Keep refrigerated or frozen between uses.

[f]Add items 1, 3–7, and 9 to the 500 ml of distilled water (item 10), which contains the calcium phosphate (item 2), and set the pH to 5.2–5.5. Adjust volume to 1000 ml with distilled water (item 10), pour the medium into a 2-l flask, and autoclave. Add the auxin (item 8) to the autoclaved medium under sterile conditions, swirl several times to mix well, and dispense into presterilized culture vessels. Do not add agar if liquid medium is being prepared.

Culture Media. Initial culture is in a liquid modification of the Vacin and Went medium (Table Arach-3). Multiplication and differentiation can take place in two different, solid modifications of the same medium (Tables Arach-4 and Arach-5). A fourth modification (Table Arach-6) is used for plantlet formation.

Procedure. Explants are first placed in a liquid medium (Table Arach-3). When PLBs have formed, they are moved to solid medium (Tables Arach-4 or Arach-5). Plantlets are cultured on the fourth medium (Table Arach-6).

Developmental Sequence. PLBs develop from the explants on the first (liquid) medium. They proliferate and start to differentiate on the second or third medium (both of which are solid) and develop plantlets on the fourth (also solid).

General Comments. *Arachnis* species often produce phenolics that may inhibit growth. For this reason it is necessary to move the explants and PLBs to fresh medium weekly for the first 6 months. Growth is "extremely slow."

TABLE ARACH-4. **Modified Vacin and Went medium (Vacin and Went, 1949) for the proliferation and differentiation of *Arachnis* explants (Lim-Ho, 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (a concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.84	2.78 g l^{-1}	10	One solution
(b)	Chelating agent, Na_2EDTA	37.24	3.72 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water	150 ml	No stock	No stock	Measure
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]The original formulation contains an iron source that is hard to dissolve. Chelated iron is preferable.

[e]Add items 1 and 3–8 to the 500 ml distilled water (item 9) that contain the calcium phosphate (item 2), set the pH to 5.2–5.5, and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. The agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar for liquid medium.

The micropropagation laboratory at the Singapore Botanic Gardens is still in existence and still under the direction of Dr. Lim-Ho. The literature search for this edition did not find any publications from it.

TABLE ARACH-5. **Modified Vacin and Went medium (Vacin and Went, 1949) for the proliferation and differentiation of *Arachnis* explants (Lim-Ho, 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock No stock		
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.84	2.78 g l^{-1}	10	One solution
(b)	Chelating agent, Na_2EDTA	37.24	3.72 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	2 g[e]	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add the other components after that.

[d]The original formulation contains an iron source that is hard to dissolve. Chelated iron is preferable.

[e]The usual amount of sugar added to this medium is 20 g.

[f]Add items 1 and 3–8 to the 500 ml distilled water (item 10) that contain the calcium phosphate (item 2). Set the pH to 5.2–5.5, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved dispense the medium into culture vessels and autoclave.

Emil Vacin, a gas mask manufacturer during World War II, and Professor Frits W. Went, a noted plant physiologist during his time and discoverer of the first plant hormone (auxin), formulated their medium at the California Institute of Technology after noting that the pH of the Knudson C solution drops considerably as a result of seedling growth. Their aim was to develop a medium which maintains its pH without change, but they did not accomplish that. Professor Knudson was not very pleased by their efforts (see Arditti, 1990 for an historical account). Vacin and Went did not name their medium after themselves. Others did that.

TABLE ARACH-6. **Modified Vacin and Went medium (Vacin and Went, 1949) for plantlet formation from *Arachnis* explants (Lim-Ho, 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock No stock		
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.84	2.78 g l^{-1}	10	One solution
(b)	Chelating agent, Na_2EDTA	37.24	3.72 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additives				
8	Coconut water	75 ml	No stock	No stock	Measure
9	Tomato juice[e]	75 ml	No stock	No stock	Measure
10	Banana homogenate[f]	75 g	No stock	No stock	Weigh
	Sugar				
11	Sucrose	2 g[g]	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[h]	To 1000 ml			
	Solidifier				
13	Agar	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 300 ml of water, and stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]The original formulation contains an iron source that is hard to dissolve. Chelated iron is preferable.

[e]Commercial juices (even those labeled "all natural") may contain preservatives that could inhibit growth. Tomato seeds may also contain inhibitors. Therefore it is advisable to prepare fresh tomato juice (item 9) by squeezing a few tomatoes and filtering to remove the seeds.

[f]Place ripe-but-still-firm banana slices in a homogenizer, and add 150 ml distilled water. Homogenize for 1–2 min, allow a 5-min interval, and homogenize again for 1 min. To prepare item 10 pour the homogenate into a beaker, wash the homogenate three times with 50-ml portions of distilled water, and combine the washings.

[g]The usual amount of sugar added to this medium is 20 g.

[h]Add items 1 and 3–10 to the 300 ml of distilled water (item 12) that contain the calcium phosphate (item 2), and set pH to 5.2–5.5. Add sugar (item 11), and adjust volume to 1000 ml with distilled water (item 12). Bring solution to a gentle boil, and add agar (item 13) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave.

Isolation of Cells from *Arachnis* Maggie Oei

Certain studies of orchid physiology can be carried out better with isolated cells since the use of whole plants or organs presents certain difficulties. To overcome these difficulties investigators at the Botany Department, National University of Singapore, isolated living cells from aerial roots, leaves, and petals of orchids (Hew and Yip, 1986).

Plant Material. Leaves, petals, and roots were taken from plants of *Arachnis* Maggie Oei (*Arachnis hookeriana* × *Arachnis flos aeris*) grown under full sunlight in Singapore.

Surface Sterilization. Since the cells were isolated for the purpose of using them in experiments and not for culture, the explants were not surface-sterilized. Those who wish to isolate cells for axenic cultures should experiment with several surface-sterilization procedures perhaps using a number of dilutions of sodium (household bleach) or calcium hypochlorite followed by several washes with sterile distilled water.

Vessels, Apparatus, and Equipment. Incubation in the enzyme mixture should be carried out in test tubes (30 mm in diameter × 60 mm in length) covered with a tight-fitting cap. Mechanical maceration can be accomplished with a round-end glass rod in 10 ml washing medium inside a Petri dish. Mortar and pestle are suitable for mechanical grinding, also in 10 ml medium.

Centrifuge tubes and a swinging-bucket centrifuge are best suited for centrifugation. Density-gradient centrifugation should be performed in centrifuge tubes layered with 1.5-ml portions of (starting from the bottom) 1.5, 1.2, 0.8, and 0.4-*M* solutions of sucrose. The cell suspensions can be filtered through a nylon sieve (which can be obtained from Hygro-Bios Apparatebau, G. M. B. H., Germany) with a pore size 2 μm smaller than the cells (Miracloth from Calbiochem is also suitable). Cells retained by the filter (nylon sieve or Miracloth) should be washed into a 50-ml beaker.

Isolation Conditions. The enzyme mixture should be infiltrated into the tissues under vacuum. This is accomplished by placing tissues in the enzyme mixture inside a test tube and infiltrating it under vacuum. Incubation should be carried out at 29°C without agitation or in a shaking water bath at 29°C. Room temperature is suitable for maceration and grinding. Culture conditions are not listed in the original paper because the cells were not cultured. It is reasonable to assume that the conditions used for the culture of *Aranda* protoplasts will also be suitable for cells.

Culture Media. The maceration medium consists of 0.5% (w/v) Macerozyme R-10 (Yakult Honsha Co., Ltd., Japan; available in the United States from Serva Biochemicals, Inc., 200 Shames Dr., Westbury, NY 11590), sorbitol, potassium dextran sulfate, and inorganic salts (Table Arach-7). Washing should be carried out with an enzyme-free solution (Table Arach-8). Incubation can be carried out in a medium containing less sorbitol plus HEPES buffer (Table Arach-9). Density-gradient centrifugation requires 1.5, 1.2, 0.8, and 0.4-*M* solutions of sucrose (513.45, 410.76, 273.84, and 136.92 g l^{-1}, respectively).

TABLE ARACH-7. **Maceration medium for isolation of cells from tissues of *Arachnis* Maggie Oei (Hew and Yip, 1986)**

		Amount per 10 ml		
Component		Weight	Percent	Molarity[a]
Calcium chloride, $CaCl_2$		0.11 mg		0.1 m*M*
Copper sulfate, $CuSO_4$		0.02 µg		0.01 µ*M*
Macerozyme R-10[b]		50 mg	0.5	
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$		0.12 mg		0.1 m*M*
Potassium iodide, KI		1.66 µg		1 µ*M*
Potassium nitrate, KNO_3		1.01 mg		1 m*M*
Monopotassium phosphate, KH_2PO_4		0.27 mg		0.2 m*M*
Potassium sulfate, K_2SO_4[c]		2.18 g		1.25 m*M*
Sorbitol		1.5 g		0.8 *M*
pH	5.8[d]			

[a]µ*M*, m*M*, and *M* stand for micromolar, millimolar, and molar, respectively, and refer to the concentration of a solution in terms of molarity; these units differ from micromoles, millimoles, and moles, which pertain to amounts (weight) of a substance. For example, a 1-m*M* solution is prepared by dissolving 1 mmol in 1 l.

[b]Yakult Honsha Co., Japan; available in the United States from Serva Biochemicals, Inc., 200 Shames Dr., Westbury, NY 11590.

[c]May be replaced with 0.3% (w/v) potassium dextran sulfate (30 mg per 10 ml).

[d]The pH can be adjusted downward (made more acidic) with a 1.0-*M* solution of hydrochloric acid (86 ml of concentrated HCl diluted to 1 l with distilled water), or upward (made more basic) with a 1.0-*M* solution of potassium hydroxide (56.11 g l^{-1}).

TABLE ARACH-8. **Washing medium for isolated cells of *Arachnis* Maggie Oei (Hew and Yip, 1986)**

		Amount per 10 ml		
Component		Weight	Percent	Molarity
Calcium chloride, $CaCl_2$		0.11 mg		0.1 m*M*
Copper sulfate, $CuSO_4$		0.02 µg		0.01 µ*M*
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$		0.12 mg		0.1 m*M*
Potassium iodide, KI		1.66 µg		1 µ*M*
Potassium nitrate, KNO_3		1.01 mg		1 m*M*
Monopotassium phosphate, KH_2PO_4		0.27 mg		0.2 m*M*
Potassium sulfate, K_2SO_4[a]		2.18 g		1.25 m*M*
Sorbitol		1.5 g		0.8 *M*
pH	5.8[b]			

[a]May be replaced with 0.3% (w/v) potassium dextran sulfate (30 mg per 10 ml).

[b]The pH can be adjusted downward with 1.0-*M* hydrochloric acid (86 ml concentrated HCl diluted to 1 l with distilled water), or upward with 1.0-*M* potassium hydroxide (56.11 g l^{-1}).

TABLE ARACH-9. **Incubation medium for isolated cells of *Aachnis* Maggie Oei (Hew and Yip, 1986)**

		Amount per 10 ml		
Component		Weight	Percent	Molarity
Calcium chloride, $CaCl_2$		0.11 g		0.1 m*M*
Copper sulfate, $CuSO_4$		0.02 µg		0.01 µ*M*
HEPES buffer		0.119 g		0.05 *M*
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$		0.12 mg		0.1 m*M*
Potassium iodide, Hl		1.66 µg		1 µ*M*
Potassium nitrate, KNO_3		1.01 mg		1 m*M*
Monopotassium phosphate, KH_2PO_4		0.27 mg		0.2 m*M*
Potassium sulfate, K_2SO_4[a]		2.18 g		1.25 m*M*
Sorbitol		0.94 g		0.5 *M*
pH	7.0[b]			

[a]May be replaced with 0.3% (w/v) potassium dextran sulfate (30 mg per 10 ml).

[b]The pH can be adjusted downward with 1.0-*M* hydrochloric acid (86 ml of concentrated HCl diluted to 1 l with distilled water), or upward with 1.0-*M* potassium hydroxide (56.11 g l^{-1}).

Procedure. Rinse freshly detached leaves with distilled water and blot them dry before removing the midribs and peeling the cuticle and lower epidermis (Fig. Arach-1). Then cut the laminas (leaf blades) into strips measuring approximately 10×15 mm. When roots are used, it is necessary to remove the velamen because only the underlying green tissue can be used. Cut it into 3×3-mm sections. Cut petals like leaves.

Place approximately 1 g tissue in 15 ml of enzyme solution inside a test tube and infiltrate it by subjecting it to a short period of mild vacuum followed by standard conditions and repeating the process two or three times. Incubate the tissue in the enzyme solution in a shaking water bath (100 oscillations or strokes per second) at 29°C for 1 h. After that, place the tissues in a Petri dish with 10 ml of washing solution and mash them by tapping lightly with a rounded glass rod until a distinctly green suspension is obtained. The suspension can be removed at this point and the tissue may be macerated again with another 10 ml of medium. This can be repeated several times, and the cell suspensions should be pooled. Separate the cells from smaller debris by filtering them through a nylon sieve that retains them. Wash cells retained by the filter into a 50-ml beaker with washing medium and centrifuge them in a swinging-bucket centrifuge at 80 g for 3 min. Resuspend the pellet containing the cells in 20 ml of washing medium, and recentrifuge under the same condition. After repeating this twice, resuspend the cells in 5 ml of incubation medium. Further purification of the cells can be accomplished by density-gradient centrifugation. This is carried out in a test tube containing 1.5-ml layers of (starting from the bottom) 1.5, 1.2, 0.8, and 0.4-*M* solutions of sucrose. At the end of all purification steps the cells can be counted with a hemocytometer, or their density can be determined by measuring the turbidity of the suspension as OD_{680}.* Chlorophyll and protein concentrations can also be measured. Since the initial isolation in the original study was intended to produce cells for physiological studies no culture procedures are given. The flowchart in Fig. Arach-1 outlines the procedure just described above as well as alternatives.

Developmental Sequence. The cells were used for physiological experiments and not cultured. For this reason no information is available regarding their development.

General Comments. This procedure is not intended to produce cells for culture. However, it can be a good starting point for procedures to produce cells and protoplasts that can be cultured.

Professor Choy sin Hew, now retired from the National University of Singapore received the Singapore National Science award in 1997 for his orchid research. He richly deserves it.

* Optical density (a measure of light transmission through a solution) at a wavelength of 680 nm.

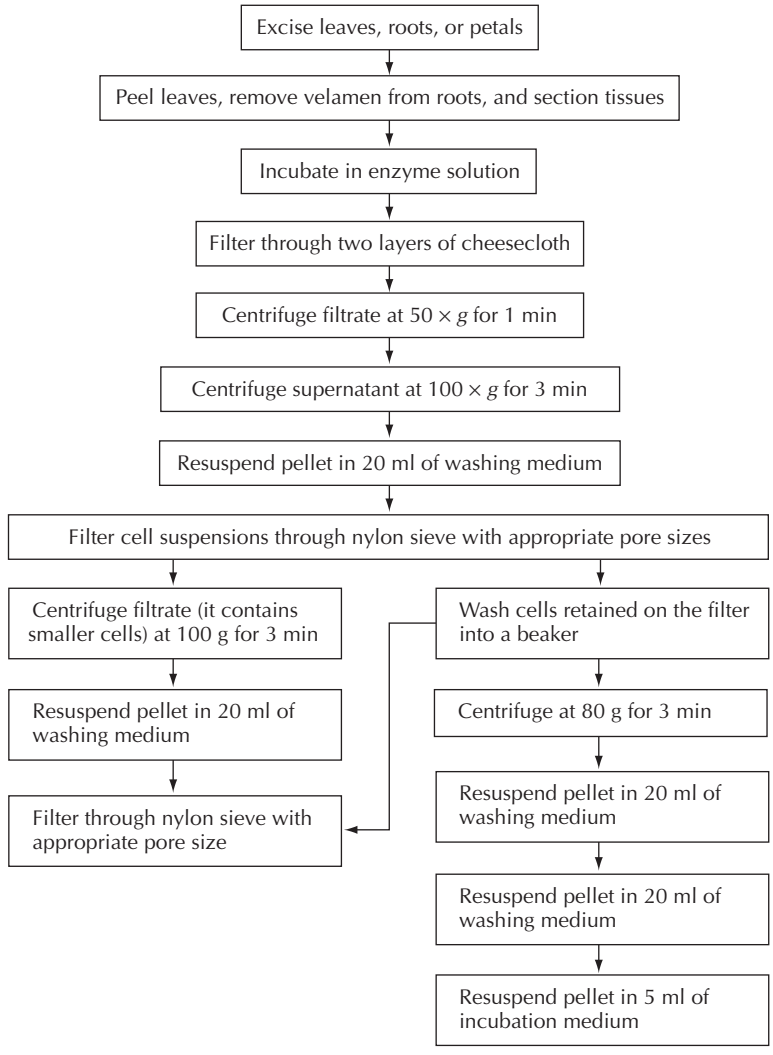


FIG. ARACH-1. **Isolation of cells for *Arachnis* (Hew and Yip, 1986; C. S. Hew, Botany Dept., National University of Singapore, personal communication).**

Arachnostylis

Apical and axillary buds of the human-made hybrid *Arachnostylis* Charcalood (*Arachnis hookeriana* × *Rhynchostylis gigantea*) were cultured at the Singapore Botanic Gardens by the methods used for *Aranda*. Explants were first cultured in a liquid modification of the MS medium (see Table Arach-6). Proliferation and differentiation occur on two solid modifications of the same medium (see Tables Arach-4 and Arach-5). A solid version of the first medium (see Table Arach-6) is used for plantlet formation. Explant growth is slow.

Agar or agar agar is a Malay/Indonesian term for an edible seaweed. In Indonesia and Malaysia agar is used for cooking and as a gelling agent for confectionary items. The agar used to gel culture media is also made from algae, more specifically *Gelidium*. Fanny Angeline "Lina" Eilhemius (b. New York in 1850), a talented artist and biological illustrator, suggested the use of agar as a gelling agent to her husband Dr. Walther Hesse (whom she met while traveling in Europe and married in 1874), who was assistant to Dr. Robert Koch (of Koch's postulates fame) having joined his laboratory in 1881.

Aranda

A hybrid genus (*Arachnis* × *Vanda*) of considerable importance, *Aranda* has been cultured by a number of investigators. Shoot tips of *Aranda* Wendy Scott 'Greenfield' (*Arachnis hookeriana* × *Vanda Rothschildiana*) were cultured successfully on the Vacin and Went medium "modified by reduction of agar to 0.9% for solid and no agar for liquid culture, occasional deletion of 2% sucrose and addition of coconut water . . . 15% by volume and green banana . . . 50 g/1" (Cheah and Sagawa, 1978). The procedure is "successful" (Sagawa and Kunisaki, 1982). In Thailand *Aranda* tissue "grew profusely without . . . auxins and cytokinins" on Schenk–Hildebrandt medium (Vajrabhaya and Vajrabhaya, 1976*b*).

Aranda has been cultured in Malaysia (Alang, 1975), and extensive studies there (Khaw et al., 1978*a*, 1978*b*) have shown that 0.25–0.5 NAA and 0.25–0.50 mg l^{-1} BA enhanced the growth of PLBs of *Aranda* Christine No. 1 (*Arachnis hookeriana* × *Vanda* Hilo Blue). Other *Aranda* hybrids and clones cultured successfully from shoot tips and/or axillary-bud explants are *Aranda* Christine Nos 5, 9, and 80; *Aranda* Wendy Scott; *Aranda* Tay Swee Eng [*Aranda* Lily Chong (*Arachnis* Ishbel × *Vanda sanderiana*) × *Vanda* Piha Moon]; *Aranda* Majulah (*Arachnis* Maggie Oei × *Vanda insignis*); *Aranda* Chong Chee Yuen; *Aranda* Noorah Alsagoff (*Arachnis hookeriana* × *Vanda* Dawn Nishimura); *Aranda* Lum Chin (*Arachnis* Maggie Oei × *Vanda* Hilo Blue); *Aranda* Lily Chong (*Arachnis* Ishbel × *Vanda sanderiana*); and *Aranda* Kooi Choo [*Aranda* Lucy Laycock (*Arachnis hookeriana* × *Vanda tricolor*) × *Vanda* Hilo Blue]. All of these *Aranda* crosses and clones were cultured on a nutrient medium consisting of the Vacin and Went macroelements (Vacin and Went, 1949) and the Heller's microelements (Heller, 1953) plus coconut water (Table Aranda-1) and hormones as indicated above (Khaw et al., 1978*a*, 1978*b*).

In Singapore *Aranda* Christine was cultured on MS medium (Singh, 1980; Lee and Mowe, 1983). The most detailed studies pertaining to *Aranda* shoot-tip and axillary-bud meristem culture were carried out at the National University of Singapore (Goh, 1973, 1981, 1983*a*; Goh and Loh, 1974–1975; Loh et al., 1975; Loh and Rao, 1985; Manorama et al., 1986). The tissues were cultured on MS, Vacin and Went, and White (White, 1943) media. These reports, pertaining to *Aranda* crosses with different *Arachnis* and *Vanda* pedigrees, suggest that the requirements of the hybrid genus *Aranda* are similar and not limited to a single formulation.

TABLE ARANDA-1. **A medium consisting of the Vacin and Went macroelements (Vacin and Went, 1949), Heller's microelements (Heller, 1953), chelated iron, and other additives for the culture of shoot-tip and auxillary-bud explants of *Aranda* (Khaw et al., 1978*a*, 1978*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated Iron**[d]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	28	2.8 g l^{-1}	10	One solution
(b)	Chelating agent, Na_2EDTA	37	3.7 g l^{-1}		
7	**Microelements**[e]				
(a)	Aluminum chloride, $AlCl_3$	0.014	1.4 mg l^{-1}	10	One solution
(b)	Ammonium molybdate, $(NH_4)_2MoO_4$	0.148	14.8 mg l^{-1}		
(c)	Boric acid, H_3BO_3	1	100 mg l^{-1}		
(d)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.03	3 mg l^{-1}		
(e)	Manganese sulfate, $MnSO_4 \cdot H_2O$	7.5	750 mg l^{-1}		
(f)	Nickel chloride, $NiCl_2 \cdot 6H_2O$	0.03	3 mg l^{-1}		
(g)	Potassium iodide, KI	0.038	3.8 mg l^{-1}		
(h)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1	100 mg l^{-1}		
	Auxin				
8	Naphthaleneacetic acid, NAA	0.25	25 mg NAA 100 ml^{-1} 95% ethanol[f]	1	
	Cytokinin				
9	Benzyladenine (benzylaminopurine), BA	0.25	25 mg BA 100 ml^{-1} 95% ethanol[f]	1	
	Complex additive				
10	Coconut water[g]	150 ml	No stock	No stock	
	Sugar				
11	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[h]	To 1000 ml			
	Solidifier				
13	Agar	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Then add other components of medium after that.

[d]This form of iron is not used in original formulation but is preferable.

[e]Add all microelements to the same 1 l of distilled water; if salts dissolve slowly, heat solution.

[f]Keep in a refrigerator or freezer between uses. The original paper suggests that 0.25–0.50 ppm of this hormone is suitable; therefore we recommend 0.25 ppm since it is always best to use the lowest hormone concentration that will accomplish the desired purpose.

[g]Keep frozen between uses.

[h]Add items 1, 3–7, and 10 to the 500 ml of distilled water (item 12) that contain the calcium phosphate (item 2), and adjust pH as required. Add sugar (item 11), and adjust volume to 1000 ml with distilled water (item 12). Bring solution to a gentle boil, and add agar (item 13) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, sterilize solution by autoclaving and add items 8 and 9 to hot, still-liquid solution under sterile conditons. Mix well, and distribute into preautoclaved culture vessels.

Clonal Propagation of *Aranda* from Apical Meristem Tissues

A method developed for *Aranda* Deborah (*Arachnis hookeriana* × *Vanda lamellata*) at the University of Singapore (Goh, 1973; Arditti, 1977*b*) was improved by the original investigator and his associates (Goh and Loh, 1974/75; Loh et al., 1978; Goh, 1981).

Plant Material. The top parts of shoots were excised from plants, and their leaves were removed to expose apical and axillary buds. Following a wash, sterilization, and rinse, a wedge-shaped section containing the bud is removed with a sterile scalpel. With apical meristems the larger leaves are removed first and the tissue is washed, sterilized, and rinsed. Then the remaining younger leaves and leaf primordia are removed, and the apical portion (about 4 mm long) is cultured.

Surface Sterilization. The excised stem sections should be washed thoroughly first with tap water and then with distilled water. After the wash the tissue is cut into sections, two to three internodes in length. Shoot tips are then sterilized in 10% Clorox (10 ml Clorox diluted to 100 ml with distilled water) for 10 min. Sections with axillary buds are sterilized in 15% Clorox (15 ml Clorox diluted to 100 ml) for an equal period. The sterilized explants are washed three times with sterile distilled water and sterilized Petri dishes. Shoot tips and axillary buds are then excised from the stem sections, rinsed again with sterile distilled water, and placed in culture.

Culture Vessels. Erlenmeyer flasks of 100-ml capacity containing 25 ml of medium are suitable.

Culture Conditions. Explants in liquid medium should be maintained under diffuse light of 1000 lx for 11-h photoperiods at 27°C on a reciprocal shaker at 80 oscillations per minute (Goh and Loh, 1974/75), but a rotary shaker at 100 rpm may also be suitable (Goh, 1973). Cultures on solid medium are maintained at 24 ± 1°C under 3000 lx and 12-h photoperiods.

Culture Media. Liquid Vacin and Went medium containing 20 g sucrose and supplemented with coconut water (Table Aranda-2) is used to proliferate callus tissues and protocorms. Solid medium (Table Aranda-2) is used for plantlet formation (Goh and Loh, 1974/75). White's medium supplemented with coconut water and peptone (Table Aranda-3) may also be suitable (Goh, 1973).

Procedure. After excision place the buds in liquid medium and culture them until enough callus or PLBs form. Then transfer these to solid medium for differentiation.

Developmental Sequence. Shoot tips swell after 3–4 weeks, the enlargement continues for a total of 1.5–2 months, and the explants triple in size and become spherical. PLBs appear near the apical dome after 3 months and proliferate. If left in liquid medium the PLBs form globular masses of yellowish callus. When moved to solid medium these PLBs produce rhizoids and plantlets 6–7 cm tall after 9 months. The callus masses form plantlets after 6–7 weeks. In all cases leaves appear before roots.

TABLE ARANDA-2. **Vacin and Went medium (Vacin and Went, 1949) as used for the culture of *Aranda* explants (Loh et al., 1978; Goh, 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Complex additive				
9	Coconut water[e]	200 ml	No stock	No stock	
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	8 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution of chelated iron, dissolve 37.3 mg of the chelating agent Na_2EDTA and 27.8 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$, in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Keep frozen between uses.

[f]Add items 1 and 3–7 to the 500 ml distilled water (item 10) that contains the calcium phosphate (item 2), adjust pH as required, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil; if agar (item 11) is to be included, add it slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar if preparing liquid medium.

Axillary buds enlarge slowly and form a dome-shaped structure after 3 months. PLBs form approximately 4 months after inoculation. Callus tissues are also formed. When moved to solid medium the PLBs and callus tissue form plantlets.

General Comments. A multitude of PLBs and plants could be produced using the original method (Goh, pers. comm. 1973; Arditti, 1977*b*). The improved procedures render this task easier and faster. These or similar procedures have been used for the culture apices of axillary buds of *Aranda* Nancy (*Arachnis hookeriana* × *Vanda dearei*) and are probably suited for other *Aranda* crosses.

TABLE ARANDA-3. **White's medium (Singh and Krikorian, 1981)[a] as used for the culture of *Aranda* explants (Goh, 1973)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[c]	288	28.8 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	737	73.7 g l^{-1}	10	
3	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	19	1.9 g l^{-1}	10	
4	Potassium chloride, KCl	65	6.5 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	80	8 g l^{-1}	10	
6	Sodium sulfate, Na_2SO_4	200	20 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	3.4	344 mg l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	4.66	466 mg l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3[f]	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$[f]	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$[f]	000.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	6.65	665 mg l^{-1}		
(e)	Potassium iodide, KI	0.75	75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$[f]	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.67	267 g l^{-1}		
	Amino acid				
9	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
13	Peptone	2 g	No stock	No stock	Weigh
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[h]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[h]	8 g	No stock	No stock	Weigh

[a]There are many inconsistencies and contradictions in the literature regarding White's medium, some of them in his own papers. This table is based on an excellent recent study, reevaluation, and modernization of the medium (Singh and Krikorian, 1981).

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]To prepare chelated iron, add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[f]Not part of White's recipe but advisable to include.

[g]Keep refrigerated or frozen between uses.

[h]Add items 1–8 and 13 to 900 ml distilled water (item 15), adjust pH to 5.2, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil and add agar (item 16) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add items 9–12 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if a liquid medium is desired.

Clonal Propagation of *Aranda* from Leaf-tip Tissues of Young Plantlets

Removal of shoot tips or axillary buds may lead to the loss of an entire plant. The excision of leaf tissues does not create such a danger, and for this reason a procedure was developed for the propagation of *Aranda* from leaf tips (Loh et al., 1975).

Plant Material. Leaf tips approximately 5 mm in length can be removed from seedlings or plantlets produced through tissue culture.

Surface Sterilization. Surface sterilization is not needed because the leaf tips are taken from axenically grown plants.

Culture Vessels. Erlenmeyer flasks of 100-ml capacity, each containing 20 ml of medium, are suitable.

Culture Conditions. Liquid cultures should be maintained on a reciprocating shaker (80 oscillations per minute) under diffuse light of approximately 1000 lx, photoperiods of 11 h, and a temperature of about 29°C. Solid cultures are maintained under the same conditions, except that shaking is not done.

Culture Medium. Liquid Vacin and Went medium with coconut water (Table Aranda-2) is used.

Procedure. Excise leaf tips, place them in medium, and culture them. When PLBs develop after about 12 weeks, remove and subculture them in liquid medium, where plants may develop. These plantlets may also develop well on solid medium.

Developmental Sequence. Leaf tips remain green and form cream- or green-colored callus bodies after 4–6 weeks. Callus formation is greater on the upper epidermis. The callus masses produce PLBs after 8–10 weeks. Clumps of PLBs form within 12–14 weeks, each body measuring 1–2 cm. Plantlets form from the PLBs.

General Comments. This is a useful procedure for the propagation of axenic plants. Leaf tips removed from plants not growing aseptically in flasks would have to be surface-sterilized. This procedure (Manorama et al., 1986) has also been used with leaf tips and other segments of *Aranda* Deborah, *Aranda* Hilda Galistan (*Arachnis hookeriana* × *Vanda suavis*), *Aranda* Lucy Laycock (*Arachnis hookeriana* × *Vanda tricolor* var. *purpurea*) and *Aranda* Mei Ling (*Arachnis hookeriana* × *Vanda coerulea*). For details see *Dendrobium* entry, Clonal Propagation of *Dendrobium crumenatum* from Leaves (p. 545, Vol. I; Manorama et al., 1986).

Clonal Propagation of *Aranda* from Leaf Tissues of Mature Plants

A problem with the culture of leaf segments of seedlings is that they are plants of unknown quality. Culturing leaves of plants obtained through the culture of shoot

tips or axillary buds may be unnecessary since a method for their clonal propagation exists already. Therefore a clonal propagation procedure that utilizes leaf tissues from adult plants is very desirable. Such a procedure was developed at the Singapore Botanic Gardens (Fu, 1978, 1979*b*).

Plant Material. Whole young leaves, 1–3 cm long and 0.5–1 cm wide, of mature plants of *Aranda* Christine Nos 27 and 130 (*Arachnis hookeriana* × *Vanda* Hilo Blue) and *Aranda* Wendy Scott (*Aranda hookeriana* × *Vanda* Rothschildiana) were used in the original experiment. Similar leaves of other *Aranda* clones and hybrids can also be used.

Surface Sterilization. The leaves are sterilized in 10% Clorox (10 ml Clorox diluted to 100 ml with distilled water) for 15–20 min. The original paper does not mention washing the sterilized leaves with sterile distilled water, but two to three such washes are advisable.

Culture Vessels. Erlenmeyer flasks or other standard vessels are suitable.

Culture Conditions. Liquid cultures are maintained on a reciprocating shaker (the number of oscillations per minute is not given, but 80–100 seems appropriate) for 16 h day^{-1} at 28°C and illuminated continuously with a "Sylvania Gro-Lux 30w tube." The information regarding the source of illumination is clearly insufficient, but it is reasonable to assume that most standard light sources and intensities would be suitable.

Culture Media. A liquid modified MS medium is used for initial culture and proliferation (Table Aranda-4). Once formed, the PLBs should be transferred to a second modification of the Vacin and Went medium (Table Aranda-5) for further growth. Another modification of the Vacin and Went medium (Table Aranda-6) or the Knudson C solution (Tables Aranda-7 and Aranda-8) with or without banana should prove suitable for subsequent plantlet regeneration.

Procedure. Remove young leaves from the plants, sterilize them, wash them after that with sterile distilled water, and then culture them in liquid medium (Table Aranda-9) until the tissues proliferate and PLBs form. Then transfer these bodies to the second solid medium for further proliferation and plantlet regeneration.

Developmental Sequence. The leaves, and particularly their bases, proliferate and form PLBs. These also proliferate further; on being transferred to solid medium the new PLBs produce plantlets.

General Comments. Since the initial medium contains high amounts of hormones, the original papers recommend that as soon as enough PLBs are produced, they should be transferred to the low-hormone medium (Table Aranda-5) and "kept in this medium to serve as stocks." This is a good suggestion, but the PLBs should not be kept as stocks for prolonged periods even in this medium since long-term culture in any solution may lead to undesirable variants.

TABLE ARANDA-4. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as used for the culture of *Aranda* leaf explants (Fu, 1978, 1979*a*, 1979*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	1	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	2,4-Dichlorophenoxyacetic acid (2,4-D)	2	200 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
11	Benzylaminopurine (benzyladenine, BA)	2	200 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.25	25 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.25	25 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	1	100 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
15	Coconut water[h]	150 ml	No stock	No stock	
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[i]	To 1000 ml			
	Solidifier				
18	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin and/or cytokinin does not dissolve, add a few drops of dilute KOH or HCl, respectively, to solubilize them.

[g]Keep refrigerated between uses.

[h]Keep frozen between uses.

[i]Add items 1–7, 9, and 15 to 900 ml distilled water (item 17), adjust pH to 5.0, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil and add agar (item 18) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add items 8 and 10–14 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

TABLE ARANDA-5. **Liquid Vacin and Went medium (Vacin and Went, 1949) as used for the maintenance of protocorm-like bodies of *Aranda* (Fu, 1978, 1979*a*, 1979*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml
	Macroelements			
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10
	Iron[d]			
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock
	Microelement			
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10
	Complex additive			
8	Coconut water[e]	150 ml		
	Solvent			
9	Water, distilled[f]	To 1000 ml		

[a]Amounts are given in mg unless indicated otherwise.

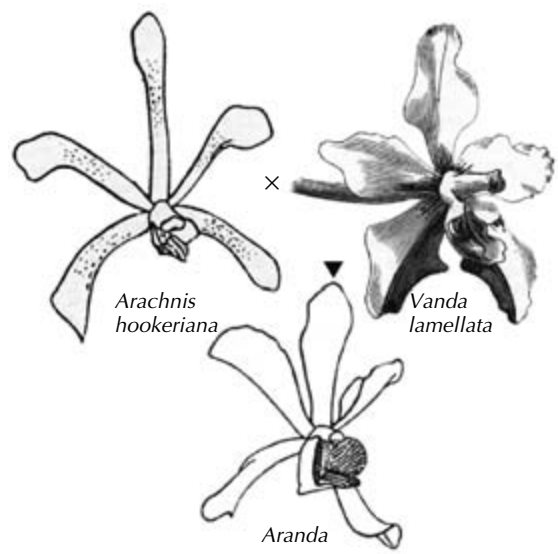
[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 37.3 mg chelating agent Na_2EDTA and 27.8 mg of ferrous sulfate, $FeSO_4 \cdot 7H_2O$, in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Keep frozen between uses.

[f]Add items 1 and 3–8 to the 500 ml distilled water (item 9) that contains the calcium phosphate (item 2). Adjust pH to 5.0, bring volume to 1000 ml with distilled water (item 9), and autoclave.



Hybrid genus *Aranda* (source: Williams and Williams, 1894; Holttum, 1964).

TABLE ARANDA-6. **Vacin and Went medium (Vacin and Went, 1949) as used for plantlet regeneration from protocorm-like bodies derived from *Aranda* leaves (Fu, 1978, 1979*a*, 1979*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution of chelated iron, dissolve 37.3 mg chelating agent (Na_2EDTA) and 27.8 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Add items 1 and 3–7 to the 500 ml distilled water (item 9) that contains the calcium phosphate (item 2), adjust pH to 5.0–5.2, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels, and autoclave.

TABLE ARANDA-7. **Knudson C medium (Knudson, 1946)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
	Iron[c]				
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Sugar				
7	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
8	Water, distilled[d]	To 1000 ml			
	Solidifier				
9	Agar[d]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. To prepare a stock solution, dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[d]Add items 1–6 to 900 ml of distilled water (item 8), adjust pH to 5.0, add sugar (item 7), and adjust volume to 1000 ml with distilled water (item 8). Bring solution to a gentle boil, and add agar (item 9) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary, adjust the pH upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. A dilute solution of hydrochloric acid (HCl) should be used to lower the pH if necessary.

TABLE ARANDA-8. **Modified Knudson C medium (Knudson, 1946)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
6	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.06	6 mg l^{-1}	10	One solution
(b)	Copper sulfate (anhydrous), $CuSO_4$	0.04	4 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}		
(d)	Molybdic acid, MoO_3	0.02	2 mg l^{-1}		
(e)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.35	35 g l^{-1}		
	Sugar				
7	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
8	Water, distilled[e]	To 1000 ml			
	Solidifier				
9	Agar[e]	12–15 g	No stock	No stock	Weigh

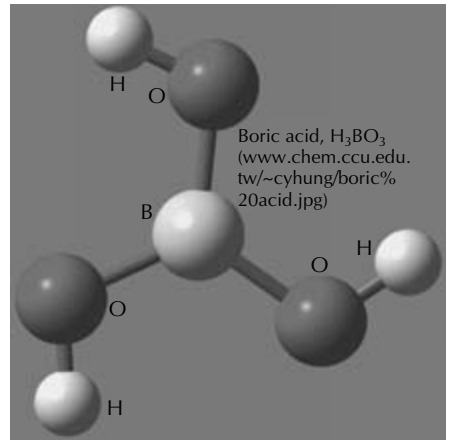
[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Add items 1–6 to 900 ml distilled water (item 8), adjust pH to 5.0–5.2, add sugar (item 7), and adjust volume to 1000 ml with distilled water (item 8). Bring solution to a gentle boil, and add agar (item 9) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When the agar is completely dissolved, pour solution into culture vessels and autoclave.



Boric acid, H_3BO_3 (www.chem.ccu.edu.tw/~cyhung/boric%20acid.jpg)

TABLE ARANDA-9. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the differentiation of *Aranda* explants (Lim-Ho, 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, monobasic KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Cytokinin-related substance				
10	Adenine sulfate[f]	40	No stock	No stock	Weigh
	Vitamins				
11	Niacin (nicotinic acid)	5.0	500 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
13	Coconut water	150 ml	No stock	No stock	Weigh
	Sugar				
14	Sucrose	2.0 g[h]	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[i]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]This compound is not soluble in alcohol and somewhat soluble in water (1 g/150 ml). Therefore weigh and suspend it in combination with items 8 and 10–12 (3 ml alcohol) and allow to stand for 20–30 min in a closed sterile container with occasional swirling. Even if most of the substance will not dissolve this treatment will sterilize it. Shake vigorously before dispensing to ensure its dispersion in the solution.

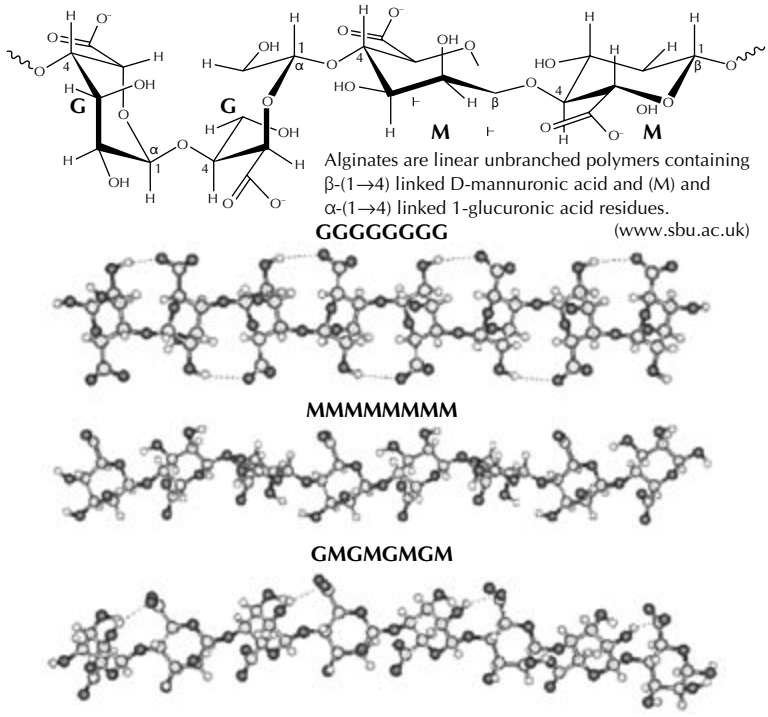
[g]Keep refrigerated between uses.

[h]The usual amount of sucrose added to this medium is 30 g.

[i]Add items 1–7, 9, and 14 to 150 ml distilled water (item 15), adjust pH to 5.2–5.7, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil, and add the agar (item 16) slowly while stirring. The agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add combined amino acid, adenine sulfate, and vitamins (items 8 and 10–12) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

Tissue Culture Propagation of *Aranda* from Buds

Thirty *Aranda* hybrids have been cultured at the Singapore Botanic Gardens (Lim-Ho, 1981). Of these, 27 were cultured from apical and axillary buds by the method used for *Arachnis* (Lim-Ho, 1981). The initial culture medium is a liquid modification of the Vacin and Went solution (see Table Arach-4). Multiplication and differentiation occur on a solid version of this medium (see Table Arach-4) or another modification (see Table Arach-5). Plantlet formation occurs on a third modification (see Table Arach-6). Growth is usually slow, and some hybrids may differentiate faster on a modification of MS medium (Table Aranda-9). Root formation may be better on a modified Knudson C medium (Table Aranda-10).



Alginates are used in some micropropagation procedures.

TABLE ARANDA-10. **Knudson C medium (Knudson, 1946) modified for root induction on plantlets of *Aranda* (Lim-Ho, 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	400	40 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	400	40 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	100	10 g l^{-1}	10	
5	**Chelated Iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
6	**Microelements**[d]				
(a)	Aluminum chloride, $AlCl_3$	0.03	3 mg l^{-1}	10	One solution
(b)	Boric acid, H_3BO_3	1.0	100 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.03	3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}		
(e)	Potassium iodide, KI	1.0	100 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4$	1.0	100 mg l^{-1}		
	Complex additives				
7	Banana homogenate[e]	75.0 g	No stock	No stock	Weigh
8	Pineapple juice[f]	150 ml	No stock	No stock	No stock
	Sugar				
9	Sucrose	2 g[g]	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[h]	To 1000 ml			
	Solidifier				
11	Agar[h]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original medium does not include chelated iron, but this form is preferable.

[d]Add all microelements to the same 1 l of distilled water, stir or heat until they are dissolved, and dispense as needed.

[e]Slice the banana (ripe but still firm) into a homogenizer, add 100 ml distilled water, and homogenize for 1–2 min. Let it stand for 5 min, and homogenize for another minute. Pour the homogenate into a beaker. Wash the homogenizer three times with 50 ml distilled water and combine the washings with the homogenate. The result is item 7.

[f]Juices, even those labeled "natural," may contain preservatives that can inhibit growth. Therefore it is preferable to use only freshly prepared juice.

[g]The usual amount of sugar added to this medium is 12–18 g.

[h]Add items 1–8 to 500 ml distilled water (item 10), adjust pH to 5.0–5.5, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary adjust pH upward with a dilute potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower the pH if necessary. Omit agar for liquid medium.

Tissue Culture Propagation of *Aranda* from Leaves

Of the 30 *Aranda* hybrids cultured in the Singapore Botanic Gardens, two were cultured from whole leaves and three from leaf bases (Lim-Ho, 1981).

Plant Material. Young leaves taken from mature plants of *Arachnis* Christine Nos 27 and 130 (*Arachnis hookeriana* × *Vanda* Hilo Blue; whole leaves cultured for both), *Arachnis* Noorah Alsagoff (*Arachnis hookeriana* × *Vanda* Dawn Nishimura; leaf base cultured), *Arachnis* Queen of Purples (*Arachnis hookeriana* × *Vanda coerulea*; leaf base cultured), and *Arachnis* Wendy Scott 'Green Field' (*Arachnis hookeriana* × *Vanda* Rothschildiana; leaf base cultured).

Surface Sterilization. The leaves are washed with detergent and water and submerged in 10–12% Clorox (10–12 ml Clorox diluted to 100 ml with distilled water). There is no mention of subsequent washing with sterile distilled water, but this step is desirable.

Culture Vessels. See *Arachnis* entry, Tissue Culture Propagation of *Arachnis* in Vitro (p. 186, Vol. I; Lim-Ho, 1981).

Culture Conditions. See *Arachnis* entry, Tissue Culture Propagation of *Arachnis* in Vitro (Lim-Ho, 1981).

Culture Media. Initial culture is in a liquid modification of the MS medium (Table Aranda-11).

Procedure. Whole leaves are placed in culture and when callus and/or PLBs form they are treated like bud explants (Lim-Ho, 1981).

Developmental Sequence. Not described in the original report.

General Comments. A method utilizing leaves is always desirable since it does not damage or endanger the plant(s) from which the explants are taken.

Aranda Deborah is the first *Aranda* raised in the Singapore Botanic Gardens. This vigorous, free flowering, and sun-loving hybrid was a popular cut flower for a long time. It was also one of the major cut flower orchids exported from Singapore. Its inflorescences are 40–50 cm long and bear up to 14 flowers on each. Flowers are 5 cm in diameter. The sepals and lateral petals are cream colored with purple streaks and irregular spots.

TABLE ARANDA-11. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for initial culture of *Aranda* leaves (Lim-Ho, 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxins				
10	Indoleacetic acid (IAA)	0.3	30 mg 100 ml^{-1} 95% ethanol[f]	1	
11	2,4-Dichlorophenoxyacetic acid (2,4-D)	1.5	150 mg 100 ml^{-1} 95% ethanol[f]	1	
	Cytokinin and related substance				
12	Adenine sulfate[g]	40	No stock	No stock	Weigh
13	6-Benzyladenine (BA)	1.25	125 mg 100 ml^{-1} 95% ethanol[f]	1	
	Vitamins				
14	Niacin (nicotinic acid)	5.0	500 mg 100 ml^{-1} 95% ethanol[f,g]	1	
15	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[f]	1	
	Complex additive				
16	Coconut water	150 ml	No stock	No stock	Weigh
	Sugar				
17	Sucrose	2.0 g[h]	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[i]	To 1000 ml			
	Solidifier				
19	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference that probably has little effect.

[e]Keep frozen between uses.

[f]Keep refrigerated or frozen between uses.

[g]This compound is not soluble in alcohol and somewhat soluble in water (1 g/150 ml). Therefore weigh and suspend it in combination with items 8 and 10–15 (7 ml alcohol), and allow to stand for 20–30 min in a closed sterile container with occasional swirling. Even if most of the substance will not dissolve, this treatment will sterilize it.

[h]The usual amount of sucrose added to this medium is 30 g.

[i]Add items 1–7, 9, and 16 to 750 ml distilled water (item 18), adjust pH to 5.2–5.7. Add sugar (item 17), and adjust volume to 1000 ml with distilled water (item 18). Bring solution to a gentle boil, and add agar (item 19) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When the agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add combined amino acid, auxins, adenine sulfate, and vitamins (items 8 and 10–15) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

Isolation and Culture of Mesophyll Protoplasts from Leaves of *Aranda* Noorah Alsagoff

The culture of isolated protoplasts offers many interesting possibilities. One is very rapid clonal propagation since it is theoretically possible to obtain microcalli and more than one plant from each protoplast (i.e., cell). Another is the selection of variants since individual protoplasts may differ genetically from each other. The third, perhaps most exciting, possibility is protoplast fusion to produce hybrids that cannot be obtained through sexual hybridization. Protoplasts of several orchids, including *Aranda*, have been isolated and cultured for short periods (Loh and Rao, 1985).

Plant Material. The original research was carried out with leaves 1–2 cm long taken from plantlets 2–4 cm tall that were grown in vitro and derived from shoot-tip cultures of *Aranda* Noorah Alsagoff (*Arachnis hookeriana* × *Vanda* Dawn Nishimura). These plantlets were maintained on a modified MS medium containing 10% coconut water, 2% sucrose, and 0.8% agar, under continuous illumination provided by white fluorescent tubes at 25°C.

Surface Sterilization. None is needed because the leaves are taken from plants grown under aseptic conditions in vitro.

Culture Vessels. Plastic Petri dishes 50 mm in diameter are used.

Culture Conditions. Both the culture of protoplasts and the incubation of tissues for the isolation of protoplasts should be conducted at 25°C in the dark.

Culture and Isolation Media. Four enzyme mixtures were screened in the original research, and the highest yield of protoplasts (1,000,000 g^{-1} of fresh weight of tissue) was obtained with a mixture of 1% Onozuka R-10 cellulase (from Yakult Honsha Co., Ltd., Japan; for a similar and reportedly improved cellulase available from Cal Biochem, see Appendix 2), 0.2% Macerozyme R-10 (from Yakult Honsha Co., Ltd., Japan; an improved enzyme of this nature, a pectinase, is available from Cal Biochem), 0.5% Driselase (from Kyowa Hakko Kogyo Co., Japan; available in the United States from Fluka Chemical Corp., 980 South Second St., Ronkonkoma, NY 11779) and 0.5-*M* mannitol (91.1 g mannitol per liter of distilled water). The enzyme mixtures must be sterilized by filtration. Protoplasts can be cultured in two media (Tables Aranda-12 and Aranda-13), both of which are modifications of the Vacin and Went medium.

Procedure. All procedures must be carried out under aseptic conditions. Cut the leaves transversely into strips about 1 mm long. Then place approximately 500 mg of these strips in 10 ml of the enzyme mixture in plastic Petri dishes and seal them with Parafilm (see Appendix 2). Incubate the dishes in the dark at 25°C for 5 h on a gyrorotatory shaker at 40 rpm. Following the incubation, filter the suspensions through a layer of Miracloth (obtainable from Cal Biochem) and centrifuge the filtrate containing the chloroplasts at 150 times the force of gravity ($\times g$) for 1 min. Resuspend the resulting pellet in a 0.4-*M* solution of mannitol (72.9 g mannitol per

TABLE ARANDA-12. **Vacin and Went macroelements (Vacin and Went, 1949) supplemented for the culture of mesophyll protoplasts of *Aranda* Noorah Alsagoff (Loh and Rao, 1985): I**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[e]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[f]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	2,4-Dichlorophenoxyacetic acid (2,4-D)	1	100 mg 100 ml^{-1} 95% ethanol[f]	1	
	Cytokinin				
11	Kinetin (6-furfurylaminopurine)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
13	Pyridoxine·HCl (Vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
14	Thiamine·HCL (Vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[f]	1	
	Sugar				
15	Glucose	72 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of the medium after that.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of water, and heat if necessary to dissolve it.

[f]Keep refrigerated or frozen between uses.

[g]Add items 1 and 3–14 to the 500 ml distilled water (item 16) that contains the calcium phosphate (item 2). Adjust pH to 5.5, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Sterilize the medium by filtration through a sterilizing filter (e.g., Millipore brand), and use as needed. This medium can probably also be autoclaved.

TABLE ARANDA-13. **Vacin and Went macroelements (Vacin and Went, 1949) supplemented for the culture of mesophyll protoplasts of *Aranda* Noorah Alsagoff (Loh and Rao, 1985): II**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[e]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[f]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxins				
10	2,4-Dichlorophenoxyacetic acid (2,4-D)	1	100 mg 100 ml^{-1} 95% ethanol[f]	1	
11	Naphthaleneacetic acid (NAA)	1	100 mg 100 ml^{-1} 95% ethanol[f]	1	
	Cytokinin				
12	Kinetin (6-furfurylaminopurine)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
14	Pyridoxine·HCl (Vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
15	Thiamine·HCL (Vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[f]	1	
	Sugar				
16	Glucose	72 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of water, and heat if necessary to dissolve it.

[f]Keep refrigerated or frozen between uses.

[g]Add items 1 and 3–15 to the 500 ml distilled water (item 17) that contain item 2. Adjust pH to 5.5, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Sterilize the medium by filtration through a sterilizing filter (e.g., Millipore brand), and use as needed. This medium can probably also be autoclaved.

liter of water), and centrifuge the suspension again at 150 × *g* for 1 min. Repeat this washing procedure three times.

For culture, suspend the washed protoplasts at a density of 100,000 ml^{-1} medium in 50-ml-capacity plastic Petri dishes. Adjust the medium (Tables Aranda-12 and Aranda-13) to pH 5.5 and filter-sterilize it.

Developmental Sequence. The isolated protoplasts may be 30–100 μm in diameter with about 60% being in the 40–50-μm range. Raphide-containing idioblasts are also released. After 7 days in culture most of the protoplasts remain intact and approximately 15% may divide or elongate. Following 10 days in culture about 12% of the protoplasts may divide. The percentage of dividing protoplasts may increase to 48% in a medium containing 2,4-D and kinetin (Table Aranda-12) and 62% when the medium contains 2,4-D, NAA, and kinetin (Table Aranda-13). Budding can also be observed in these media. Small cell clusters may be observed after 20 days in culture, but at this point the cells die and lyse.

Ceneral Comments. When this was written there were no reports of fully successful protoplast cultures of orchids. This procedure was a major advance at the time. Media and procedures have improved and protoplasts are now kept alive much longer.

Isolation and Culture of Leaf Protoplasts of *Aranda* Tay Swee Eng and *Aranda* Noorah Alsagoff

Aranda protoplasts were first isolated and cultured at the Botany Department, National University of Singapore (Loh and Rao, 1985) by C. S. Loh (who obtained her undergraduate degree there, a Ph.D. elsewhere, and is now a faculty member at NUS) and by A. N. Rao (the department's long-time effective leader and excellent chairman). Loh was also responsible for an attempt to develop a second procedure (Koh et al., 1988).

Plant Material. Plantlets of *Aranda* Tay Swee Eng (*Aranda* Lily Chong × *Vanda* Piha Moon) and *Aranda* Noorah Alsagoff (*Arachnis hookeriana* × *Vanda* Dawn Nishimura), through the culture of shoot tips as described in the previous method (Loh and Rao, 1985), were the source of the leaves from which the protoplasts were isolated for the original research. Callus tissues and PLBs were also produced from leaves (0.5–1 cm long) of *Aranda* Tay Swee Eng through methods developed for *Vanda* (Loh et al., 1975). Young leaves (< 1–3 cm in length), taken from plantlets 2–5 cm in height, were used for protoplast isolation.

Surface Sterilization. Plantlets were cultured in vitro under axenic conditions; there was no need for surface sterilization.

Culture Vessels. Standard vessels can be used to culture the plants that are used as a source of explants. Test tubes, vials, Petri dishes, centrifuge tubes, and other suitable laboratory glassware may be used for the isolation of protoplasts. The isolated protoplasts should be cultured in 14-μl droplets of medium in sealed plastic Petri dishes.

Culture and Protoplast Isolation Conditions. Plantlets used as sources of protoplasts should be maintained under conditions of 22–24°C and 16-h photoperiods. Incubation of leaf explants must be at 30°C in the dark for 7 h (for *Aranda* Noorah Alsagoff) or 10 h (for *Aranda* Tay Swee Eng) on a gyrorotatory shaker at 40 rpm. Protoplasts ($0.8–1.5 \times 10^5$ ml^{-1}) are cultured in the dark at 23 ± 2°C.

Culture Media. Media used for the culture of the donor plants are listed in the procedures for *Aranda* and *Vanda* (Loh et al., 1975; Loh and Rao, 1985). The enzyme mixture for the isolation of protoplasts consists of 1.5% (w/v) Onozuka R-10 cellulase, 0.5% (w/v) Driselase, and 136.92 mg l^{-1} sucrose (0.4-*M* solution). This mixture must be sterilized through a filter. Isolated protoplasts are washed with: (1) a filter-sterilized 0.4-*M* (136.92 mg l^{-1}) sucrose solution, and (2) a filter-sterilized culture medium. Protoplasts are reported to survive for 9 weeks in a modification of the Kao–Wetter 8p medium (Table Aranda-14).

Procedure. Leaves of *Aranda* Tay Swee Eng, less than 1 cm in length, released the largest number of protoplasts [1.97×10^6 g^{-1} fresh weight (FW)]. Leaves 1–3 cm and 3–6 cm in length released a smaller number (1.69×10^6 and 0.93×10^6, respectively). Callus masses can also be used; those of *Aranda* Tay Swee Eng release 0.5×10^4 protoplasts g^{-1} FW. Excise the leaves at their bases, and cut them transversely into 1-mm strips. Also section callus masses into small pieces. Immerse approximately 1.5 g of leaves (and presumably the same amount of callus) in 10 ml of the filter-sterilized enzyme mixture, and incubate them in the dark with agitation on a gyrorotatory shaker at 40 or 50 rpm (the original paper gives both figures), at 30°C for 7 h (for *Aranda* Noorah Alsagoff) or 10 h (for *Aranda* Tay Swee Eng). Following incubation, filter the enzyme–explant–protoplast mixture through Miracloth (obtainable from Cal Biochem; see Appendix 2) to remove large debris. After that centrifuge the filtrate for 5 min at 90 g. The green pellet at the bottom of the centrifuge tube will contain the protoplasts. Wash it twice with the 0.4-*M* sucrose solution and once with the filter-sterilized culture medium by resuspending and centrifuging.

The number of protoplasts (i.e., their density) can be determined by counting with an American Optical (AO) hemocytometer.

In the original research protoplasts were cultured in 14-µl drops of filter-sterilized medium with a density of 0.8 to 1.5×10^5 ml^{-1} in sealed plastic Petri dishes (i.e., a strip of Parafilm was wrapped around the circumference) at 23 ± 2°C in the dark. Staining with fluorescein acetate can be used to determine viability. However, this step can be omitted since viable protoplasts that are not dividing and slowly dying are simply lingering on. Cultures of such protoplasts can be discerned easily with a minimum of practice. Two signs of such cultures reported and/or observed by other workers involve many protoplasts that (1) are plasmolysed and/or (2) have burst and (3) are in aggregates. Only cultures of appropriate density that contain many dividing protoplasts are of any significance unless the purpose of the isolation is to obtain material for short-term experiments.

Developmental Sequence. The protoplasts seem to have survived for approximately 60 days, and only 2% divided.

TABLE ARANDA-14. **Kao–Wetter 8p medium (Kao and Wetter, 1977) modified for the culture of *Aranda* protoplasts (Koh et al., 1988)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	600	60 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot H_2O$	600	60 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	300	30 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
6	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.75	75 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	3	300 mg l^{-1}	10	One solution
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2	200 mg l^{-1}		
	Polyol				
7	*myo*-inositol	100	No stock	No stock	Weigh
	Hormone				
8	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.2	20 mg 100 ml^{-1} distilled water[e,f]	1	
	or benzyladenine (BA)	0.5	50 mg 100 ml^{-1} distilled water[e,f]		
	Vitamins				
9	Ascorbic acid	1	100 mg 100 ml^{-1} distilled water[e]	1	
10	*P*-Aminobenzoic acid	0.01	1 mg 100 ml^{-1} distilled water[e]	1	
11	Biotin	0.005	0.5 mg 100 ml^{-1} distilled water[e]	1	
12	D-calcium pantothenate	0.5	50 mg 100 ml^{-1} distilled water[e]	1	
13	Folic acid	0.2	20 mg 100 ml^{-1} distilled water[e]	1	
14	Niacinamide (nicotinamide)	1	100 mg 100 ml^{-1} distilled water[e]	1	
15	Pyridoxine (vitamin B_6)	1	100 mg 100 ml^{-1} distilled water[e]	1	
16	Thiamine (vitamin B_1)	10	1 g 100 ml^{-1} distilled water[e]	1	
	Organic acids				
17	Citric acid	10	1 g 100 ml^{-1} distilled water[e]	1	
18	Fumaric acid	10	1 g 100 ml^{-1} distilled water[e]	1	
19	Malic acid	10	1 g 100 ml^{-1} distilled water[e]	1	
20	Sodium pyruvate	5	500 mg 100 ml^{-1} distilled water[e]	1	
	Complex additives				
21	Casein hydrolysate	1	No stock	No stock	Weigh
22	Coconut water	100 ml	No stock	No stock	Measure
	Sugar alcohols				
23	Sorbitol	125	No stock	No stock	Weigh
24	mannitol	125	No stock	No stock	Weigh
	Sugars				
25	Cellobiose	125	No stock	No stock	Weigh
26	Fructose	125	No stock	No stock	Weigh
27	Glucose	68,400	No stock	No stock	Weigh
28	Mannose	125	No stock	No stock	Weigh
29	Rhamnose	125	No stock	No stock	Weigh
30	Ribose	125	No stock	No stock	Weigh
31	Sucrose	136,920	No stock	No stock	Weigh
32	Xylose	125	No stock	No stock	Weigh
	Solvent				
33	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original medium (Kao and Wetter, 1977) and the modification (Koh et al., 1988) use 28 mg F330 per liter of medium. This commercial preparation contains the ferric sodium salt of EDTA, which is essentially similar to the chelated iron listed here. To prepare chelated iron add chelating agent (item 5a) and iron salt (item 5b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If 2,4-D or BA does not dissolve, add a few drops of dilute KOH or HCl, respectively.

[g]Add all items to 700 ml of distilled water (item 33), set pH to 5.6, and adjust volume to 1000 ml with distilled water (item 33). A solution of this type should be sterilized by filtration. Agar is not added to liquid media. This medium can probably also be autoclaved.

General Comments. Like most other attempts to isolate orchid protoplasts this procedure did not result in the formation of callus or the regeneration of plants. However, there can be no doubt that with time these workers or others will be able to isolate orchid protoplasts and regenerate plants from them.

Carbon–Nitrogen Ratios, Protocorm Formation, and Chlorophyll Content in Callus Tissues of *Aranda* Tay Swee Eng

After 31 days in culture on Vacin and Went medium containing 0–2% fructose, the number of PLBs that developed from callus tissue of *Aranda* Tay Swee Eng (*Aranda* Lily Chong × *Vanda* Piha Moon) was inversely proportional to the residual carbon–nitrogen (C/N) ratio. Chlorophyll content of the protocorms was directly proportional to the initial C/N ratio (Table Aranda-15; Chia et al., 1988).

TABLE ARANDA-15. **Relation between carbon/nitrogen ratio, formation of protocorm-like-bodies and chlorophyll content in callus of *Aranda* Tay Swee Eng (Chia et al., 1988)**[a]

	Fresh weight of tissues, g			Carbon–nitrogen ratios × 10			Chlorophyll content, µg per gram of fresh weight tissue	Protocorm-like bodies, % per flask
Fructose, % w/v	Initial	Final	Gain[b]	Initial	Residual	Difference[b]		
0	1.09	3.78	2.69	14.5	2.1	–12.4	179	146
0.25	1.01	2.55	1.54	21.4	6.9	–14.5	133	74
0.50	1.05	2.40	1.35	27.9	25.8	–2.1	119	45
1.00	1.03	2.42	1.39	41.4	30.1	–11.3	106	37
2.00	1.04	2.35	1.31	68.2	122.8	+54.6	59	13

[a]The tissues were cultured on Vacin and Went medium containing 10% (v/v) coconut water. Data were taken after 31 days in culture and are the means of several replicas.

[b]Recalculated for this table from the original paper.

Utilization of Glucose by Callus Tissues of *Aranda* Noorah Alsagoff

Glucose uptake by callus tissues of *Aranda* Tay Swee Eng (*Arachnis* Lily Chong × *Vanda* Piha Moon) and *Aranda* Noorah Alsagoff (*Arachnis hookeriana* × *Vanda* Dawn Nishimura) follows linear kinetics and is a function of the initial glucose concentration. Only the peripheral cell layers seem to be involved in the uptake (Hew et al., 1987).

Micropropagation of *Aranda* Deborah through the Culture of Inflorescence-tip Explants

In monopodial orchids, excision of a shoot-tip explant can endanger or even destroy the donor plant itself. Therefore methods utilizing other explants were developed for several of these orchids. One of these procedures utilized inflorescence tip explants to propagate a well-known *Aranda* hybrid (Goh and Wong, 1990).

Plant Material. Tips, 0.5–1.0 cm long, should be excised from developing inflorescences, 20–40 cm in length.

Surface Sterilization. The explants must first be washed with liquid detergent (2% Teepol, a petrochemically based detergent first produced in 1938 by Shell in Great Britain, was used in the original research, but baby shampoo or almost any mild detergent can be employed) and rinsed with distilled water several times. After that the explants should be immersed in 8% Clorox (80 ml of this or any other household bleach that contains 5.25% sodium hypochlorite adjusted to 1000 ml with distilled water; only 70 ml of 6% Clorox should be used) plus a few drops of Tween 20 (or baby shampoo or a mild household detergent) for 20 min. Following a few rinses with sterile distilled water, the explants should be immersed for 15 min in 4% Clorox (40 ml of this or any other household bleach that contains 5.25% sodium hypochlorite adjusted to 1000 ml with distilled water; only 35 ml of 6% Clorox should be used). This should be followed by several rinses with sterile distilled water.

Culture Vessels. Photographs in the original paper show liquid cultures in 100-ml Erlenmeyer flasks.

Culture Conditions. In the original research the cultures were maintained at 26 ± 1°C under 14-h photoperiods and a light intensity of 20 $\mu E\ m^{-2}\ s^{-1}$ produced by cool white fluorescent lamps. Other conditions are also suitable. Liquid cultures should be placed on 80 rpm rotary shakers.

Culture Media. Three media are required for this procedure. The first or initiation medium is liquid Knudson C supplemented with 15% (v/v) coconut water and 1 mg BA l^{-1} (Table Aranda-16). Liquid Vacin and Went (VW) medium containing 10% (v/v) coconut water and 2% sucrose (Table Aranda-17) is used for subsequent growth and proliferation. Solid VW with 10% (v/v) coconut water, 2% sucrose, and 0.03% (w/v) activated charcoal (Table Aranda-18) is suitable for rooting and growth. Plantlets, 2–4 cm high, taken from the agar medium (Table Aranda-18) should be potted in charcoal chips in community pots.

Procedure. Inflorescence tips are placed initially in the liquid Knudson C (Table Aranda-16) and cultured in this medium for about 12 weeks to 4 months. PLBs or shoots that form in this medium should be transferred to the liquid VW (Table Aranda-17). Plantlets that form in this medium within 2–3 months should be excised and moved to the solid medium (Table Aranda-18).

Developmental Sequence. Inflorescence tips remain green in the first medium (Table Aranda-16) and elongate 2–3 times during the first 4 weeks. Some floral buds may also develop during this period, but they die eventually. Papillae-like projections form after 2 weeks and develop into PLBs within approximately 6–8 weeks. Callus forms on the cut ends of the explants in 4-week cultures. PLBs and shoots are produced by the end of 12 weeks. Development slows down in the first medium after 4 months. Growth, rooting, and differentiation become much better following transfer to the second medium (Table Aranda-17) and clumps of plantlets (about

TABLE ARANDA-16. **Knudson C medium (Knudson, 1946) as modified for the culture of *Aranda* Deborah inflorescence tips (Goh and Wong, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 1H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Cytokinin				
7	Benzyladenine[d]	1.0	100 mg 100 ml^{-1} 95% ethanol	1	
	Complex additive				
8	Coconut water[e]	150.0 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Knudson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$, l^{-1} but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible, will make no difference, and can be ignored.

[d]If the benzyladenine fails to dissolve add a few drops of 0.1 N HCl. The stock solution should be stored in a freezer.

[e]Water from green (immature) coconuts is preferable. However water from mature coconuts can also be used. In areas where coconuts do not grow, green and/or mature ones can sometimes be found in markets which specialize in Asian food. These stores may also carry frozen water from green coconuts. However the frozen water may also contain sugar and preservatives, both of which must be avoided. The same is true for canned coconut water. Water from more mature (brown) coconuts in stores can also be used. Regardless of the source (green or brown coconuts) the water should be drained from the nuts, filtered and kept frozen between uses. Coconut water can be autoclaved. The correct name for this liquid is coconut water, not coconut milk.

[f]Add items 1–6 and 8 to 750 ml of distilled water (item 10), adjust pH to 5.2, add sugar (item 9), and raise volume to 1000 ml with distilled water (item 10). Pour the solution into a 2-l flask and autoclave. Using a sterile pipette add the cytokinin to the autoclaved medium, swirl well to mix, and dispense into culture vessels. There are no indications in the original paper whether microelements other than manganese (item 6) were added to the medium and it is not clear if the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the Murashige–Skoog medium will probably prove to be suitable. It is not clear from the original paper if the cytokinin was added before or after the medium was autoclaved. Since it is much easier and simpler to add it before autoclaving, users of this medium may wish to experiment by adding this hormone before and after autoclaving.

1 cm high) form after 2–3 months. Growth is enhanced on the third medium (Table Aranda-18) and 2–4-cm-tall plants are obtained in approximately 2 months.

General Comments. *Aranda* Deborah (*Arachnis hookeriana* × *Vanda lamellata*) is the first hybrid in this monopodial genus produced by the Singapore Botanic Gardens. It was introduced in 1945 and is named after the daughter of Professor Eric Holttum, director of the gardens before and after World War II and later Professor of Botany at the University of Singapore. Being vigorous and free flowering, it became one of the major cut flower orchids exported from Singapore.

TABLE ARANDA-17. **Vacin and Went medium (Vacin and Went, 1949) modified for the culture of protocorm-like bodies derived from inflorescence-shoot-tip explants of *Aranda* Deborah (Goh and Wong, 1990): I**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additive				
9	Coconut water[e]	100.0 ml	No stock	No stock	Measure
	Solvent				
10	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve. Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original recipe of this medium calls for 28 mg ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, l^{-1} but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible. See also footnote *c* under Table Aranda-16.

[e]Water from green (immature) coconuts is preferable. However water from mature coconuts can also be used. In areas where coconuts do not grow, green and/or mature ones can sometimes be found in markets which specialize in Asian food. These stores may also carry frozen water from green coconuts. However the frozen water may also contain sugar and preservatives, both of which must be avoided. The same is true for canned coconut water. See also footnote *e* under Table Aranda-16.

[f]Add items 1–7 and 9 to 500–700 ml of distilled water (item 10), adjust pH to 5.2, add sugar (item 8), and raise volume to 1000 ml with distilled water (item 10). Pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is unclear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary or desirable, the formulation used as part of the Murashige–Skoog medium will probably be suitable.

This procedure seems effective. However a statement in the original paper that "by repeated subculture of . . . proliferating protocorm-like bodies, plantlet production could be maintained continuously for as long as desired" is very ill advised because such a practice can lead to undesirable mutations, malformed flowers, and unproductive plants.

TABLE ARANDA-18. **Vacin and Went medium (Vacin and Went, 1949) modified for the culture of protocorm-like bodies derived from inflorescence-shoot-tip explants of *Aranda* Deborah (Goh and Wong, 1990): II**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additive				
9	Coconut water[e]	100.0 ml	No stock	No stock	Measure
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar	12–15 g	No stock	No stock	Weigh
	Darkening agent				
12	Activated charcoal (AC)	300.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve. Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original recipe of this medium calls for 28 mg ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, l^{-1} but chelated iron as suggested here is preferable. A small difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible. See also footnote *c* under Table Aranda-16.

[e]Water from green (immature) coconuts is preferable. However water from mature coconuts can also be used. In areas where coconuts do not grow, green and/or mature ones can sometimes be found in markets which specialize in Asian food. These stores may also carry frozen water from green coconuts. However the frozen water may also contain sugar and preservatives, both of which must be avoided. The same is true for canned coconut water. See also footnote *e* under Table Aranda-16.

[f]Add items 1–7 and 9 to 500–700 ml of distilled water (item 10), adjust pH to 5.2, add sugar (item 8), and raise volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved add the charcoal (item 12) with vigorous stirring, pour the solution into culture vessels, and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary or desirable, the formulation used as part of the Murashige–Skoog medium will probably be suitable.

Aranda Deborah was named for one of the daughters of Professor Eric Holttum who was Director of the Singapore Botanic Gardens and Professor of Botany at the University of Singapore.

Thin Section Culture of *Aranda* Deborah

Development of PLBs from shoot-tip explants of *Aranda* is "slow, taking 9–12 months" (Laksmanan et al., 1995). Therefore, a method using thin sections was developed at the National University of Singapore to accelerate the process (Laksmanan et al., 1995; Prakash et al., 1996).

Plant Material. Top portions of shoots should be cut and the leaves removed carefully without damaging the shoot tips. Thin sections, 0.6–0.7-mm thick, are cut by transversely sectioning the apical 6–7-mm part of the shoot tips. Very sharp razor blades or scalpels must be used for all sectioning and cutting.

Surface Sterilization. A surface sterilization procedure is not described in the original paper (Laksmanan et al., 1996). The procedure used for inflorescence-shoot-tip explants (Goh and Wong, 1990; see above) is probably suitable.

Culture Vessels. Erlenmeyer flasks, 100-ml capacity, containing 20 ml of medium should be used for liquid VW. GA7 containers (Magenta Corporation, 3800 North Milwaukee Ave., Chicago, IL 60641-2874, USA, telephone 1-773-777-5050, fax 1-773-777-4055, www.magentacorp.com) containing 50 ml of solid medium are suitable for plantlet development. Other vessels can also be used.

Culture Conditions. In the original research the cultures were maintained at 26 ± 1°C under 16-h photoperiods of 30 $\mu E\ m^{-2}\ s^{-1}$ intensity provided by cool white fluorescent tubes. Standard laboratory conditions will probably also be suitable. Liquid cultures should be agitated on a gyrorotatory shaker at 120 rpm.

Culture Media. The thin sections produce PLBs on liquid VW medium containing 20% (v/v) coconut water (Table Aranda-19). PLBs proliferate on the same medium (Table Aranda-19). Plantlets develop from the PLB on VW which contains 10% (v/v) coconut water and 500 $mg\ l^{-1}$ activated charcoal (Table Aranda-20).

Procedure. Thin sections cut under sterile conditions are placed in liquid VW medium (Table Aranda-19). Once PLBs are formed they can be proliferated by subculturing them in the same medium (Table Aranda-19), or transferred to solid VW medium (Table Aranda-20) for plantlet formation.

Developmental Sequence. The thin sections form PLBs within 45 days of being placed in liquid culture (Table Aranda-19). PLBs proliferate on the same medium (Table Aranda-19). Plantlets develop on solid VW medium (Table Aranda-20).

General Comments. Like the inflorescence-shoot-tip culture method for the propagation of *Aranda* Deborah (Goh and Wong, 1990) the thin section procedure is described as "rapid" and "efficient" (Laksmanan et al., 1995; Prakash et al., 1996) because "over 80,000 plantlets can be produced from [thin section] explants obtained from a single shoot tip in a year" especially in the presence of NAA.

TABLE ARANDA-19. **Vacin and Went medium (Vacin and Went, 1949) modified for the culture of thin sections of *Aranda* Debora shoot tips and protocorm-like body proliferation (Laksmanan et al., 1995)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additive				
9	Coconut water[e]	200.0 ml	No stock	No stock	Measure
	Solvent				
10	Water, distilledf[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve. Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original recipe of this medium calls for 28 mg ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, l^{-1} but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible. See also footnote *c* under Table Aranda-16.

[e]Water from green (immature) coconuts is preferable. However water from mature coconuts can also be used. In areas where coconuts do not grow, green and/or mature ones can sometimes be found in markets which specialize in Asian food. These stores may also carry frozen water from green coconuts. However the frozen water may also contain sugar and preservatives, both of which must be avoided. The same is true for canned coconut water. See also footnote *e* under Table Aranda-16.

[f]Add items 1–7 and 9 to 500–700 ml of distilled water (item 10), adjust pH to 5.2, add sugar (item 8), and raise volume to 1000 ml with distilled water (item 10). Pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is unclear whether the addition of other nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary or desirable, the formulation used as part of the Murashige–Skoog medium will probably be suitable.

What this statement does not take into account is that overproliferation of explants, especially in the presence of NAA (and/or other synthetic hormones), can result in undesirable mutations (for example, plants which produce malformed blossoms or inflorescences with missing flowers that have no commercial value). These and other mutations can lead to major losses for cut flower growers. The value of the thin section method as a means of freeing orchids from viruses is also questionable because "the percentage of regenerants free from [cymbidium mosaic virus] and [odontoglossum ringspot virus] . . . varied." Altogether, the "80,000 plants" statement, the use of NAA in addition to coconut water for excessive proliferation, and the uncertainty about freeing plants from virus point to a need for cautious formulation of media, conservative and better informed assertions, as well as for wiser, more realistic and

TABLE ARANDA-20. **Vacin and Went medium (Vacin and Went, 1949) modified for differentiation and growth of plantlets produced by protocorm-like bodies (PLB) derived from thin sections of *Aranda* Deborah shoot tips (Lakshmanan et al., 1995)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additive				
9	Coconut water[e]	100.0 ml	No stock	No stock	Measure
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar	12–15 g	No stock	No stock	Weigh
	Darkening agent				
12	Activated charcoal (AC)	500.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve. Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original recipe of this medium calls for 28 mg ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, l^{-1} but chelated iron as suggested here is preferable. A difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible. See also footnote *c* under Table Aranda-16.

[e]Water from green (immature) coconuts is preferable. However water from mature coconuts can also be used. In areas where coconuts do not grow, green and/or mature ones can sometimes be found in markets which specialize in Asian food. These stores may also carry frozen water from green coconuts. However the frozen water may also contain sugar and preservatives, both of which must be avoided. The same is true for canned coconut water. See also footnote *e* under Table Aranda-16.

[f]Add items 1–7 and 9 to 500–700 ml of distilled water (item 10), adjust pH to 5.2, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved add the charcoal (item 12) with vigorous stirring, pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary or desirable, the formulation used as part of the Murashige–Skoog medium will probably be suitable.

better conceived advice by those who develop micropropagation methods. This is especially true for researchers who have been part of the field for a long time (Goh, 1970, 1973, 1989, 1996) and perceive themselves as leaders (Goh headed the Department of Botany when this research was carried out) despite modest contributions of their own.

Aranthera

One of the first crosses in this hybrid genus, *Aranthera* Caprice (*Arachnis maingayi* × *Renanthera storiei*) was produced in 1939 by J. van Brero in Bandung (then spelled Bandoeng), Indonesia. The second and presently better-known cross *Aranthera* James Storei (*Arachnis hookeriana* × *Renanthera storiei*) originated at the Singapore Botanic Gardens also in 1939. (The name is sometimes misspelled in the literature as Storiei, probably because this is the spelling of the specific epithet of one of its parents.) Many additional hybrids have been produced since then involving as parents *Arachnis flos-aëris*, *Arachnis clarkei*, *Arachnis* Maggie Oei (*Arachnis hookeriana* × *Arachnis flos-aëris*), *Renanthera coccinea*, *Renanthera elongate*, and *Renanthera monachica* among others. Some of the hybrids are of commercial importance, and therefore several micropropagation procedures were developed for *Aranthera* (Alang, 1975; Sagawa and Kunisaki, 1982).

Tissue Culture of *Aranthera* James Storie

The first method for mass rapid clonal propagation of *Aranthera* was developed at the famed Bogor Botanical Gardens in Indonesia in view of its export potential at the time (Irawati et al., 1977).

Plant Material. Apical and axillary buds from developing shoots of *Aranthera* James Storei are used. Buds are excised in cubes of tissue measuring 0.5 × 0.5 × 0.3 cm.

Surface Sterilization. Following removal of the leaves the explants should be submerged for 15 min in a 0.5% calcium hypochlorite solution. (In making up this solution from 0.5 g calcium hypochlorite, it is important that the powder be truly dry to begin with, as it can absorb water from the air in humid climates, and wet powder will produce an ineffective sterilant; dissolve the powder in enough water for a final volume of 100 ml, stir or shake the solution about every 5 min for 15–20 min, and then filter or decant it.) It is advisable to wash the sterilized explants two to three times with sterile distilled water before the sheaths that cover the buds are removed. After that, soak the exposed buds for an additional 15 min in 0.2% calcium hypochlorite (0.2 g per 100 ml of water, or 40 ml of the 0.5% solution diluted to 100 ml with distilled water).

Culture Vessels. Culture tubes and Erlenmeyer flasks are suitable.

Culture Conditions. Cultures should be maintained under a constant illumination of 1000–1100 lx (which in the original research was provided by Philips fluorescent tubes; other light sources are also suitable) and 24 ± 2°C. Liquid cultures require agitation. (A 2-rpm rotary shaker was used by Irawati et al., 1977, but other shakers can also be employed.)

Culture Medium. Liquid-modified Knudson C medium (Table Arnth-1) is suitable for the initial explants. PLBs produced under these conditions should be placed on

TABLE ARNTH-1. **Modified Knudson C medium (Knudson, 1946) for the culture of *Aranthera* James Storie buds (Irawati et al., 1977)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	**Chelated Iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
6	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.06	6 mg l^{-1}		
(b)	Copper sulfate (anhydrous), $CuSO_4$	0.04	4 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	One solution
(d)	Molybdic acid, MoO_3	0.02	2 mg l^{-1}		
(e)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.35	35 g l^{-1}		
	Sugar				
7	Sucrose	20 g	No stock	No stock	Weigh
	Complex additive				
8	Coconut water	150 ml	No stock	No stock	Measure
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 5a) and iron salt (item 5b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved. Only ferrous sulfate, $FeSO_4 \cdot 7H_2O$ (25 mg l^{-1}), was used in the original formulation, but the chelated form is preferable.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The amounts listed here have been rounded up from those in the original paper (Irawati et al., 1977).

[e]Add items 1–6 and 8 to 800 ml distilled water (item 9), adjust pH to 5.3, add sugar (item 7), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into culture vessels and autoclave. Omit agar if liquid medium is being prepared.

a solid version of this solution (Table Arnth-1) or Knudson C medium (see Tables Aranda-7 and Aranda-8) with or without banana homogenate. Modified Vacin and Went medium (Table Arnth-2) can also be used in either liquid or solid form.

Procedure. Place the sterilized buds in liquid medium (Tables Arnth-1 and Arnth-2), and culture them until PLBs form. Transfer these to solid medium (see Tables Aranda-7, Aranda-8, Arnth-1, and Arnth-2) for plantlet regeneration.

Developmental Sequence. Explants change in color from white, yellowish-green or pale green to green within 1–3 weeks and the tissues turn dark green in approximately 7 weeks. The tissues become swollen after the first week of culture and may produce abnormal leaves subsequently. However, with time the explants produce PLBs that regenerate plantlets that appear normal.

TABLE ARNTH-2. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of buds of *Aranthera* James Storie (Irawati et al., 1977)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Complex additive				
9	Coconut water	150 ml	No stock	No stock	Measure
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier				
11	Agar[e]	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 37.3 mg chelating agent (Na_2EDTA) and 27.8 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Add items 1, 3–7, and 9 to the 500 ml distilled water (item 10) that contain item 2. Adjust pH to 5.3, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 10). To prepare solid medium bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar if liquid medium is desired.

General Comments. Explant survival rate is low but sufficient for a practical mass rapid clonal propagation procedure. It is not clear at present whether this procedure can be used with *Aranthera* crosses involving other parents.

In Vitro Propagation of *Aranthera* James Storie

This procedure was developed because "*Aranthera* James Storie [is an] important commercial cut [flower] . . . exported from Singapore and . . . Thailand [but] . . . methods of vegetative propagation [were] inadequate to provide the volume of plants required for establishment of new fields" (Cheah and Sagawa, 1978).

Plant Material. Soft upper nodes should be used as the source of explants. The apical bud explants used in the original research were 5–6 mm long and included

three to four internodes, an equal number of leaf primordia, and the apical meristem. Axillary-bud explants consisted of a single bud and a portion of the stem.

Surface Sterilization. All leaf portions except the sheaths should be removed before surface-sterilizing the sections in 10% Clorox (10 ml Clorox diluted to 100 ml with distilled water) for 10 min. The sheaths are removed after that, and the sections are submerged in 5% Clorox (5 ml Clorox diluted to 100 ml) for 5 min. Buds are excised from the sterilized stem sections under aseptic conditions with sterile tools, rinsed with distilled water, and placed into culture.

Culture Vessels. Erlenmeyer flasks, culture tubes, and other suitable containers can be used.

Culture Conditions. During the original research the tissues were maintained on a New Brunswick Model V shaker at 160 rpm under conditions of 2100 lx of continuous illumination (provided by General Electric Power Groove white fluorescent light) and 26 ± 3°C. Similar, though not identical, conditions are also suitable.

Culture Medium. The media consist of the basal salts of Vacin and Went medium, a reduced amount of agar, "occasional deletion of 2% sucrose," 150 ml coconut water, and 50 g banana homogenate (Tables Arnth-3 to Arnth-5).

Procedure. First place the explants on a liquid medium that contains 15% coconut water (Table Arnth-3). After 1 week of culture remove the outer bud scales. Then transfer the PLBs to a liquid medium containing sugar-free coconut water (Table Arnth-4). After further proliferation move them to solid sugar-free medium (Table Arnth-5). To produce roots, transfer shoots from this medium to a medium containing banana (Table Arnth-6).

Developmental Sequence. Both apical and axillary buds become swollen and green after 2 weeks in culture. PLBs are produced at the nodes, bases, and middle and upper parts of explants after 6–8 weeks. These bodies multiply rapidly on the medium containing sugar-free coconut water. They die in solutions containing sugar. The PLBs produce shoots on a sugar-free medium (Table Arnth-5). When these shoots reach a height of 2.5 cm and are transferred to banana-containing medium (Table Arnth-6) they develop roots.

General Comments. This procedure is complicated due to the use of several media. However, it is a useful and productive one. As with the previous method it is not clear whether it can be used with *Aranthera* crosses involving other parents.

TABLE ARNTH-3. **Vacin and Went medium (Vacin and Went, 1949) for the initial culture of apical and axillary buds of *Aranthera* James Storie (Irawati et al., 1977)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Complex additive				
9	Coconut water	150 ml	No stock	No stock	Measure
	Solvent				
10	Water, distilled[e]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Then add other components of the medium.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 37.3 mg chelating agent (Na_2EDTA) and 27.8 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Add items 1, 3–7, and 9 to the 500 ml distilled water (item 10) that contain item 2. Adjust pH to 5.3, add sugar (item 8), and bring volume to 1000 ml with distilled water (item 10). Dispense into culture vessels and autoclave.

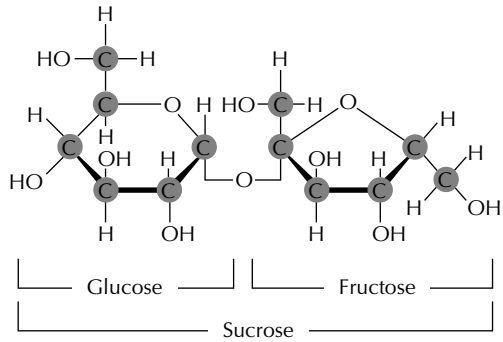


TABLE ARNTH-4. **Modified Vacin and Went medium (Vacin and Went, 1949) for the rapid multiplication of protocorm-like bodies produced by apical and axillary buds of *Aranthera* James Storie (Cheah and Sagawa, 1978)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water	150 ml	No stock	No stock	Measure
	Solvent				
9	Water, distilled[e]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate it. To prepare a stock solution of chelated iron, dissolve 37.3 mg chelating agent (Na_2EDTA) and 27.8 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Add items 1 and 3–8 to the 500 ml distilled water (item 9) that contain item 2. Adjust pH to 5.3, bring volume to 1000 ml with distilled water (item 9), dispense into culture vessels, and autoclave.

TABLE ARNTH-5. **Vacin and Went medium (Vacin and Went, 1949) for shoot production from protocorm-like bodies of *Aranthera* James Storie (Cheah and Sagawa, 1978)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Solvent				
8	Water, distilled[e]	To 1000 ml			
	Solidifier				
9	Agar[e]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Then add other components of the medium.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 37.3 mg of the chelating agent (Na_2EDTA) and 27.8 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Add items 1 and 3–7 to the 500 ml distilled water (item 8) that contain item 2. Adjust pH to 5.3, and adjust volume to 1000 ml with distilled water (item 8). Bring solution to a gentle boil, and add agar (item 9) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave.

TABLE ARNTH-6. **Vacin and Went medium (Vacin and Went, 1949) for root induction on shoots of *Aranthera* James Storie (Cheah and Sagawa, 1978)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additives				
8	Coconut water	150 ml	No stock	No stock	Measure
9	Green banana homogenate[e]	50	No stock	No stock	Weigh
	Sugar				
10	Sucrose	20 g	No stock	No stock	Weigh
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Agar[f]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Then add other components of the medium.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 37.3 mg of the chelating agent (Na_2EDTA) and 27.8 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]To prepare the banana homogenate place the pulp in a homogenizer (e.g., a household blender) with 100–150 ml water, and homogenize for 1–2 min.

[f]Add items 1 and 3–9 to the 500 ml distilled water (item 11) that contain item 2. Set pH to 5.3, and adjust volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil, and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water, which is then brought to a boil and stirred. When the agar is completely dissolved, dispense the medium into culture vessels and autoclave.

Apical Meristem Culture of *Aranthera* James Storie

Cymbidium mosaic virus (CymV) is the most widespread orchid virus in Malaysia and most probably other areas. A method to free infected *Aranthera* James Storie plants of this virus was developed at the Malaysian Agricultural Research and Development Institute (MARDI) in Selangor (Ong and Chua, 1980). This method can also be used for mass rapid clonal propagation.

Plant Material. Shoot tips of mature plants are removed, surface-sterilized, and rinsed before removal of the leaves to expose the apical meristem. Meristem domes (0.5–1.0 mm) with two leaf primordia should be excised aseptically with a sharp needle or microscalpel.

Surface Sterilization. Excised shoot tips should be rinsed with water, submerged in 10% Clorox (10 ml of Clorox diluted to 100 ml with distilled water) for 10 min and rinsed several times with sterile distilled water.

Culture Vessels. Erlenmeyer flasks (of 50- or 125-ml capacity, containing medium equal to one-fifth of their volume), culture tubes, or other containers can be used.

Culture Conditions. Few details are given in the original paper beyond the suggestion that PLBs that form on the apical meristem were cultured in liquid medium agitated at 120 rpm on an orbital shaker. The culture conditions used in conjunction with the other methods for *Aranthera* will most probably prove suitable for this procedure.

Culture Medium. A modified solid Vacin and Went (VW) medium containing coconut water (Table Arnth-7) is used for the initial culture. PLBs are cultured on liquid sucrose-free VW medium (Table Arnth-8). For shoot and root formation the PLBs should be transferred to solid VW without sucrose (Table Arnth-8).

Procedure. Place excised apical meristem domes on solid modified VW medium (Table Arnth-7) for approximately 10 months or until PLBs form. Transfer these to a liquid sugar-free VW medium (Table Arnth-8) for proliferation. When enough PLBs are available, culture them on solid sucrose-free VW medium (Table Arnth-8) for plantlet regeneration. Plantlets with at least two roots can be transplanted into thumb pots with charcoal as a potting medium.

Developmental Sequence. Apical meristem domes form PLBs after 10 months on the first medium. The PLBs proliferate in the liquid VW medium and form plantlets when moved to solid sucrose-free VW medium.

General Comments. Bacterial contamination can be a problem for this procedure. In the original research only three of ten apical meristems survived contamination and developed PLBs. Not all plantlets produced by this method can be expected to be free of CymV. For this reason the authors (Ong and Chua, 1980) wisely state that their study "shows that it is necessary to keep mericlones for a prolonged probation period and that thorough testing is an essential prerequisite before any clone produced by this technique is accepted as virus free."

The name *Aranthera* James Storie is sometimes misspelled *Aranthera* James Storei or *Aranthera* James Storiei.

TABLE ARNTH-7. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of apical meristems of *Aranthera* James Storie (Ong and Chua, 1980)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water	200 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier				
11	Agar	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Then add other components of the medium.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 37.3 mg chelating agent (Na_2EDTA) and 27.8 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Add items 1 and 3–8 to the same 500 ml distilled water (item 10) that contains item 2. Set pH to 5.3, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave.

TABLE ARNTH-8. **Modified Vacin and Went medium (Vacin and Went, 1949) for the proliferation of protocorm-like bodies and plantlet production of *Aranthera* James Storie (Ong and Chua, 1980)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water	200 ml	No stock	No stock	Measure
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Then add other components of the medium.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 37.3 mg chelating agent (Na_2EDTA) and 27.8 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Add items 1 and 3–8 to the same 500 ml distilled water (item 9) that contain item 2. Set pH to 5.3 and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave.

Clonal Propagation of *Aranthera* from Bud Explants

Bud explants of six *Aranthera* hybrids were cultured at the Singapore Botanic Gardens by the method used for *Arachnis* (Lim-Ho, 1981). The explants are cultured initially on a liquid modification of the Vacin and Went medium (see Table Arach-4). Multiplication and differentiation occur on a solid version of this medium (see Table Arach-4) and a second modification (see Table Arach-6) is suitable for plantlet formation. Growth is slow, and differentiation may be better on a modification of the MS medium (see Table Aranda-9). The explants may produce phenolics that could inhibit growth or kill the explant. For this reason the explants must be transferred to fresh medium every few days during the first 6 months.

Clonal Propagation of *Aranthera* from Leaf Bases

Aranthera Beatrice Ng 'Yellow' can be propagated from leaf bases. The procedure is the same as the one for leaves and leaf bases of *Aranda* (Lim-Ho, 1981) except that the initial medium is a different liquid modification of the Vacin and Went solution (Table Arnth-9).

TABLE ARNTH-9. **Vacin and Went medium (Vacin and Went, 1949) modified for initial culture of *Aranthera* leaves (Lim-Ho, 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxins				
8	Indoleacetic acid (IAA)	0.3	30 mg 100 ml^{-1} 95% ethanol[e]	1	
9	Naphthaleneacetic acid (NAA)	0.2	20 mg 100 ml^{-1} 95% ethanol[e]	1	
10	2,4-Dichlorophenoxyacetic acid (2,4-D)	1.5	150 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin				
11	N^6-benzyladenine (BA)	1.25	125 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
12	Coconut water	150 ml	No stock	No stock	Measure
	Solvent				
13	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Then add other components of the medium.

[d]The original formulation contains a different iron salt, but the chelated form is preferable.

[e]Keep refrigerated or frozen between uses.

[f]Add items 1, 3–7, and 12 to the 500 ml distilled water (item 13) that contains item 2. Set pH to 5.2–5.5, adjust volume to 1000 ml with distilled water (item 13); pour into a 2-l flask, and autoclave. Add hormones (items 8–11) while medium is still hot and liquid, and swirl several times to mix well. Do not add agar since this is a liquid medium.

Arundina

Distributed throughout much of Asia, *Arundina bambusifolia* is an attractive, easy-to-grow terrestrial orchid whose flowers last up to 5 days. Two micropropagation procedures are available for it (Mitra, 1971; Banik et al., 1986).

Shoot-tip and Stem-section Culture of *Arundina bambusifolia*

An investigation of seed germination, seedling development, and morphogenesis of *Arundina bambusifolia* led to the development of tissue culture methods that can be used for clonal propagation (Mitra, 1971).

Plant Material. Use shoot tips 1–2 mm long taken from seedlings (Table Arnd-1) that are 6–8 mm high (only two to three internodes visible), and stem disks (1–2 mm in diameter and thickness). Removal of the tissues appears to involve merely cutting under sterile conditions. The explants are then placed on the appropriate culture medium.

TABLE ARND-1. **Effects of media used in the culture of shoot tip and stem sections of *Arundina bambusifolia* (Mitra, 1971)**

Raghavan and Torrey (1964) medium	Seedling shoot tips, 8 mm long, containing 2 nodes, "one only" visible	Seedling shoot tips, 1–2 mm long	Seedling stem disks, 1–2 mm in diameter and thickness
Plus:			
Urea, 25–50 mg l^{-1} (Table Arnd-2)	Development of leaf primordia Root formation at basal end Protocorm formation at basal end Development of shoot buds		Plantlet formation on 25 mg l^{-1}
Peptone, 1 g l^{-1} (Table Arnd-2)	Protocorm formation at basal end Development of shoot buds Slight development of leaf primordia		Plantlet formation
Vitamin-free casein hydrolysate, 400 mg l^{-1} (Table Arnd-2)	Development of leaf primordia Root formation at basal end		
Yeast extract, 200 mg l^{-1} (Table Arnd-2)			Plantlet formation
Ribonucleic acid, 25–50 mg l^{-1} (Table Arnd-2)	Slight development of leaf primordia Root formation at basal end Development of shoot buds		
Coconut water, 100 mg l^{-1} (Table Arnd-2)	Development of leaf primordia Root formation at basal end		
Coconut water, 200 ml l^{-1}	Development of shoot buds Development of leaf primordia Root formation at basal end Development of individual shoot tips into "full-fledged plantlets"		
Control, no additives (Table Arnd-2)	Development of leaf primordia Root formation at basal end Development of shoot buds		
Modified (Tables Arnd-3 and Arnd-4)		Complete plant formation	

TABLE ARND-2. **Raghavan and Torrey (1964) medium as used for the culture of *Arundina bambusifolia* 6–8-mm-long shoot tips and stem disks (Mitra, 1971)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements[b]				
1	Potassiuim phosphate, KH_2PO_4	270	27 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	240	24 g l^{-1}	10	
3	Calcium sulfate, $Ca_3(PO_4)_2$[c]	80	8 g l^{-1}	10	
4	Calcium phosphate, $Ca_3(PO_4)_2$[c]	100	10 g l^{-1}	10	
5	Ammonium nitrate, NH_4NO_3	80	8 g l^{-1}[b]	10[b]	Or weigh[b]
6	**Microelements**[c]				
(a)	Boric acid, H_3BO_3	0.60	600 mg l^{-1}		
(b)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.40	400 mg l^{-1}		
(c)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	50 mg l^{-1}		
(d)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	50 mg l^{-1}	1	One solution
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	50 mg l^{-1}		
(f)	Potassium iodide, KI	0.03	30 mg l^{-1}		
(g)	Cobaltous nitrate, $Co(NO_3)_2$	0.05	50 mg l^{-1}		
	Iron[d]				
7	Ferric tartrate, $Fe_2(C_4H_4O_6)_3$	3	300 mg l^{-1}[d]	10[d]	
	Organic additives				
8	Urea	25 or 50	2.5 g l^{-1} or 5 g l^{-1}[b]	10[b]	Use as needed
9	Ribonuclei acid[b]	25 or 50	2.5 g l^{-1} or 5 g l^{-1}[b]	10[b]	(see Table Arnd-2)
	Complex additives[b,e]				
10	Peptone	1 g	No stock[b]	No stock[b]	Weigh
11	Vitamin-free casein hydrolysate	400 or 1 g	No stock[b]	No stock[b]	Weigh
12	Yeast extract	200	No stock[b]	No stock[b]	Weigh
13	Coconut water[e]	100 ml or 200 ml			
	Sugar[e]				
14	Sucrose	20 g	No stock	No stock	Weigh
15	Water, distilled[e]	To 1000 ml			
	Solidifier[e]				
16	Agar	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions of nitrogenous salts or organic substances may become contaminated. Therefore, stock solutions should not be prepared. If made, they must be kept frozen between uses.

[c]Add all microelements to the same 1 l, stir and/or heat until dissolved. Add 1 ml to culture medium.

[d]Ferric tartrate is relatively insoluble. Grinding it with a mortar and pestle before dissolving helps. The addition of a pellet or two of KOH to the solution will increase solubility, but a precipitate may form nevertheless. To insure equal distribution, shake stock solution well before dispensing.

[e]Dissolve items 1–13 as needed in 500 ml distilled water (item 15) adjust the pH to 5.2–5.5, and add the sugar (item 14) and the coconut water (item 13), if used; bring volume up to 1000 ml with distilled water. Add agar (item 16) slowly, while stirring, to the gently boiling solution. When fully dissolved, dispense into culture vessels and autoclave.

Surface Sterilization. This is not required since explants are obtained from seedlings grown under aspectic conditions.

Culture Vessels. Wide-mouth, 150-ml Erlenmeyer flasks and 25 × 180 mm culture tubes containing 50 and 25 ml medium, respectively, were used in the original work. Other vessels could no doubt also be used.

Culture Conditions. Maintain cultures at 26 ± 1°C under a light intensity of 3000 lx and 12-h photoperiods. Philips "Natural" fluorescent tubes were originally used,

TABLE ARND-3. **A medium (Raghavan and Torrey, 1964) modified for the culture of 1–2-mm-long stem tips of *Arundina bambusifolia* (Mitra, 1971)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	50	5 g l^{-1}	10	Or weigh
2	Potassium phosphate, KH_2PO_4	100	10 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	240	24 g l^{-1}	10	
4	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	100	10 g l^{-1}	10	Or weigh
5	Potassium nitrate, KNO_3[b]	85	8.5 g l^{-1}	10	Or weigh
6	Potassium chloride, KCl	65	6.5 g l^{-1}	10	
7	Calcium phosphate, $CaH_4(PO_4)_2$	100	10 g l^{-1}	10	
8	**Chelated iron**[d]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	2.78	278 mg l^{-1}	10	One solution
(b)	Na_2EDTA	3.73	374 mg l^{-1}		
9	**Microelements**[c]				
(a)	Boric acid, H_3BO_3	0.60	600 mg l^{-1}		
(b)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.40	400 mg l^{-1}		
(c)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	50 mg l^{-1}		
(d)	Copper sulfate, $CuSO_4 \cdot H_2O$	0.05	50 mg l^{-1}	1	One solution[c]
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	50 mg l^{-1}		
(f)	Potassium iodide, KI	0.03	30 mg l^{-1}		
(g)	Cobaltous nitrate, $Co(NO_3)_2$	0.05	50 mg l^{-1}		
	Sugar				
10	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[e]	To 1000 ml			
	Solidifier				
12	Agar	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, they must be kept frozen between uses.

[c]Add all microelements to the same 1 l, stir and/or heat until dissolved. Add 1 ml per liter of culture medium.

[d]Add items a and b to the same 1 l, dissolve. Add 10 ml per liter of culture medium.

[e]Dissolve items 1–9 in 900 ml distilled water (item 11), adjust pH to 5.2–5.5; add sugar (item 10), and bring volume up to 1000 ml with distilled water. Add agar (item 12) slowly, while stirring, to the gently boiling solution. When fully dissolved, dispense into vessels and autoclave.

but equivalent ones (e.g., Sylvania Gro Lux) would probably prove to be equally suitable.

Culture Media. Enriched Raghavan–Torrey medium (Raghavan and Torrey, 1964), which is itself a modification of a medium first used by Spoerl (1948), is employed for 6–8-mm shoot tips and stem disks (Tables Arnd-1 and Arnd-2). A modified version of the same medium is used for 1–2-mm shoot tips (Tables Arnd-1 and Arnd-3).

Procedure. Place explants on media under the appropriate culture conditions.

Developmental Sequence. Leaf development on shoot tips was invariably associated with root production. When leaves did not develop, roots were not formed.

General Comments. Since seedlings are the tissue source, this method does not allow for the propagation of proven plants. However, it is entirely possible that one of the media employed could be useful to culture shoot tips or stem disks from mature plants. The method could prove useful in cases where only a few seedlings of a valuable cross can be obtained from a capsule.

Clonal Propagation of *Arundina bambusifolia*

A tissue culture method for clonal propagation of *Arundina bambusifolia* was developed at the Department of Botany, University of Dakka, Bangladesh (Banik et al., 1986).

Plant Material. Shoots bearing 10–15 leaves taken from mature plants are used as a source for apical and axillary bud explants.

Surface Sterilization. After removal of the leaves to expose the apical and axillary buds, the shoots should be washed with running water, scrubbed with a mild detergent, and surface-sterilized by immersion in 10% household bleach for 15–20 min. (Domestos was used in the original research, but other household bleaches containing approximately 5% sodium hypochlorite are suitable; 10% is obtained by diluting 10 ml of bleach to 100 ml with distilled water.) The original paper also recommends using 5% calcium hypochlorite (5 gm calcium hypochlorite diluted to 100 ml with distilled water; the solution should be stirred several times and decanted or filtered before the liquid portion is used) or 0.1% mercuric chloride ($HgCl_2$, a toxic, dangerous compound to be avoided if at all possible) for an equal period. All tissues should be rinsed several times with sterile distilled water to remove traces of the sterilant.

Culture Vessels. Erlenmeyer flasks, culture tubes, and other containers are suitable.

Culture Conditions. Cultures should be maintained under 12-h photoperiods provided by fluorescent tubes and 25 ± 2°C.

Culture Medium. Modified Vacin and Went medium should be used (Table Arnd-4).

Procedure. Place explants on the medium, and culture them until PLBs form. When subcultured the PLBs produce plantlets. Remove plantlets from the flasks when they are 7–8 cm tall, pot them in sterilized vermiculite, and maintain them under high humidity for several days.

Developmental Sequence. Formation of PLBs takes place 30–40 days after the explants are placed in culture. Plantlet production from PLBs occurs 30–40 days following the subculture. The regenerated plants grow to a height of 4–5 cm in 7–8 months.

General Comments. This is a useful procedure for the propagation of an attractive orchid.

TABLE ARND-4. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of *Arundina bambusifolia* shoot tips (Banik et al., 1986)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Hormones				
8	Napthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
9	6-Furfurylaminopurine (kinetin)	0.2	20 mg l^{-1} 5% ethanol[e]	1	
	Complex additives				
10	Coconut water	200 ml	No stock	No stock	Measure
11	Tomato juice[f]	35 ml	No stock	No stock	Measure
	Sugar				
12	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
13	Water, distilled[g]	To 1000 ml			
	Solidifier				
14	Agar	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Then add other components of the medium.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 37.3 mg chelating agent (Na_2EDTA) and 27.8 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Keep refrigerated or frozen between uses.

[f]The original paper (Banik et al., 1986) states that "5% T[omato] J[uice] (70%) was used." Assuming a liquid containing 70% tomato juice, the actual amount added was probably 35 ml. To prepare tomato juice, squeeze ripe tomatoes (do not homogenize) into a container; then filter the juice to remove pulp and seeds. This is important because the seeds may contain germination inhibitors, which could have a deleterious effect on the cultures. Use only the juice. Store unused juice in a freezer.

[g]Add items 1 and 3–11 to the 500 ml distilled water (item 13) that contains item 2. Set pH to 5.3, add sugar (item 12), and adjust volume to 1000 ml with distilled water (item 13). Bring solution to a gentle boil, and add agar (item 14) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. Sterilize solution by autoclaving, add hormones (items 8 and 9) under sterile conditions to the warm, still liquid solution, and dispense medium into preautoclaved culture vessels.

Micropropagation and Artificial Seed Technology of *Arundina graminifolia*

An endangered orchid species, *Arundina graminifolia* (Don) Hoechr. (*Arundina bambusifolia* Lindl.), is found in China, India, the Himalayas, Malaysia, Myanmar, the Philippines, and Sri Lanka. Micropropagation and artificial seed production methods for this species were developed at the Molecular Cytogenetics and Tissue Culture Laboratory, Department of Botany, North Bengal University, India and the Department of Botany, University College, Raiganj, India (Kanjilal et al., 2000, pers.

comm.; I thank Professors K. B. Data and B. Kanjilal for providing me with a reprint, unpublished data, and photographs as well as permission to use them).

Plant Material. Seeds for the production of protocorms utilized in the artificial seed research were from mature undehisced capsules. Plantlets which served as sources of explants were grown from the same seeds. Protocorms, 20–30 days old, were used for the production of artificial seeds. The thin sections employed for tissue culture experiments were taken from seedlings raised in vitro.

Surface Sterilization. Undehisced capsules should be washed with a mild detergent under running water before being surface-sterilized with "3% [v/v] sodium hypochlorite solution" [this is presumably 57 ml household bleach which contains 5.25% sodium hypochlorite (or a different volume if the bleach contain less or more sodium hypochlorite) brought up to 1000 ml with distilled water], washed with sterile distilled water, and blotted dry with sterilized filter paper. There is no need to surface-sterilize in-vitro-grown protocorms and seedlings.

Culture Vessels. Erlenmeyer flasks can be used for seed germination, seedling and plantlet growth, and regeneration from encapsulated protocorms. Test tubes are suitable for explant culture. Petri dishes, test tubes, and Erlenmeyer flasks were used in the original research for plantlet regeneration from artificial seeds.

Culture Conditions. Cultures of seeds, seedlings, explants, plantlets, and artificial seeds (i.e., encapsulated protocorms) should be matained at $25 \pm 2°C$ and 16-h photoperiods of 1000 lx provided by cool white tubes. Standard culture room conditions are also suitable.

Culture Media. Vacin and Went medium supplemented with 15% coconut water (Table Arnd-5) is suitable for germination of natural and artificial seeds as well explant culture. Additions of 0.5 mg NAA l^{-1} or 1 mg IAA l^{-1} can increase the production of PLBs on explants. However, it is better not to force excessive proliferation through the use of these auxins or by any means because this can bring about undesirable mutations, malformed flowers, and uneven inflorescences.

Procedure. After splitting the sterilized seed capsules horizontally, the seed should be removed with a sterile spatula or knife and placed on the culture medium (Table Arnd-5). Few details are given about the excision of explants beyond "thin sections of stems of *in vitro* raised plants of *A. graminifolia* when cultured . . . produce . . . PLBs." It is safe to assume that the shoot tips are sectioned and the sections are placed on the culture medium (Table Arnd-5).

To prepare artificial seeds, 20–30-day-old protocorms (Fig. Arnd-1F) are mixed with 3% alginate solution, which also contains 4 mg Bavastin l^{-1} and 20 mg sodium bicarbonate l^{-1}, and then dropped in an autoclaved 100-m*M* solution of $CaCl_2$ l^{-1}. Calcium alginate beads (Fig. Arnd-1G) form 10–15 min after the mixture is placed on an 80-rpm shaker. The beads should be washed with sterile distilled water to remove excess $CaCl_2$. Encapsulated PLBs resume growth when placed on culture medium or soil (Fig. Arnd-1H–J).

TABLE ARND-5. **Vacin and Went medium (Vacin and Went, 1949) modified for seed germination, and the culture of thin stem sections and encapsulated protocorms of *Arundina graminifolia* (Kanjilal et al., 2000 and unpublished)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monosobsic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additive				
9	Coconut water[e]	150.0 ml	No stock	No stock	Measure
	Solvent				
10	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve. Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original recipe of this medium calls for 28 mg ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, l^{-1} but chelated iron as suggested here is preferable. A difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible.

[e]The correct term for the liquid endosperm of coconuts is coconut water (CW), not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Filtered CW must be stored in a freezer until it is used. Canned or commercially frozen CW must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Add items 1–7 and 9 to 500–700 ml of distilled water (item 10), adjust pH to 5.2, add sugar (item 8), and raise volume to 1000 ml with distilled water (item 10). Pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is unclear whether the addition of such nutrients would be of benefit. If incorporation of additional microelements becomes necessary or desirable, the formulation used as part of the Murashige–Skoog medium will probably be suitable.

Developmental Sequence. Seeds germinate and produce seedlings when placed on culture medium. Stem sections swell and produce plantlets (Fig. Arnd-1A) after being placed in culture. Plantlets develop from encapsulated PLBs (Fig. Arnd-1H–J). These plants continue to develop normally when moved to a potting mix (Fig. Arnd-1K).

General Comments. The seed germination, tissue culture, and encapsulation methods for *A. graminifolia* which are described here (Kanjilal et al., 2000) provide effective means for sexual and clonal propagation of this species and should serve as models for other orchids.

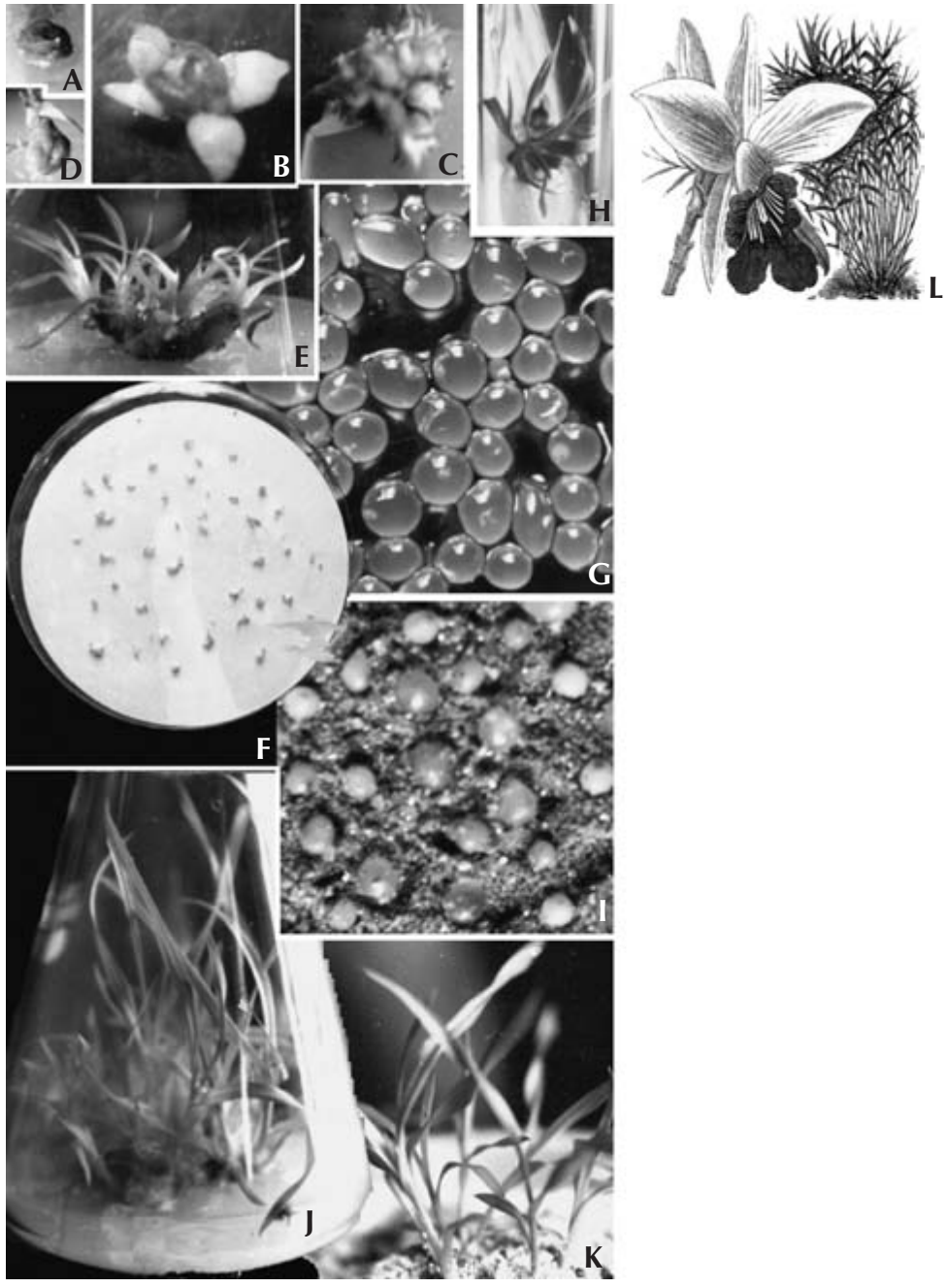


FIG. ARND-1. ***Arundina graminifolia*** **and its propagation. A. Stem disk expanding on culture medium. B, C. Formation of multiple protocorm-like bodies (PLBs) on a stem disk. D. Single PLB developing into a plantlet. E. Multiple shoots forming from a PLB cluster. F. Protocorms selected for encapsulation. G. Encapsulated protocorms. H. Plantlet arising from encapsulated protocorms. I. Growth of encapsulated protocorms on soil. J. Plantlets produced in vitro from encapsulated protocorms. K. Plantlets produced from encapsulated protocorms and growing in a pot. L.** ***Arundina graminifolia*** **flower and plant. (Sources: A–K, courtesy B. Kanjilal, K. B. Datta, and D. De Sarker; L, Williams and Williams, 1894.)**

Ascocenda

The first cross of this hybrid genus *Ascocenda* Portia Doolittle [*Ascocentrum* (*Saccolabium*) *curvifolium* × *Vanda lamellata*] was registered in 1949. *Ascocenda* Chryse (*Ascocentrum miniatum* × *Vanda lamellata*) was registered in 1951. A hybrid registered as *Ascocenda* Meda Arnold [*Ascocentrum* (*Saccolabium*) *curvifolium* × *Vanda* Rothschildiana] in 1950 is presently the trigeneric cross *Schlechterara* Meda Arnold (Garay and Sweet, 1974) since *Vanda* Rotschildiana (originally listed as being of the parentage *Vanda coerulea* × *Vanda sanderiana*) is now considered to be the bigeneric hybrid *Vandanthe* Rotschildiana (*Vanda coerulea* × *Euanthe sanderiana*). In some of the horticultural literature and Sander's List of Orchid Hybrids, *Schlechterara* Meda Arnold is still being referred to as *Ascocenda* Meda Arnold. One paper reports that it can be cultured by a method that is also suitable for other *Ascocenda* clones and a species of *Vanda*. For this reason the culture of *Schlechterara* and *Ascocenda* will be treated together under discussion of the latter.

Propagation of *Ascocenda* from Leaf Tissue

Monopodial orchids are generally more difficult to culture in vitro than sympodial ones. For this reason an attempt was made at the Singapore Botanic Gardens to culture leaf tissue of the hybrid *Ascocenda* Hilo Rose (*Vanda* Hilo Queen × *Ascocenda* Meda Arnold) × *Vanda* Miss Joaquim (*Vanda hookeriana* × *Vanda teres*, the National Flower of Singapore) listed in this case under its synonym, *Vanda* Josephine (Fu, 1978, 1979*b*). A check of Sander's List of Orchid Hybrids for 1971–1975, 1976–1980, and 1981–1985 does not show such a hybrid. The list for 1971–1975 includes the hybrid *Ascocenda* Heah Hock Heng, which was registered from Singapore in 1971. Its parentage is *Ascocenda* Hilo Rose × *Vanda* Josephine van Brero (*Vanda insignis* × *Vanda teres*). *Vanda* Josephine van Brero was registered from Indonesia in 1936 by J. van Brero.

Plant Material. Whole young leaves, 1–3 cm long and 0.5–1 cm wide, from mature plants are cultured.

Surface Sterilization. The leaves are immersed in 10% Clorox (10 ml Clorox diluted to 100 ml with distilled water) for 15–20 min and then rinsed two to three times with sterile distilled water.

Culture Vessels. Erlenmeyer flasks, culture tubes, and other containers can be used.

Culture Conditions. Cultures should be maintained on a reciprocal shaker 16 h per day (and turned off for the other 8 h), under continuous illumination (which in the original research was provided by a Sylvania Gro Lux 30-W tube, but other sources are also suitable) and a temperature of 28°C.

Culture Media. A liquid modified MS medium is used for initial culture and proliferation (see Table Aranda-4). Once formed, the PLBs should be transferred to a

second medium (see Table Aranda-5). A third medium, a modification (see Table Aranda-6) of the Knudson C solution (see Tables Aranda-7 and Aranda-8) with or without 100 g banana l^{-1}, should prove suitable for subsequent regeneration of plantlets.

Procedure. Remove young leaves from mature plants, surface-sterilize them, preferably after that wash them with sterile distilled water, and culture them in liquid medium (see Table Aranda-4) until the tissues proliferate and PLBs form. Transfer these bodies to the second medium (see Table Aranda-6) for further growth and to a third solution (see Tables Aranda-6 to Aranda-8) for plantlet regeneration.

Developmental sequence. The leaves and especially their bases proliferate and form PLBs. These proliferate further and on being transferred to an appropriate medium produce plantlets.

General comments. Clonal propagation from leaf tissue offers two important advantages. One is the fact that removal of explants does not endanger the source plant. The second is availability of a large number of explants. This should eliminate the need for extensive proliferation, which leads to undesirable variants.

Propagation of *Ascocenda* through Shoot Meristem Culture

Shoot-tip meristems of *Schlechterara* (*Ascocenda*) Tan Chai Beng [*Schlechterara* (*Ascocenda*) Meda Arnold × *Vandanthe* (*Vanda*) Rothschildiana], *Schlechterara* (*Ascocenda*) Meda Arnold, and *Schlechterara* (*Ascocenda*) Carnival [*Ascocentrum curvifolium* × *Vandanthe* (*Vanda*) Rothschildiana]

> "were cultured successfully on medium RM-1962 containing 0.1 parts per million of NAA [naphthaleneacetic acid]. . . . The cultures [also] grew well . . . on Hyponex [3 g l^{-1}] medium containing 2 g l^{-1} Trypton. . . . [But in the case of medium RM-1962 the cultures were inhibited even at a low Trypton levels.] Optimal level of sucrose was 2% [but] development of shoots was accelerated at lower levels. . . . Cultures grew better on liquid medium than on solid medium . . . [and] pH 7. . . ." (Ichihashi, 1979)

The quote and information presented above are taken from the summary of a paper in Japanese. Additional information gleaned from the paper is that the cultures should be maintained under an illumination of 700 lx at 25°C. Several illustrations in the paper indicate that this procedure is an effective one.

Clonal Propagation of *Ascocenda* from Bud Explants

Two unregistered *Ascocenda* hybrids were propagated from bud explants at the Singapore Botanic Gardens by the methods used for *Arachnis* (Lim-Ho, 1981). The explants are cultured first in a liquid modification of the Vacin and Went medium (see Table Arach-3). When callus and/or PLBs form, they should be transferred to a solid version of the same medium (see Table Arach-3). Two other modifications of the Vacin and Went medium (see Tables Arach-4 and Arach-5) are used for

proliferation and differentiation. Plantlet formation occurs on a fourth modification (see Table Arach-6). Plantlet growth is slow, and differentiation of one hybrid was better on a modified MS medium (see Table Aranda-9). Explant growth is also slow.

Clonal Propagation of *Ascocenda* from Whole Leaves

Whole young leaves of *Ascocenda* Heah Hock Heng have been cultured at the Singapore Botanic Gardens by the method used for *Aranda* leaves (Lim-Ho, 1981). Explants are first cultured in a liquid modification of the MS medium (see Table Aranda-4). Two modifications of the Vacin and Went medium can be used for multiplication and differentiation (see Tables Arach-4 and Arach-5). A third modification of the same medium (see Table Arach-6) is suitable for plantlet formation. Growth of the explants is fast.

Rapid Clonal Propagation of *Ascocenda* through the Culture of Leaf Explants

As with other monopodial orchids, *Ascocenda* has only one shoot tip, which must be excised in attempts to culture excised apices. This means that the donor plant (often an expensive one-of-a-kind hybrid) itself may be endangered or even lost. Methods that utilize explants "whose excision does not jeopardize the survival of the mother plant, [are] thus desirable" (Vij and Kaur, 1999). One such method was developed at the Orchid Laboratory, Department of Botany, Panjab University, Chandigarh-160 014, India by its long-time leader Professor Suraj Prakash Vij and one of his associates (Vij and Kaur, 1999).

Plant Material. In the original research 5-mm explants were taken from mature leaves (10–30 mm long) or those of 25-week-old plantlets obtained in vitro through the culture of flower stalks.

Surface Sterilization. Explants from in-vitro-grown plants do not require surface sterilization. The surface sterilization process for leaves from greenhouse-grown plants involves four steps. Step 1: immersion in 70% ethanol (74 ml 95% ethanol diluted to 100 ml with distilled water) for 10 s. Step 2: soaking in 0.1% streptomycin (100 mg streptomycin sulfate dissolved in 100 ml distilled water) for 30 min. Step 3: mild agitation in 7% calcium hypochlorite (suspend 7 g calcium hypochlorite in 100 ml distilled water, stir or shake well, allow precipitate to settle, and repeat the stirring and settling several times before decanting the yellowish supernatant for use as a sterilant that must be used within a few hours of preparation because it can lose its potency) for 15 min. Step 4: wash three times with sterile distilled water to remove traces of the sterilants.

Culture Vessels. Erlenmeyer flasks (50–250-ml capacity), test tubes, or other containers are suitable. They should be filled with culture medium to approximately one-fifth of their capacity

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx [the illumination source(s) is/are not listed in the original paper, but is it is reasonable to assume that cool white fluorescent tubes alone or mixed with incandescent bulbs, or plant growth lights such as Gro Lux are suitable]. Standard culture room conditions are probably also suitable.

Culture Media. The MPR (Mitra, Prasad, and Roychowdhury) medium (Mitra et al., 1976) supplemented with 1 mg BA l^{-1} (Table Ascda-1) should be used to bring about the formation of PLBs by explants. Each explant can produce nearly 60 plantlets with 2–3 leaves and 2–4 roots in 20 weeks on this medium. According to the original paper these plantlets "were gradually hardened *in vitro* by sequential removal of growth adjuvants, vitamins and minor and major salts from the nutrient mix" (Vij and Kaur, 1999). No details are given about this hardening process. However, it is safe to assume that one or more of the media used in other methods for the culture of *Ascofinetia* leaf sections (see Tables Arach-4 to Arach-6, Aranda-4 to Aranda-8) or other explants (see Tables Arach-3 to Arach-6) will prove suitable.

Procedure. Explants should be cultured on the modified MPR medium (Table Ascda-1) for about 20 weeks or until they develop 2–3 leaves and an equal number of roots. After that they should be grown on one of the other media (see Tables Arach-3 to Arach-6, Aranda-4 to Aranda-8). Following further development, the plants should be potted in 6-cm-diameter clay pots in a potting mix consisting of charcoal chips, pine bark, and brick pieces (in the ratio 1 : 1 : 1, v/v/v) topped with moss. The potted plants should be mist irrigated and hardened for 4–6 weeks in a humidity chamber. After that they can be repotted in 15-cm-diameter clay pots and grown outdoors.

Developmental Sequence. PLBs form on the explants on the first medium (Table Ascda-1) and develop into plantlets. These plantlets harden on the second medium (see one of the following: Tables Arach-3 to Arach-6, Aranda-4 to Aranda-8) and grow to maturity after being transferred to pots.

General Comments. This procedure is easy to use and makes possible the propagation of a monopodial orchid from leaves without endangering the donor plant.

***Ascocenda* Fiftieth State Beauty**

TABLE ASCDA-1. **Mitra, Prassad, and Roychowdry medium (Mitra et al., 1976) as modified for the culture of leaf explants of *Ascocenda* Fiftieth State Beauty (Vij and Kaur, 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Cytokinin				
14	Benzylaminopurine	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Agar[f]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

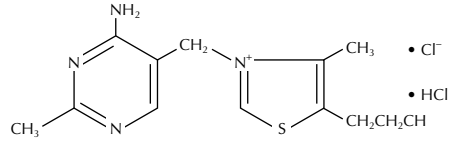
[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original recipe calls for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses. If the cytokinin fails to dissolve add a few drops of 0.1N HCl to the stock solution.

[f]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred (agar is not used in liquid media). When the agar is completely dissolved pour the solution into a 2-l Erlenmeyer flask and autoclave. As a rule, media which contain vitamins, hormones, and/or other heat labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).



Thiamine/vitamin B_1

Ascocentrum

Ascocentrum as a genus was segregated from *Saccolabium* by the German orchid taxonomist Rudolph Schlechter (Grove, 1995). The plants are small epiphytes which may have strap or semi-terete leaves. Flowers are brightly colored, small, resemble miniature *Vanda* blossoms, and are arranged tightly on the racemes (Grove, 1995). *Ascocentrum* species have been crossed with *Vanda* to produce the hybrid genus *Ascocenda* which combines some of the best characteristics of the parent genera and produces spectacular flowers.

Micropropagation of *Ascocentrum ampullaceum*

Ascocentrum ampullaceum is distributed from the Himalaya hills in India eastward to Burma, the northwestern mountains of Thailand, Laos, and southern Yunnan in China (Grove, 1995). The micropropagation procedure described here is the result of research on the effects of pH and micronutrients on the growth of PLBs (Wannakrairoj and Tanyasonti, 1996).

Plant Material. PLBs obtained from axillary bud cultures (by a method which was not described) were used in the original research.

Surface Sterilization. No surface sterilization is needed for PLBs taken from in vitro cultures.

Culture Vessels. Erlenmeyer flasks, of 50-ml capacity, containing 20 ml of medium are suitable.

Culture Conditions. Cultures should be placed on 120 rpm orbital shakers at $26 \pm 2°C$ under continuous illumination of 25 μmol m^{-2} s^{-1}.

Culture Media. Explants should be cultured in liquid Vacin and Went medium (Vacin and Went, 1949) containing the micronutrients of the MS solution (Murashige and Skoog, 1962) [a mixture referred to as the WS (perhaps standing for the first author's initials with the initial of the last name first) medium], 15% (v/v) coconut water (CW), 50 μmol chelated iron (Fe_2EDTA), 25 μmol manganese, and 0.075 μmol copper (Table Asctm-1). This "formula, [is] currently called 'KU [presumably referring to the authors' institution, Kasetsart University] medium'."

Procedure. Protocorms from in vitro cultures are sectioned and placed in the liquid culture medium (Table Asctm-1).

Developmental Sequence. The fresh weight of PLBs increased, but the original paper does not provide further details.

General Comments. This procedure was used to study the effects of pH and several micronutrients on PLB development. The PLBs used in this research were taken

TABLE ASCTM-1. **Vacin and Went (VW) medium (Vacin and Went, 1949) with the microelements of the Murashige–Skoog (MS) solution (Murashige and Skoog, 1962) as modified for the culture of *Ascocentrum ampullaceum* protocorm-like bodies, and referred to as the WS medium (Wannakrairoj and Tanyasonti, 1996)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monosobsic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	40.0	4.0 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	28.0	2.8 g l^{-1}		
7	**Microelements**[e]				
(a)	Boric acid, H_3BO_3	6.2	62.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	16.9	1.69 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Complex additive				
8	Coconut water[f]	150.0 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve. Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference between the amount suggested here and that in the original formulation is negligible.

[e]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[f]The correct term for the liquid endosperm of coconuts is coconut water (CW), not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through Whatman No. 1 filter before it is added to culture media. Filtered CW must be stored in a freezer until it is used. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[g]Add items 1–8 to 500–700 ml of distilled water (item 10), adjust pH to 5.6–5.8, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Pour the solution into culture vessels and autoclave.

from in vitro cultures of axillary buds (using a method which was not described). Since the original cultures already produced PLBs, another procedure does not seem to be necessary unless there was no growth and/or differentiation on the medium used to culture the axillary buds.

Micropropagation of *Ascocentrum* through the Culture of Flower Bud Explants

The methods used for *Vandofinetia* (Kishi et al., 1997*a*, 1997*b*) can also be used for *Ascocentrum*.

Origin of the term protocorm

An incorrect but widespread and oft repeated (for one example see Cribb, 1999) assertion is that the term *protocorm* was coined by Noël Bernard (1874–1911), the French botanist (left) who discovered the role of mycorrhiza in orchid seed germination. Actually the term was proposed in 1890 (9 years before Bernard made his discovery) by Melchior Treub (1851–1910), the Dutch botanist (right) who was the long time director of the famed Bogor Botanical Gardens, to describe an early stage in the germination of Lycopods.

Ascofinetia

Ascofinetia is a hybrid genus involving *Ascocentrum curvifolium, Ascocentrum miniatum, Ascocentrum pumilum, Ascocentrum ampullaceum*, or *Ascocentrum* hybrids and *Neofinetia falcata*. It is a monopodial orchid, so removal of apical buds may cause the loss of a plant. Therefore a tissue culture procedure was developed for young inflorescences (Intuwong and Sagawa, 1973; Khaw et al., 1978*a*, 1978*b*; Sagawa and Kunisaki, 1982).

Plant Material. Inflorescences, 1.5 cm long or less, with flower primordia are used. Those 3 cm or longer elongated and produced flowers that either aborted and turned brown or developed normally and opened (Table Ascf-1).

Surface Sterilization. Inflorescences are removed and sterilized for 10 min with 100 ml of 10% Clorox plus one drop of Tween 20. The bracts are then removed and the inflorescences sterilized again, this time with 100 ml of 5% Clorox (5 ml Clorox diluted to 100 ml with distilled water) plus one drop of Tween 20. This is followed by a 3-min rinse in sterile distilled water.

Culture Vessels. Use 125- or 250-ml Erlenmeyer flasks containing 25 or 50 ml medium, respectively.

Culture Conditions. Maintain cultures at 26 ± 3°C with continuous illumination of about 200 ft-c provided by General Electric Power Groove white fluorescent lamps. Liquid cultures should be agitated at approximately 160 rpm (a New Brunswick Scientific Co. Model V shaker was used in the original research).

Culture Media. Vacin and Went liquid medium (VW; Vacin and Went, 1949) containing 20 g sucrose (Su) and 15% coconut water (CW) is used for the initial culture (VW + Su + CW; Table Ascf-1). The medium will become turbid-brown within 10–14 days and inhibit the growth of PLBs. Therefore the medium must be changed every 10–14 days. Even when the medium is changed regularly the PLBs will turn yellow if left in this formulation. To ensure the survival of PLBs and cause them to proliferate, they must be transferred to sugar-free liquid medium [Table Ascf-1 (VW – Su) + CW]. For differentiation of plantlets, the PLBs are transferred onto potato

TABLE ASCF-1. **Effects of four culture media on inflorescence explants and tissues of *Ascofinetia, Neostylis,* and *Vascostylis* (Intuwong and Sagawa, 1973)**

Culture medium[a]	Inflorescence, ≤ 1.5 cm long	Inflorescence, ≤ 3 cm long	Protocorm-like bodies	Plantlets
VW + Su + CW	Proliferation	Flowering in vitro or aborted flowers	Yellow color	
(VW – Su) + CW			Multiplication	
(VW – Su) + CW + PE + GB			Differentiation into plantlets	
(VW – Su) + CW + PE + RB				Maintenance of growth

[a]CW, coconut water; GB, 100 g green banana homogenate; PE, liquid obtained after boiling 100 g freshly diced potatoes in 200 ml distilled water for 5 min; RB, 100 g ripe banana homogenate; Su, sucrose; VW, Vacin and Went medium (Vacin and Went, 1949).

TABLE ASCF-2. **Modified Vacin and Went medium for the culture of *Ascofinetia* inflorescences (Intuwong and Sagawa, 1973)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Tricalcium phosphate, $Ca_3(PO_4)_2$[b]	200	20 g l^{-1}	10	Or weigh
2	Potassium nitrate, KNO_3[c]	525	52.5 g l^{-1}	10	Or weigh
3	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
4	Ammonium sulfate, $(NH_4)SO_4$[c]	500	50 g l^{-1}	10	Or weigh
5	Ferric tartrate, $Fe_2(C_4H_4O_6)_3$[d]	28	2.8 g l^{-1}	10	
6	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additives				
8	Coconut water (CW)	150 ml	No stock	No stock	Fresh
9	Potato extract (PE)[e]	200 ml	No stock	No stock	
10	Ripe banana (RB)[f], or green banana (GB)[f]	100 g	No stock	No stock	Weigh
	Sugar				
11	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[g]	To 1000 ml			
	Solidifier[g]				
13	Agar	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]If salt does not dissolve completely, shake stock solution well before dispensing to insure an even suspension; or better yet, weigh out each time.

[c]Solutions containing ammonium and nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between use.

[d]Ferric tartrate is relatively insoluble. Grinding it with a mortar and pestle before dissolving helps. The addition of a pellet or two of KOH to the solution will increase solubility, but a precipitate may form nevertheless. To insure equal distribution, shake stock solution well before dispensing.

[e]Liquid PE is obtained by boiling 100 g freshly diced potato (1-cm cubes) in 200 ml distilled water for 5 min.

[f]Homogenize 100 g banana (RB or GB) with 200 ml water in a blender for 30–60 s at high speed.

[g]To prepare basic medium (VW + Su), mix items 1–7 with 750 ml distilled water (item 12), adjust pH to 5.5, add sugar (item 11), and adjust volume to 1000 ml with more distilled water (item 12). If liquid medium is desired, dispense solution and autoclave. When solid medium is needed, dissolve agar (item 13) by adding it slowly while stirring to the gently boiling solution. Then dispense and autoclave. When sugar is not desired (VW – Su), omit item 11 and prepare as before. To prepare media containing RB, GB, CW, or PE, use homogenate of liquid instead of an equal volume of water. Media prepared are VW + Su + CW, (VW – Su) + CW, (VW – Su) + CW + PE + GB, and (VW – Su) + CW + PE + RB.

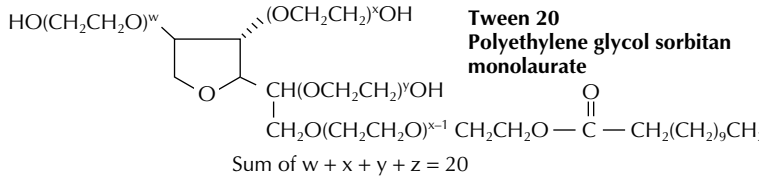
extract (PE) and green banana (GB)-containing solid medium [(VW – Su) + CW + PE + GB; Tables Ascf-1 and Ascf-2]. Growth of the plantlets is maintained on a solid medium containing ripe banana (RB), CW, and PE [(VW – Su) + CW + PE + RB; Tables Ascf-1 and Ascf-2].

Procedure. Remove inflorescences 1.5 cm long or less, sterilize, and place in liquid VW + Su + CW (Table Ascf-1). Change medium every 10–14 days or when it becomes turbid-brown. When PLBs are produced, transfer them to liquid (VW – Su) + CW (Table Ascf-1). To obtain differentiation, subculture PLBs onto solid (VW – Su) + CW + PE + GB (Table Ascf-1). Once plantlets are formed, maintain their growth on (VW – Su) + CW + PE + RB (Table Ascf-1).

Developmental Sequence. The explants proliferate in liquid VW + Su + CW (Table Ascf-1) within 20–51 days. Proliferation is acropetal. Further multiplication occurs

on liquid (VW – Su) + CW (Table Ascf-1). Differentiation takes place on solid (VW – Su) + CW + PE + GB (Table Ascf-1) and growth is maintained on (VW – Su) + CW + PE + RB (Table Ascf-1).

General Comments. This is a very effective method for clonal propagation and does not require sacrificing or even setting back a plant.



Tween 20
Polyethylene glycol sorbitan monolaurate

Barlia

As is the case with so many other orchids, there is no agreement regarding the nomenclature of this genus and its species. *Barlia longibracteata* has also been referred to as *Himantoglossum bracteatum, Orchis longibracteata, Aceras longibracteata, Loroglossum longibracteatum*, and *Barlia robertiana*. The last name in this list appears in several recent European orchid floras and will be used here.

Isolation of Protoplasts of *Barlia robertiana*

Protoplasts of *Barlia robertiana* (*Barlia longibracteata*) were isolated to "provide [a system for] the study of the [orchid] endophyte/host interactions" (Pais et al., 1982) as well as for studies of protoplast fusion (Pais et al., 1983).

Plant Material. Youngest of first leaves of 2–3-month-old plants are used.

Surface Sterilization. A procedure for the surface sterilization of the leaves is not given in the original papers. It would seem that such sterilization is desirable, but an appropriate method can be developed only with trial and error. Methods used for surface-sterilizing the leaves of *Aranda* and other orchids that can be propagated from leaf tissues are a good starting point.

Culture Conditions. There is no information regarding temperature and illumination in the original papers. However, the original reports (Pais et al., 1982, 1983) refer to standard procedures, which suggests that the conditions used for other orchid protoplasts may be suitable. The osmotic concentration of the isolation solution is given in one paper as being equivalent to a 0.6-*M* (109.3 g l^{-1}) solution of mannitol (Pais et al., 1982) and in another as 0.55-*M* (100.2 g l^{-1}) sorbitol (Pais et al., 1983).

Culture Medium. No culture medium is used. The protoplasts are obtained by placing the leaf tissues in an enzyme solution consisting of 1% cellulase (1 g per 100 ml) and 0.5% (0.5 g per 100 ml) Macerozyme (both obtainable from Cal Biochem and Fluka chemical companies) at pH 5.7 for 3 h (Pais et al., 1983) to 4 h (Pais et al., 1982). Protoplast fusion is carried out in a solution consisting of 4 g l^{-1} sodium chloride, 1 g l^{-1} sodium acetate trihydrate, 294 mg l^{-1} calcium chloride (presumably dihydrate), 63.8 g l^{-1} sorbitol, and either 50 g l^{-1} sodium alginate or 150 g l^{-1} dextran with the pH adjusted to 5.5.

Procedure. Place the leaf tissue in the enzyme solution for the required time. Isolate the protoplasts by centrifugation, and wash several times with osmoticum. To induce fusion add 0.5 ml of protoplast suspension (containing 10^8 protoplasts) of each species to 0.1 ml of fusion medium and incubate.

Developmental Sequence. Fusion occurs within approximately 30 min, but there are no reports of further development despite the fact that isolated protoplasts and even fusion product may remain alive for some time.

General Comments. Several authors have reported on the isolation and fusion of orchid protoplasts, but callus cultures and/or plants have yet to be obtained with these procedures. However, it is reasonable to assume that plantlet regeneration will become possible in the future. The methods described here are also suitable for *Ophrys bombyliflora* and *Ophrys lutea*.

Bletilla

A genus consisting of nine species; *Bletilla* is found in Asia.

Plantlet Regeneration from Root Tips of *Bletilla striata*

In Hong Kong *Bletilla striata* grows in open grasslands. It is used medicinally and therefore is overcollected. A method for plantlet regeneration from root tips was developed as part of research associated with a Ph.D. dissertation (Yam, 1989; Yam and Weatherhead, 1991*b*).

Plant Material. In the original research, root tips 1–1.5 mm in length were taken from 6-month-old seedlings. It is important to use a sharp and sterile scalpel blade. Tips should be cut in a sliding action to prevent pressure on the tissues. The seeds were germinated and the seedlings were grown on Knudson C medium supplemented with 20% (v/v) coconut water from mature nuts. They were maintained under 25 ± 2°C and 16-h photoperiods of 2000 lx provided by four 40-W Sylvania Gro Lux fluorescent tubes placed 60 cm above the cultures.

Surface Sterilization. Since the tips are taken from aseptic seedlings, there is no need to surface-sterilize them.

Culture Vessels. Wide-mouth, γ-irradiated plastic flasks of 75-ml capacity (from Johns Mallinkrodt, Johns Division, Mallinkrodt Australia, Pty., Ltd.) were used in the original research. Other containers like Erlenmeyer flasks, various bottles, and test tubes are also suitable.

Culture Conditions. In the original research the flasks were maintained under conditions of 16-h photoperiods of 2000 lx (provided by 40-W Gro Lux lamps 60 cm above the flasks) and 25 ± 2°C.

Culture Media. Explants were placed on modified half-strength MS medium (Table Ble-1). Plantlets were moved to modified Knudson C medium (Table Ble-2) 3 months after they form.

Procedure. Cut root tips and place them flat on the medium. Examine the cultures at regular intervals, and move plantlets to the second medium 3 months after their formation.

Developmental Sequence. The root tips swell and form a callus, which produces multiple shoots 7 months after the start of culture. Roots form only following transfer to the second medium.

General Comments. This method can be used with other orchids and can be employed to increase the number of plantlets in cases where only a few seedlings are available. Only a very small percentage (less than 10%) of the root tips produces plantlets.

TABLE BLE-1. **Murashige–Skoog medium (Murashige and Skoog, 1962) as modified for the culture of root tips of *Bletilla striata* (Yam, 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220	22 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950	95 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Napthaleneacetic acid (NAA)	1	100 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
11	Benzyladenine (BA)	0.2	20 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh
	Darkening agent				
18	Graphite[h]	3 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of dilute KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–15 and 18 to 900 ml distilled water (item 16), adjust pH to 5.8, and adjust volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into culture flasks and autoclave. (In general, solutions that may contain labile organic additives should not be autoclaved without first determining whether this will not have a deleterious effect; this medium can be autoclaved.) Omit agar if liquid medium is desired.

TABLE BLE-2. **Knudson C medium for the germination of orchid seeds (Knudson, 1946) as modified for the culture of root-tip-derived plantlets of *Bletilla striata* (Yam, 1989)**

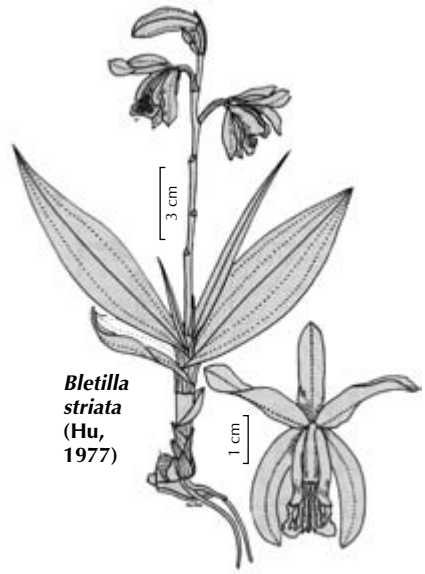
Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
	Iron[c]				
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
7	Coconut water	200			
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[d]	To 1000 ml			
	Solidifier				
10	Agar[d]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. To prepare a stock solution, dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[d]Add items 1–8 to 700 ml distilled water (item 9), adjust pH to 5.6, and adjust volume to 1000 ml with distilled water (item 9). Bring the solution to a gentle boil and add the agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into culture flasks and autoclave. Omit agar if preparing liquid medium. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be 4.6–4.7. If necessary adjust pH upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower the pH if necessary.



***Bletilla striata* (Hu, 1977)**

Plantlet Regeneration from Stem Nodes of *Bletilla striata*

Bletilla striata is very rare in Hong Kong, where it grows in open grasslands. It is used medicinally in Chinese herbal medicine, which leads to excessive collecting. A method for plantlet production from stem nodes was developed at the Botany Department, University of Hong Kong (Yam, 1989).

Plant Material. In the original research nodes, 1-cm-long stem sections with a bud in the middle were taken from seedlings 3–5 nodes long. It is important to use a sharp, sterile scalpel blade. Tips should be cut in a sliding action to prevent pressure on the tissues. The seeds were germinated and the seedlings were grown on Knudson C medium supplemented with 20% (v/v) coconut water from mature nuts. They were maintained under conditions of 25 ± 2°C and 16-h photoperiods of 2000 lx provided by four 40-W Gro Lux fluorescent tubes placed 60 cm above the cultures.

Surface Sterilization. Since the sections are taken from aseptic seedlings, there is no need to surface-sterilize them.

Culture Vessels. Wide-mouth, γ-irradiated plastic flasks of 75-ml capacity (from Johns Mallinkrodt) were used in the original research. Other containers like Erlenmeyer flasks, various bottles, and test tubes are also suitable.

Culture Conditions. In the original research the flasks were maintained under conditions of 16-h photoperiods of 2000 lx (provided by 40-W Gro Lux lamps 60 cm above the flasks) and 25 ± 2°C.

Culture Media. Explants are placed on modified Knop's medium (Table Ble-3). Three months after plantlets form, they were moved to modified Knudson C medium (Table Ble-2) for root initiation.

Procedure. Cut stem sections, free them of leaves, and place them horizontally on the medium. Examine the cultures at regular intervals, and move plantlets to the second medium 3 months after their formation.

Developmental Sequence. The buds on the stem sections swell after 2–3 weeks, and multiple shoots form after an additional 2–4 weeks.

General Comments. This method can be used with other orchids and can be employed to increase the number of plantlets in cases where only a few seedlings are available. The success rate is very high.

TABLE BLE-3. **Knop's medium (Knop, 1884) modified for the culture of *Dendrobium* stem nodes and used to culture stem nodes of *Bletilla striata* (Yam, 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2$[b,c]	500	50 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4$[c]	125	12.5 g l^{-1}	10	
3	Monopotassium phosphate, KH_2PO_4	125	12.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	125	12.5 g l^{-1}	10	
	Iron				
5	Ferric citrate[d]	10	1 g l^{-1}	10	
6	**Microelements**				
(a)	Boric acid, H_3BO_3	0.056	56 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2$	0.02	20 mg l^{-1}		
(c)	Copper chloride, $CuCl_2 \cdot 2H_2O$	0.054	54 mg l^{-1}		
(d)	Ferric cloride, $FeCl_3 \cdot 6H_2O$	0.5	500 mg l^{-1}		
(e)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.036	36 mg l^{-1}	1	One solution
(f)	Sodium EDTA, Na_2EDTA	0.8	800 mg l^{-1}		
(g)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2 2O$	0.025	25 mg l^{-1}		
(h)	Zinc chloride, $ZnCl_2$	0.152	152 mg l^{-1}		
	Polyol				
7	*myo*-inositol	100	No stock	No stock	Weigh
	Vitamin				
8	Thiamine·HCl	0.4	40 mg 100 ml^{-1} 95% ethanol	1	
	Anti-auxin				
9	*trans*-cinnamic acid (tCA)	70	7 g 100 ml^{-1} 95% ethanol	1	
	Sugar				
10	Sucrose[e]	30 g	No stock	No stock	Weigh
	Solvent				
11	Distilled water[e,f]	To 1000 ml			
	Solidifier				
12	Agar[e]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The number of waters of hydration is not given.

[d]This compound may not dissolve easily but can be solubilized by adding a few drops of dilute KOH. It is preferable to chelate the iron. Use 37.3 mg chelating agent (Na_2EDTA) and 27.8 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$). To prepare a stock solution add 3.73 g l^{-1} of chelating agent and 2.78 g l^{-1} of iron salt. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]As originally formulated the solution does not contain sugar or agar.

[f]Add items 1–9 to 900 ml distilled water (item 11), adjust pH to 5.6, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring solution to a gentle boil, and add agar (item 12) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into culture vessels and autoclave.

Micropropagation of *Bletilla striata* through the Culture of Young Pseudobul Segments

A native to China and Japan, *Bletilla striata* is a terrestrial orchid that produces attractive flowers ranging from white (rarely) through rose-pink to purple (Hu, 1977; Bechtel et al., 1981, 1986, 1992; Vij and Dhiman, 1997). This species was discovered in Japan by Carl Thunberg who described it as *Limodorum striatum* in his *Flora Japonica* in 1784 (Bechtel et al., 1981, 1986, 1992). Heinrich Gustav Reichenbach (Reichenbach *f*, 1823–1889) transferred it to the genus *Bletilla* in 1878 as *Bletilla striata* (Bechtel et al., 1981, 1986, 1992). Like many other orchids, *B. striata* was kicked around by taxonomists during the last two centuries and as a result it suffers from an overabundance of synonyms including *Bletia gebina*, *Bletia striata*, *Bletilla gebina*, *Cymbidium hyacinthinum*, *Cymbidium striatum*, *Epidendrum tuberosum*, *Guyas humilis*, and *Jimensia striata*. There is no telling how many additional synonyms the future holds.

In addition to being an ornamental plant *B. striata* is used as a herb to treat gastrointestinal problems, malaria, respiratory ailments, and maglinancy, and as an embolizing agent (Lawler, 1984; Vij and Dhiman, 1997). These multiple uses have lead to overcollection of the species. A micropropagation method was developed at the Orchid Laboratory, Department of Botany, Pamjab University, Chandigarh to alleviate the pressure on native populations (Vij and Dhiman, 1997).

Plant Material. Pseudobulbs (5–7.5 mm in size) of asymbiotically produced seedlings were sectioned into single node segments (Fig. Ble-1).

Surface Sterilization. Explants are taken from in vitro cultures and do not require surface sterilization.

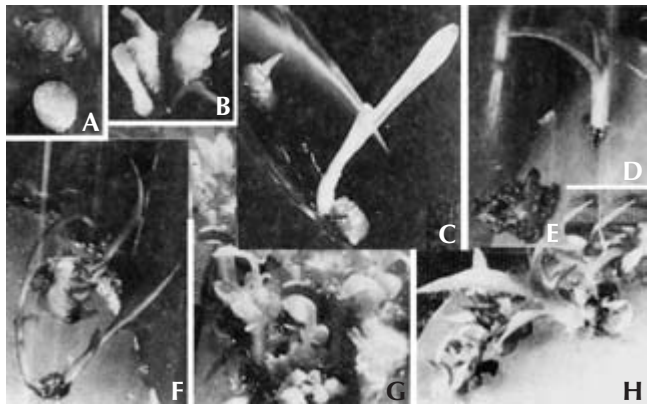


FIG. BLE-1. **Culture *of Bletilla striata* explants. A. Explant in culture. B. Shoot and root formation. C. Shoot initiation. D. Plantlet. E. Callus. F. Formation of multiple shoots. G, H. Plantlet formation. (Source: Vij and Dhiman, 1997.)**

Culture Vessels. Photographs in the original paper (Fig. Ble-1D–H) indicate that culture tubes were used. They should be filled with medium to approximately 20% of their volume.

Culture Conditions. Standard laboratory conditions or 25 ± 2°C under 12-h photoperiods of 3500 lx are suitable.

Culture Media. Seeds were germinated on Ichihashi medium (Ichihashi, 1989) supplemented with 1 mg casein hydrolysate l^{-1} (the same medium as the one in Table Ble-4, but with 20 g sucrose l^{-1} and 10 g agar l^{-1} rather than glucose and 8.5 g agar l^{-1}as in the modification; the recipe is not given here in detail because this book does not deal with seed germination). Explants should be cultured on a modified Ichihashi medium (Ichihashi, 1989), which contains BA, glucose instead of sucrose, and less agar than in the original formulation (Table Ble-4). Roots are formed when plantlets produced on the first medium (Table Ble-4) are moved to a NAA-containing modification of the Ichihashi solution (Table Ble-5).

Procedure. Single node segments are taken from pseudobulbs of young seedlings and placed on the first medium (Table Ble-4). Plantlets from this medium should be moved to the second solution (Table Ble-5) for development. Well-developed plants can be potted in a potting mix.

Developmental Sequence. Callus-mediated PLBs form within 4 weeks on modified Ichihashi medium containing 2 mg BA l^{-1} (Table Ble-4). Shoots develop on this medium within an additional 2 weeks (i.e., a total of 6 weeks after the start of culture). These shoots do not develop roots. Rhizogenesis occurs only on a medium that contains 1 mg BA l^{-1} and an equal amount of NAA (Table Ble-5).

General Comments. This method can be very effective as a means of increasing the number of seedlings. It can not be used to propagate outstanding cultivars because the quality of seedlings is not known.

TABLE BLE-4. **Modified Ichihashi (MI) medium (Ichihashi, 1989) as used for the culture of *Bletilla striata* sections of young pseudobulbs (Vij and Dhiman, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Monoammonium phosphate, $NH_4H_2PO_4$[b]	460.0	46.0 g l^{-1}	10	
2	Potassium nitrate, KNO_3[b]	808.0	80.8 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	708.5	70.85 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	246.5	24.65 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
6	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	62.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
7	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Gibberellin				
9	Gibberellic acid (GA_3)	3.5 µg	0.350 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
10	Benzylaminopurine	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Glucose	15.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar	8.5 g	Agar[g]	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the gibberellin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH 5.1 ± 1, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave.

TABLE BLE-5. **Modified Ichihashi (MI) medium (Ichihashi, 1989) as used for the culture of *Bletilla striata* plantlets obtained from sections of young pseudobulbs (Vij and Dhiman, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Monoammonium phosphate, $NH_4H_2PO_4$[b]	460.0	46.0 g l^{-1}	10	
2	Potassium nitrate, KNO_3[b]	808.0	80.8 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	708.5	70.85 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	246.5	24.65 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
6	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	62.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
7	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
9	Naphthaleneleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
10	Benzylaminopurine	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Gibberellin				
11	Gibberellic acid (GA_3)	3.5 µg	0.350 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Glucose	15.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar	8.5 g	Agar[g]	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the gibberellin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH as required, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 7), hormones (items 9–11) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

Brassavola

Protoplasts were isolated from leaves of *Brassavola nodosa* using the procedures developed for *Acampe praemorsa*. The yield was 3.6×10^4 protoplasts per gram of fresh weight of tissue.

Brassia

A South American genus, *Brassia*, is named after William Brass, a botanical illustrator who collected plants in Guinea and South Africa around 1790 for Sir Joseph Banks. Several species in this genus have been used as parents for intergeneric hybrids involving one to five other genera. For this reason alone a useful method for the isolation of protoplasts would be valuable since it could be used in fusion experiments involving the genera employed in sexual hybridization. A method for the preparation of *Brassia* protoplasts was developed as a step in the isolation of orchid nuclei (Capesius and Meyer, 1977).

Plant Material. Virus-free protocorms of *Brassia maculata* grown in liquid medium at 25°C and continuous illumination were used in the original work. The protocorms were sliced into thin disks for the purpose of protoplast isolation.

Surface Sterilization. It is not necessary to surface-sterilize the protocorms since they are grown under sterile conditions. However, the culture medium must be washed off, first with sterile distilled water and then with a sterile solution consisting of 10 mg l^{-1} magnesium sulfate ($MgSO_4 \cdot 7H_2O$) and 25 mg l^{-1} potassium chloride (KCl).

Culture Vessels. The tissue and protoplasts were not cultured in the original research. If attempting to culture the protoplasts use small Petri dishes or multiple-well plates. Erlenmeyer flasks, Petri dishes, tubes, or beakers can be used for the isolation procedure. Use centrifuge tubes for centrifugation.

Culture Conditions. The original investigators made no attempt to culture the protoplasts since their aim was to isolate nuclei.

Culture Media. A culture medium was not formulated since the protoplasts were not cultured. The tissues are plasmolysed in the sterile $MgSO_4$–KCl solution and pretreated in 0.1% (0.1 g per 100 ml) Pectinol fest (from Fa. Röhm, Darmstadt, Germany). Protoplast separation occurs in the $MgSO_4$–KCl solution containing 1% (1 g per 100 ml) cellulase (probably also from Röhm, but other companies like CalBiochem or Sigma Chemical Co. sell this enzyme too) and 0.3% Pectinol fest (300 mg per 100 ml), with a pH of 5.8. The protoplasts were washed with the sterile $MgSO_4$–KCl solution.

Procedure. Plasmolyse the tissues for 30 min and pretreat them for 1 h. They should be subjected to the protoplast separation solution for 3–4 h at room temperature

(22°C) with occasional gentle manual shaking. Collect the protoplasts by low-speed centrifugation (50× *g*) for 3 min, and wash three times.

Developmental Sequence. The protoplasts were not cultured.

General Comments. With this procedure the protoplast yield is high. Protoplasts from protocorms tend to fuse. It is possible therefore that this procedure may be useful in attempts to develop protoplast-based propagation and bioengineering methods for orchids. This method was also used to isolate protoplasts from leaves of *Cymbidium* Ceres (*Cymbidium i'ansonii* × *Cymbidium insigne*), protocorms of *Cymbidium pumilum*, and *Cattleya*. (The last genus is not clear since the paper lists *Cattleya schombocattleya* as one of the plants; this could have been a *Cattleya* species, a *Schombocattleya*, or a hybrid between the two.)

Brassocattleya

Orchids are unusual in that it is easily possible to make intergeneric crosses within the family. Some of the earliest crosses were made between *Brassavola* and *Cattleya* at the famed Veitch and Sons establishment in the UK. The resulting hybrid genus is *Brassocattleya*.

Micropropagation of *Brassocattleya* through the Culture of Buds from Shoots and Pseudobulbs

Because of the large interest in species and hybrids of the *Cattleya* alliance, there are reports of experiments with several members of the group. One procedure was developed using *Brassocattleya* Princess Patricia as the source of explants (Kako, 1973).

Plant Material. Best results are obtained with the third bud from 15-cm-long, newly growing shoots. However, other buds also grow. When using back bulbs, it is best to remove buds that are just starting to grow.

Surface Sterilization. The procedure used for *Cattleya* (Scully, 1967) is suitable.

Culture Vessels. This information was not given. However, it seems that 50-ml Erlenmeyer flasks containing 5 ml of medium should be appropriate (Scully, 1967).

Culture Conditions. Explants are placed in liquid medium that is not shaken or rotated ["liquid standing medium" (Kako, 1973)] and maintained at 25°C under continuous illumination.

Culture Medium. Liquid modified MS medium (Table Bc-1) is used as the starting medium. A different modification, this time solid, of the same medium (Table Bc-2) is used for callus induction.

Procedure. Excise buds like those of *Cattleya* (Scully, 1967) and place them into the culture medium, which is allowed to stand (not shaken). Within a month they grow and must be subcultured by cutting them in half and transferring the sections to liquid medium for a month and subculturing again. After 3 months, transfer halves to a solid medium. Addition of 2,4-D and kinetin enhances callus formation.

When transferred to Knudson C medium (see Tables Aranda-7 and Aranda-8) the callus forms plantlets.

Developmental Sequence. Explants swell after a few days in culture. Larger cells are formed at the cut sides 2–4 weeks later, and vascular bundles develop. Active cell division can be noted among epidermal cells.

General Comments. An interesting aspect of this procedure is that the cultures need not be shaken. This reduces costs without eliminating quick proliferation. When whole PLBs instead of halves are transferred to solid medium, survival rates are better.

TABLE BC-1. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for the culture of *Brassocattleya* buds (Kako, 1973)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[h]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin does not dissolve, add a few drops of dilute KOH to solubilize it.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 15), set pH to 5.3–5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Pour solution into a 2-l flask, and autoclave. Add amino acid, auxin, and vitamins (items 8, 10–13) into the hot solution under sterile conditions with sterilized pipettes, mix well, and dispense into preautoclaved culture vessels.

TABLE BC-2. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for callus induction from buds of *Brassocattleya* (Kako, 1973)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Dichlorophenoxyacetic acid (2,4-D)	10	1000 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
11	Kinetin	10	1000 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin and/or the cytokinin do not dissolve, add a few drops of dilute KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml of distilled water (item 16), set pH to 5.3–5.5, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Either bring the solution to a gentle boil and add the agar slowly with stirring, or, add agar to cold solution and then bring it to a boil with stirring. Pour solution into a 2-l flask and autoclave. Add amino acid, hormones, and vitamins (items 8, 10–14) into hot solution under sterile conditions with sterilized pipettes, mix well, and dispense into preautoclaved culture vessels.

However, since halving doubles the number of sections, even with higher losses more calli are obtained.

Micropropagation of *Brassocattleya* through Axillary Bud Culture

Brassocattleya Pastoral flowers range from deep purple-magenta to cream tinged with purplish pink. There is also a pure white form named *Brassocattleya* Pastoral 'Innocense'. A tissue culture method for axillary buds of this hybrid was developed by Fumiaki Kishi of the Kirin Brewery Company and Koujirou Takagi of the Takagi Orchid Nursery in Japan (Kishi and Takagi, 1997*a*, 1997*b*).

Plant Material. In the original research, axillary buds were excised from 15-cm-long pseudobulbs of *Brassocattleya* Pastoral 'Innocense'.

Surface Sterilization. Buds must first be washed with 10% (v/v) benzalkonium chloride for 10 min (household detergent and water can probably also be used). The washed buds should be dipped in 70% (v/v) ethanol (74 ml 95% ethanol diluted to 100 ml with distilled water) for 30 sec. This should be followed by a 5-min submersion in 0.5% (v/v) sodium hypochlorite (8–10 ml Clorox or another household bleach that contains 6–5.25% sodium hypochlorite diluted to 100 ml with distilled water) plus 0.05% (v/v; this really amounts to a few drops per 100 ml) Tween 20 (or a mild household detergent). After that the buds should be washed three times with autoclaved distilled water.

Culture Vessels. Test tubes (3×20 cm) containing 20 ml of medium were used originally. Other vessels can also be used.

Culture Conditions. In the original research the cultures were placed on a drum-type rotary shaker tilted at 15° and rotating at 1 rpm. A different type of shaker may also be suitable but should be tested with a few explants before being put into large-scale use. Cultures should be maintained at 25 ± 2°C under illumination of 56.2 ± 5.2 μmol m^{-2} s^{-1} or standard laboratory conditions.

Culture Media. Liquid (this is not stated directly in the materials and methods section of the original paper, but is implied by the use of a shaker), one-quarter strength modified MS medium (Murashige and Skoog, 1962) should be used (Table Bc-3) for the production of PLBs. For plantlet production, the PLBs should be cultured on modified full-strength MS (Table Bc-4).

Procedure. Explants are cultured on the first medium (Table Bc-3). PLBs that form on it are moved to the second medium (Table Bc-4) for plantlet formation.

Developmental Sequence. PLBs form on the first medium (Table Bc-3) and plantlets develop on the second one (Table Bc-4).

General Comments. This procedure may be suitable for other *Brassocattleya* hybrids.

TABLE BC-3. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the culture of axillary buds of *Brassocattleya* Pastoral 'Innocense' (Kishi and Takagi, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	412.5	41.25 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	110.0	11.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	92.5	9.25 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	475.0	47.5 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	42.5	4.25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (IAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	If necessary[e]
	Cytokinin				
11	Kinetin	1–10.0	10–100 mg 100 ml^{-1} 95% ethanol[e,f]	1	If necessary[e]
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to 1 l of distilled water and stir and/or heat until dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect.

[e]Keep frozen between uses. There is no indication in the original paper whether auxin and/or cytokinin were used and if so how much. The auxin concentration suggested here is the same as that in Table Bc-1. There is no cytokinin in the medium outlined in Table Bc-1 and none is being suggested here. Should a cytokinin prove to be necessary 1 or 10 mg kinetin l^{-1} medium may be appropriate. These suggestions are speculative. Therefore those who plan to use this procedure are advised to test the medium as described here with a few buds before employing any formulations on a large scale.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or 0.1N HCl, respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.4, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11) if necessary (see footnote *e*) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. It is also possible to add items 1–14 before the addition of sugar, complete preparation of the medium, distribute to culture vessels, and autoclave the mixture.

TABLE BC-4. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for plantlet production from protocorm-like bodies of *Brassocattleya* Pastoral "Innocense" (Kishi and Takagi, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indoleacetic acid (IAA)	1.0–3.0	100–300 mg 100 ml^{-1} 95% ethanol[e,f]	1	If necessary[e]
	Cytokinin				
11	Kinetin	0.04–1.0	4–100 mg 100 ml^{-1} 95% ethanol[e,f]	If necessary[e]	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Gelrite[h]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect.

[e]Keep frozen between uses. There is no indication in the original paper whether auxin and/or cytokinin were used and if so how much. The auxin and cytokinin concentration suggested here are estimates based on other media. These suggestions are speculative. Therefore those who plan to use this procedure are advised to test the medium as described here with a few protocorm-like bodies before employing any formulations on a large scale. In many instances PLBs form plantlets without the addition of hormones to culture media. Therefore the Knudson C medium (see Tables Aranda-7 and Aranda-8) with or without ripe banana may also prove to be suitable.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.4, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Add the Gelrite in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11) if necessary (see footnote *e*) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar or Gelrite are not added to liquid media. It is also possible to add items 1–14 before the addition of sugar, complete preparation of the medium, distribute to culture vessels, and autoclave the mixture.

[h]Gellan gum (available as such from www.caissonlabs.com) as Phytagel (www.sigmaaldrich.com) or Gelrite is an agar substitute which produces a firm, clear, and colorless solid substrate at lower concentrations than agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} may have to be used for media that must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

Brassolaeliocattleya

A hybrid genus *Brassolaeliocattleya* consists of *Brassavola*, *Laelia*, and *Cattleya*. The first hybrid, *Brassolaeliocattleya* Lawrencei was described in the *Gardeners' Chronicle* (series 3, vol. 40, p. 201, 1906).

Although specific procedures for the culture of *Brassolaeliocattleya* do not seem to have been published, shoot tips ("meristems") of this hybrid genus (from *Brassavola*, *Laelia*, and *Cattleya*) are being cultured. In at least one case, that of *Brassolaeliocattleya* Greenwich 'Cover Girl,' clonal propagation through the culture of shoot tips resulted in a splash petal mutation that was named *Brassolaeliocattleya* Greenwich 'Irish Flair' (Green, 1983). It is reasonable to assume that procedures that are suitable for *Brassavola*, *Brassocattleya*, *Brassolaelia*, *Brassolaeliocattleya*, *Cattleya*, *Laelia*, and *Laeliocattleya* would also be suitable for this trigeneric hybrid.

Leaf explants from *Brassolaeliocattleya* seedlings produced PLBs when cultured on Knudson C medium (Knudson, 1946) supplemented with 3–5 mg BA l^{-1}. When moved to Knudson C medium containing 10% banana homogenate, the PLBs produced plantlets (Ding et al., 2002).

Bulbophyllum

One of the largest orchid genera, *Bulbophyllum*, was established by Louis-Marie Aubert Du Petit-Thouars in his *Histoire Particulière des Plantes Orchidées Recueillies sur les Trois Îsles Australes d'Afrique* which was published after he returned to France following his sojourn (1792–1802) in Madagascar and other islands in the Mascarene archipelago (Siegerist, 2001). Estimates of the number of species in *Bulbophyllum* vary from "as many as a 1000" (Bechtel et al., 1992) to "thousands" (Romero-Gonzalez, 2001). The genus is distributed worldwide, but most of its species are found in Asia.

Micropropagation of *Bulbophyllum careyanum*

Bulbophyllum careyanum is native to India, Myanmar (formerly Burma), Sikkim, and Thailand (Kataki, 1986; Vij et al., 2000*a*; Siegerist, 2001). Its racemes are arched, arise from the bases of the pseudobulbs, and produce tightly packed yellowish flowers. The pseudobulbs, borne on scale-covered rhizomes, are approximately 3.5 cm high (Kataki, 1986; Siegerist, 2001). Reverend Charles Samuel Parish (1822–1897), Chaplain to the British forces in Burma who collected and drew orchids there, is reported to have called it "a worthless plant" (Siegerist, 2001). In 1888, Ernst Hugo Heinrich Pfitzer (1846–1906), Professor of Botany at Heidelberg, used it as the type genus for *Bulbophyllum* section *Careyana*. A micropropagation method for this species was developed at the Orchid Laboratory, Department of Botany, Panjab University, Chandigarh, India (Vij et al., 2000*a*).

Plant Material. In the original research pseudobulbs from both greenhouse- and in-vitro-grown plants were used as explant sources. Only the use of explants from greenhouse-grown plants will be described here because they can be utilized for clonal propagation of desirable forms. The pseudobulbs should be sectioned into two halves (Fig. Bulb-1A) – distal and proximal (top and bottom, respectively).

Surface Sterilization. Pseudobulbs should be washed with running tap water and soaked in a detergent (Teepol was used in the original research, but any mild household brand is suitable) for 10 min and rinsed thoroughly. The actual sterilization procedure consists of several steps: (1) a 5-s dip in 70% ethanol (74 ml 95% ethanol diluted to 100 ml with distilled water) followed by a thorough rinse with sterile distilled water; (2) a 5-min soak in 0.01% streptomycin (100 mg l^{-1} distilled water, w/v) and again thorough rinsing with sterile distilled water; and (3) a 5-min soak in a mixture of 0.1% $HgCl_2$ (1 g l^{-1} distilled water, w/v; this is a highly toxic substance that should be handled with care or avoided altogether) and a few drops of surfactant (Teepol or any mild detergent) and yet another through rinsing with sterile distilled water. The cut ends of each section must be severed and discarded before placing the sections in culture.

FIG. BULB-1. **Tissue culture of *Bulbophyllum careyanum*. A. Sections. B. Buds on sections starting to grow. C–K. Plantlets. L. Plant in pot.**

Culture Vessels. The culture vessels used in the original research are not described, but the photographs show test tubes. They should contain 20–30% their volume of medium.

Culture Conditions. Illumination of 3500 lx, 12-h photoperiods, and 25 ± 2°C or standard culture room conditions are suitable.

Culture Media. Several media, all based on the Vacin and Went medium (Vacin and Went, 1949), are suitable. However, only a medium which does not contain hormones as such (and therefore has the lowest mutation-inducing potential) will be presented here (Table Bulb-1).

Procedure. The sections are placed in culture after surface sterilization (Fig. Bulb-1A). When plantlets form and proliferate (Fig. Bulb-1B–K) they should be moved to a new culture vessel for further growth. Plantlets that are large enough can be potted in a potting mix in a pot (Fig. Bulb-1L).

Developmental Sequence. After being placed in culture (Fig. Bulb-1A), buds on the sections start to grow (Fig. Bulb-1B, C) and produce plantlets (Figs Bulb-1D–K), which grow to maturity in pots (Fig. Bulb-1L).

TABLE BULB-1. **Vacin and Went (VW) medium (Vacin and Went, 1949) as modified for the culture of *Bulbophyllum* (Vij et al., 2000*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additive				
9	Coconut water	100.0 ml	No stock	No stock	Measure
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier				
11	Agar[e]	12–15 g	No stock	No stock	Weigh
	Darkening agent				
12	Activated charcoal	2.0 g	No stock	No stock	

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve. Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original recipe of this medium calls for 28 mg ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, l^{-1} but chelated iron as suggested here is preferable. A difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible.

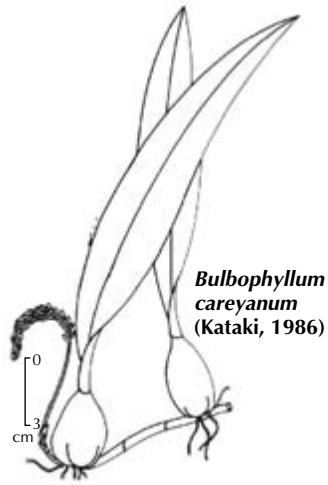
[e]Add items 1–7 and 9 to 500–700 ml of distilled water (item 10), adjust pH to 5.2–5.4 or as required, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, add the charcoal (item 12) with vigorous stirring and then pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of other nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

General Comments. This is the first tissue culture method to be developed for a *Bulbophyllum* species. It may be suitable for additional bulbophyllums and/or serve as a starting point for methods for additional *Bulbophyllum* or orchid species. Also, the use of pseudobulbs as explants merits further attention because these organs may be able to survive for longer periods following excision due to the nutrients and other substances they store (Ng and Hew, 2000).

Isolation of *Bulbophyllum neilgherrense* protoplasts

Protoplasts were isolated from the leaves and roots (4.2×10^4 and 2.7×10^4 g fresh weight, respectively) of *Bulbophyllum neilgherrense* with the procedure developed for *Acampe praemorsa* (Seeni and Abraham, 1986). See *Acampe* entry, Isolation of Protoplasts from *Acampe praemorsa* (p. 139, Vol. I).

Bulbophyllum, from the Greek βολβός (bolbos: bulb) and ψύλλον (phyllon: leaf) in reference to the thick and fleshy leaves; meaning bulb-leaf (Schultes and Pease, 1963).



***Bulbophyllum careyanum* (Kataki, 1986)**

Burkillara

Three British families produced fathers and sons who were both directors of the same botanical garden. Sir William Jackson Hooker (1785–1865, knighted in 1836) was director of Kew Gardens from 1841 until his death. His son Sir Joseph Dalton Hooker (1817–1911, knighted in 1877) assumed his father's position as Director of Kew Gardens and held it until 1885. He was interested in orchids and wrote about them. The names of several orchid species honor him.

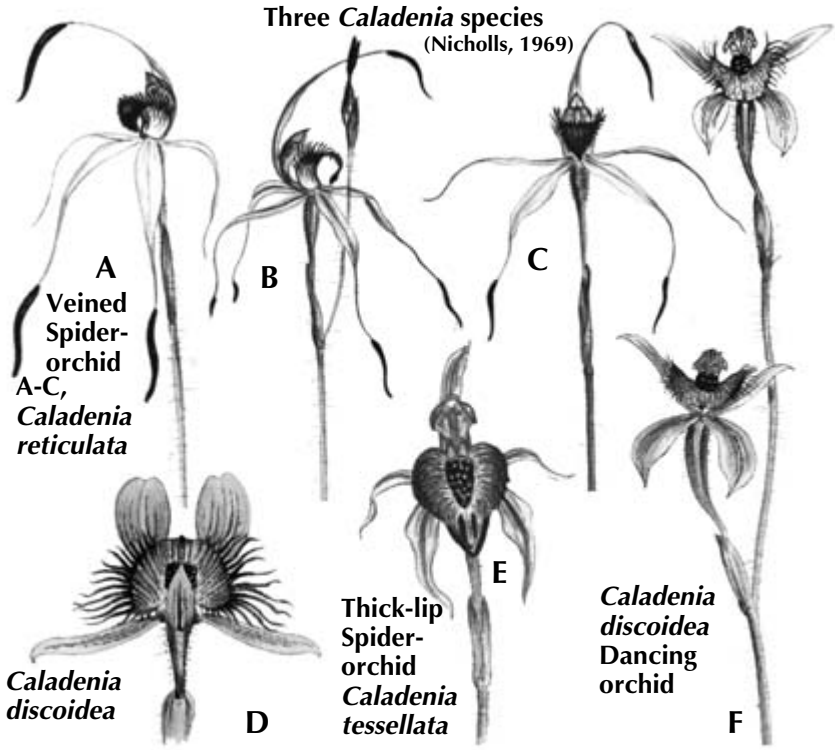
In Ireland, David Moore (1807–1879) was director of the Glasnevin Botanical Gardens and the first to germinate seeds of tropical orchids under horticultural conditions. His son, Frederick William Moore (1857–1950), also became director of the Glasnevin Botanical Gardens and established an impressive orchid collection there. The genus *Neomoorea* is named after him.

Isaac Henry Burkill was director of the Singapore Botanic Gardens from 1912 to 1925, and his son Humphrey Morrison Burkill held the same post between 1957 and 1969. The hybrid genus *Burkillara* [*Aeridachnis* Mandai (*Arachnis hookeriana* × *Aerides augustianum*, registered in 1963 by Singapore Orchids) × *Vanda tricolor* (registered in 1972 by Singapore Orchids)] honors Burkill. And fittingly, the first tissue culture procedure for *Burkillara* was developed at the Singapore Botanic Gardens (Lim-Ho, 1981).

Apical and axillary buds of *Burkillara* Ong Thye Chiew were cultured at the Singapore Botanic Gardens. The procedure and media sequence are exactly the same as those used for *Aranda* (Lim-Ho, 1981): liquid (see Table Arach-3); solid (see Table Arach-4 or Arach-5); and solid (see Table Arach-6). Modified MS medium (see Table Aranda-9) can also be used for proliferation and differentiation.

Caladenia

Consisting of about 80 species, *Caladenia* is found primarily in Australia, with several species extending into Indonesia (Java), New Caledonia, and New Zealand. The common names of some species include the word "spider" due to the shape of their flowers. The micropropagation method developed for *Diuris* (Collins and Dixon, 1992) can also be used for *Caladenia* (K. W. Dixon, Kings Park and Botanic Garden, West Perth, Australia, pers. comm.).



Calanthe

The earliest report regarding micropropagation of *Calanthe* is that "within one year [the *Cymbidium* tissue culture] technique [was extended] to *Calanthe*" (Bertsch, 1967). The contents of the articles (Bertsch, 1966, 1967) suggest that the techniques in question may have been those used for *Cattleya* and/or *Cymbidium* (Vacherot, 1966; Morel, 1974). The development of young *Calanthe* plants was studied in the late 1980s (Buyun and Budak, 1986).

Micropropagation of *Calanthe sieboldii*

Plantlets were produced from apical buds of *Calanthe sieboldii* cultured on a medium consisting of half-strength macroelements and quarter-strength microelements of the B5 medium and 2 mg BA l^{-1} in "slanting rotary culture" (Yamamoto, 1990 cited by Ichihashi, 1994).

Micropropagation of *Calanthe discolor*

Calanthe discolor is native to Japan and the Ryukyu Islands. It was first described and named in the West by John Lindley in 1838.

Plant Material. Axillary buds from mature plants are cultured (Ichihashi, 1994).

Surface Sterilization. Buds were sterilized by submerging and shaking them "with 1% benzalkonium chloride [www.sigma-aldrich.com, www.fishersci.com] and 1% sodium hypochlorite [20 ml of a household bleach containing 5–5.25% sodium hypochlorite diluted to 100 ml with distilled water]." No information is given on how to prepare the sterilant. A logical assumption is that 1 g benzalkonium chloride was dissolved in 100 ml of 1% sodium hypochlorite. The buds should be washed three times with sterile distilled water after the surface sterilization and before their apical meristems, 1 mm long, are excised and placed in culture.

Culture Vessels. Test tubes, 30 mm in diameter (length not given; 15–25 cm will probably be suitable), containing 20 ml medium should be used for the initial cultures. Petri dishes, 9 cm in diameter (height not given), containing 20 ml of medium are appropriate for PLBs. Other containers can also be used for PLBs.

Culture Conditions. Initial cultures should be maintained on slanted rotary shakers (type and degree of slant are not indicated) at 20–24°C under continuous illumination of 3000–5000 lx provided by fluorescent lamps (wattage and source not given). PLBs should be cultured at 15–20°C under 18-h photoperiods of approximately 3000 lx (light sources are not described).

Culture Media. To induce PLBs and multiple roots, explants should be placed on an induction medium that consists of half-strength macroelements and organic components and quarter-strength microelements of the B5 medium (Gamborg et al., 1968) plus 2 mg NAA l^{-1} and 0.02 mg BA l^{-1} (Table Cal-1). Callus can be induced by culturing explants on a different medium, CM (Table Cal-2). Despite an assertion that the paper reports "on . . . the condition of plant regeneration" a medium for

TABLE CAL-1. **B5 medium (Gamborg et al., 1968) modified for the induction of protocorm-like bodies from apical meristems of *Calanthe discolor* buds (Ichihashi, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	67.0	6.7 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	75.0	7.5 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	125.0	12.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1250.0	125.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	75.0	7.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	9.33	0.93 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	6.95	0.7 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	1.5	150.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.006	0.63 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.006	0.63 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	0.63	62.5 g l^{-1}		
(e)	Potassium iodide, KI	0.19	18.75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.06	6.25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	50.0	No stock	No stock	Weigh
	Auxin				
9	Naphthaleneacetic acid (NAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
10	Benzylaminopurine (BAP)	0.02	2 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	5 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	5 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	5.0	500 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–8 to 900 ml of distilled water (item 15), adjust pH to 5.7, add sugar (item 14), adjust volume to 1000 ml with distilled water (item 15), pour the solution into culture vessels, and autoclave. As a rule hormones and vitamins (items 9–13 in this case) are added after autoclaving to the still warm and liquid solution under sterile conditions with sterilized pipettes, mixed well, and the medium distributed to preautoclaved culture vessels. However this does not seem to be indicated for the present medium.

TABLE CAL-2. **B5 medium (Gamborg et al., 1968) modified for callus induction from apical meristems of *Calanthe discolor* buds (Ichihashi, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	40.02	4.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	29.4	2.94 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	98.59	9.86 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1277.0	127.7 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	55.2	5.52 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	9.33	0.93 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	6.95	0.7 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	1.5	150.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.006	0.63 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.006	0.63 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	0.63	62.5 g l^{-1}		
(e)	Potassium iodide, KI	0.19	18.75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.06	6.25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	50.0	No stock	No stock	Weigh
	Auxin				
9	Naphthaleneacetic acid (NAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
10	Benzylaminopurine (BAP)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	5 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	5 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	5.0	500 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	15.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise. It is not clear from the original paper if this medium is considered by the author as being a new medium or a modification of the B5 solution. The macroelements are present each in a different fraction of their level in the B5 medium and NH_4NO_3 is not a component of that solution. On the other hand the microelement concentration is one-quarter that in B5 and the vitamins are included at half their B5 levels. Iron and *myo*-inositol are not mentioned as being part of this medium (table 2 in the original paper), but they are included here on the assumption that if they are required and not present the medium may not be effective, but if added even if not needed they will cause no harm.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–8 to 900 ml of distilled water (item 15), adjust pH to 5.7, add sugar (item 14), adjust volume to 1000 ml with distilled water (item 15), pour the solution into culture vessels, and autoclave. As a rule, hormones and vitamins (items 9–13 in this case) are added after autoclaving to the still warm and liquid solution under sterile conditions with sterilized pipettes, mixed well, and the medium distributed to preautoclaved culture vessels. However this does not seem to be indicated for the present medium. Since there is no indication in the original paper whether this medium should be liquid or solid, both forms should be tested. If agar is needed, bring the solution to a gentle boil and add 8–10 g agar slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into preautoclaved culture vessels.

this is not suggested directly. Multiple roots and shoots are produced on medium (SRM) containing 0.2 mg NAA l^{-1} and 0.2 mg BA l^{-1} (Table Cal-3). Therefore, it is reasonable to assume that if root-bearing shoots are separated and cultured on a hormone-free medium they will grow into plantlets. Both NAA and BA are available from www.sigmaaldrich.com, www.fishersci.com, and www.caissonlabs.com.

Procedure. Buds are taken from plants and surface-sterilized before their meristems are excised and cultured on induction medium (Table Cal-1). If the intent is to induce callus, meristems should be cultured on CM (Table Cal-2). If shoots and roots do not form on PLBs cultured on induction medium (Table Cal-1), they should be transferred to SRM (Table Cal-3).

Developmental Sequence. PLBs and possible shoots and roots form on induction medium (Table Cal-1). Multiple shoots and roots develop on SRM (Table Cal-3). Callus develops on CM (Table Cal-2).

General Comments. *Calanthe* is a popular orchid in Japan. This can be very useful, especially since it can be employed with other species and hybrids.

Calanthe discolor **(www.hokudai.ac.jp/fsc/bg/news/news/1994/1994.html)**

Calanthe aristulifera **(Ito and Karasawa, 1969)**

TABLE CAL-3. **B5 medium (Gamborg et al., 1968) modified for the induction of multiple shoots and roots on tissues or protocorm-like bodies derived from meristems of *Calanthe discolor* buds (Ichihashi, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	40.02	4.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	29.4	2.94 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	98.59	9.86 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1277.0	127.7 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	55.2	5.52 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	9.33	0.93 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	6.95	0.7 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	1.5	150.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.006	0.63 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.006	0.63 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	0.63	62.5 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.19	18.75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.06	6.25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	50.0	No stock	No stock	Weigh
	Auxin				
9	Naphthaleneacetic acid (NAA)	0.2	20 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
10	Benzylaminopurine (BAP)	0.2	20 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	5 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	5 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	5.0	500 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	15.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise. It is not clear from the original paper if this medium is considered by the author as being a new medium or a modification of the B5 solution. The macroelements are present each in a different fraction of their level in the B5 medium and NH_4NO_3 is not a component of that solution. On the other hand, the microelement concentration is one-quarter that in B5 and the vitamins are included at half their B5 levels. Iron and *myo*-inositol are not mentioned as being part of this medium (table 2 in the original paper), but they are included here on the assumption that if they are required and not present the medium may not be effective, but if added even if not needed they will cause no harm.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–8 to 900 ml of distilled water (item 15), adjust pH to 5.7, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15); pour the solution into culture vessels and autoclave. As a rule hormones and vitamins (items 9–13 in this case) are added after autoclaving to the still warm and liquid solution under sterile conditions with sterilized pipettes, mixed well, and the medium distributed to preautoclaved culture vessels. However this does not seem to be indicated for the present medium. Since there is no indication in the original paper whether this medium should be liquid or solid, both forms should be tested. If agar is needed, bring the solution to a gentle boil and add 8–10 g agar slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into preautoclaved culture vessels.

Micropropagation of *Calanthe* × bicolor, *Calanthe* × satuma, and *Calanthe sieboldii*

The *Calanthe discolor* method (Ishihashi, 1994) can be used.

Micropropagation of *Calanthe aristulifera*, *Calanthe* × *Higo*, *Calanthe* × *Hizen*, and *Calanthe* × *Kouzo*

The *Calanthe discolor* method (Ishihashi, 1994) can be used, but the induction medium must be modified to contain 0.01 mg NAA l^{-1} and 0.5 mg BA^{-1}.

Isolation of Mesophyll Protoplasts of *Calanthe*

A method for the isolation of protoplasts of *Calanthe discolor* was developed at the Department of Horticulture, University of Minamikyushu, Takanabe-cho, Miyazaki Prefecture, Japan (Yasugi et al., 1986).

Plant Material. Excised young leaves were used in the original research. They were taken from plants grown in the greenhouse of Minamikyushu University.

Surface Sterilization. The leaves should be immersed in 0.5% sodium hypochlorite (10 ml of household bleach diluted to 100 ml with distilled water) for 10 min, transferred to 70% ethanol (74 ml of 95% ethanol diluted to 100 ml with distilled water) for 1 min and washed three times with sterile distilled water. No mention is made in the original paper of washing the leaves before placing them in the hypochlorite solution. This may be advisable and can be done by gently washing the leaves with a mild soap, very soft brush (a used, soft toothbrush is suitable), and copious amounts of tap or distilled water.

Culture Vessels. Beakers would probably be most suitable for the surface sterilization steps. Sterile Petri dishes and Erlenmeyer flasks are appropriate for cutting the leaves and storing the sections. Erlenmeyer flasks should be used for incubation.

Isolation Conditions. Incubation should be at 28°C on a shaker set at 40 strokes (2.2-cm amplitude) per minute. The original paper does not indicate whether the incubation was in the light or dark. Therefore it may be wise to attempt isolation both in the dark and under illumination.

Isolation Medium. The enzyme mixture (Table Cal-4) consists of 1% (w/v) Onozuka cellulase R-10 (from Yakult Honsha Co., Ltd., Japan), 0.2% (w/v) Macerozyme R-10 (Yakult Honsha), and 0.01% (w/v) Pectolyase (Kikkoman Shyoyo Co., Japan; for US sources of all enzymes see *Aranda* entry, sections on the isolation and culture of mesophyll and leaf protoplasts), 0.5 mol (91.1 g) l^{-1} mannitol, 5% (w/v) potassium dextran sulfate, and 10 mmol (1.47 g) l^{-1} $CaCl_2 \cdot 2H_2O$ adjusted to pH 5.8.

TABLE CAL-4. **Isolation medium for *Calanthe* protoplasts (Yasugi et al., 1986)**[a]

Characteristic or component	Amount		
	Molarity	Weight per 10 ml solution	Percent
Enzymes			
Onozuka cellulase R-10[b]		100 mg	1
Macerozyme R-10[b]		20 mg	0.2
Pectolyase[c]		1 mg	0.01
Osmoticum			
Mannitol[d]	*0.7 M*	1.28 g	12.8
Viscocity agent			
Potassium dextran sulfate[e]			0.5
Mineral			
Calcium chloride, $CaCl_2 \cdot 2H_2O$	10 m*M*	1.47 g	14.7
pH 5.8			

[a]Yakult Honsha, Japan. These enzymes are available in the United State from Serva Biochemicals, Inc., 200 Shames Drive, Westbury, NY 11590.

[b]Kikkoman Shyoyo Co., Japan. Available in the United States from Serva Biochemicals (see footnote *a* for address).

[c]Driselase, if needed, can be obtained in the United States from Fluka Chemical Corp., 980 S. Second St., Ronkonkoma, NY 11779.

[d]A general assumption is that plants cannot utilize mannitol. This may not be true for some orchids, therefore the initial and final concentrations of mannitol in this solution may be different.

[e]The available dextran sulfates differ in molecular weight. The original paper does not indicate which one was used. Sodium dextran sulfates are available in the United States from Fluka Chemical Corp. (see footnote *c*) and Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178, Tel. 1-800-325-3010 (toll-free for calls from within the U.S.A.) and 314-771-5750 (from anywhere in the world). Sigma also sells enzymes.

Procedure. Place 2×20-mm leaf sections weighing approximately 1 g (fresh weight) in 10 ml enzyme mixture in a 50-ml Erlenmeyer flask and incubate for 90 min under the conditions listed above. The number of protoplasts can be determined with a hemocytometer.

Developmental Sequence. There is no mention of development in the original paper Bertsch, 1966).

General Comments. The purpose of this research was only "to find . . . [a] suitable enzyme mixture and treatment time [for] high yield of mesophyll protoplasts from . . . mature leaves. . . ." (Bertsch, 1967). Therefore the paper does not provide information on culture, division, and fusion. However, it should be noted that S. Yasugi did produce embryoids from mesophyll protoplasts of *Dendrobium*.

Isolation of Protoplasts from Leaves of *Calanthe masuca*

Protoplasts, 21.7×10^4 g of fresh weight tissue, were isolated using the procedure developed for *Acampe praemorsa*.

Calanthe, from the Greek καλός kalos (beautiful) and ἄνθη anthe (flower) in reference to the beautiful flowers produced by this genus (Schultes and Pease, 1963), and *discolor*, from the Latin for "of a different color; different; different from".

Catasetum

The genus *Catasetum* was established in 1822 by Karl Sigismund Kunth (1788–1850) on the basis of notes by Louis Claude Marie Richard (1754–1821) who collected in French Guiana from 1781 to 1789 (Holst, 1999). Kunth based his description on *Catasetum macrocarpum* and *C. maculatum*, both of which have two antennae attached to the gynostemium that extend downward. These antennae led to the name of the genus, which is derived from the Greek *cata* (downward) and the Latin *saeta* or *seta* (bristles). *Catasetum* is one of very few orchid genera to produce separate male, female, and hermaphroditic flowers. This resulted in the creation of three genera: *Monachanthus*, *Myanthus*, and *Catasetum*. The trimorphism became apparent by 1837 but the first effort by Rudolf Mansfeld (1901–1960) to put the taxonomy of *Catasetum* in order took place in 1932. A more recent revision is by Calaway H. Dodson (b. 1928) in 1975 (Bechtel et al., 1992; Holst, 1999).

Culture of *Catasetum* Root Tips

A study of the potential of root tips of some Brazilian orchids as suitable explants for micropropagation resulted in the method for the clonal propagation of a *Catasetum* hybrid (Kerbauy, 1984*a*).

Plant Material. The original research was carried out with root tips 1.5 mm in length, taken from seedlings 6–7 cm high, and growing in vitro, of *Catasetum trulla* × *Catasetum* Berthrand.

Surface Sterilization. There was no need to surface-sterilize the root tips because the seedlings were growing aseptically.

Culture Vessels. Erlenmeyer flasks of 50-ml capacity and containing 20 ml medium were used in the original research.

Culture Conditions. The original conditions were 16-h photoperiods of 500 lx produced by Sylvania Gro Lux fluorescent lamps at 25 ± 1°C. Other light sources and photoperiods may also be suitable.

Culture Media. Several culture media were screened initially, all based on Knudson C, and each produced different results (Table Ctsm-1). Root tips developed on a medium containing peptone, coconut water, thiamine, and NAA (Table Ctsm-2). Both PLBs and callus were produced on a medium containing thiamine only (Table Ctsm-3). PLBs and healthy plants developed without callus formation on a medium containing thiamine, peptone, and coconut water but not NAA (Table Ctsm-4).

Procedure. Place the root tips on the culture medium.

Developmental Sequence. Callus masses, PLBs, roots, and plantlets may be produced (Table Ctsm-1).

TABLE CTSM-1. **Effect of culture media on *Catasetum* root tips (Kerbauy, 1984*a*)**

Item	Culture medium[a]	Type of growth[b]
1	BM + CW	Mostly roots, 5.5% small dark callus masses
2	BM + PP	Dark-green callus masses, PLBs, Plantlets
3	BM + TM	40% PLBs, 60% callus masses
4	BM + NAA	Small callus masses, few PLBs
5	BM + CW + NAA	More vigorous callus masses, 32% PLBs, etiolated plantlets that also produced PLBs
6	BM + PP + NAA	Vigorous callus masses, no PLBs
7	BM + TM + NAA	Vigorous callus masses no PLBs
8	BM + CW + PP + TM	54% direct formation of PLBs
9	BM + CW + PP + TM5	Long, thick pilose roots, lateral roots, PLBs

[a]BM, basal medium (Knudson C) with chelated iron and 8% agar (see Table Ctsm-2); CW, 15% coconut water; NAA, 5 mg l^{-1} naphthaleneacetic acid; PP, 1 g l^{-1} peptone; TM, 0.5 mg l^{-1} thiamine; TM5, 5 mg l^{-1} thiamine.

[b]Percentages refer to proportion of all cultures; PLBs, protocorm-like bodies.

TABLE CTSM-2. **Knudson C medium (Knudson, 1946) as modified for the culture of *Catasetum* root tips (Kerbauy, 1984*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	**Iron**[c]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8	27.8 g l^{-1}	10	
(b)	Chelating agent, Na_2EDTA	37.3	37.3 g l^{-1}	10	
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additives				
7	Peptone	1 g	No stock	No stock	Weigh
8	Coconut water	150 ml	No stock	No stock	Measure
	Vitamin				
9	Thiamine (vitamin B_1)	5	500 mg 100 ml^{-1} 95% ethanol[d]	1	
	Hormone				
10	Naphthaleneacetic acid (NAA)	5	500 mg 100 ml^{-1} 95% ethanol[d]	1	
	Sugar				
11	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[e]	To 1000 ml			
	Solidifier				
13	Agar[e]	8 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The iron in the original formulation is not chelated, but the form used here is preferable.

[d]Keep refrigerated between uses. If NAA fails to dissolve, add a few drops of dilute KOH.

[e]Add items 1–8 to 900 ml distilled water (item 12), set pH to 5.5, add sugar (item 11), and adjust volume to 1000 ml with distilled water (item 12). Bring solution to a gentle boil, and add agar (item 13) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add vitamin (item 9) and hormone (item 10) to the warm, still liquid solution, mix well, and distribute into culture vessels. Do not add agar to liquid medium. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower the pH if necessary.

TABLE CTSM-3. **Knudson C medium (Knudson, 1946) as modified for the production of callus and protocorm-like bodies from *Catasetum* root (Kerbauy, 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	**Iron**[c]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8	27.8 g l^{-1}	10	
(b)	Chelating agent, Na_2EDTA	37.3	37.3 g l^{-1}	10	
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Vitamin				
7	Thiamine (vitamin B_1)	5	500 mg 100 ml^{-1} 95% ethanol[d]	1	
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	8 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The iron in the original formulated is not chelated, but the form used here is preferable.

[d]Keep refrigerated between uses.

[e]Add items 1–6 to 900 ml distilled water (item 9), set pH to 5.5, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). If solid medium is desired, bring solution to a gentle boil, and add agar (item 10) slowly while stirring. The agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add vitamin (item 7) to the warm, still liquid solution. Mix well, and distribute into culture vessels. Do not add agar if preparing liquid medium. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower the pH if necessary.

General Comments. Like other procedures developed with explants from seedlings it is not clear if this one can be used for mature plants.

TABLE CTSM-4. **Knudson C medium (Knudson, 1946) as modified for the protocorm and plantlet production from root tips of *Catasetum* root tips (Kerbauy, 1984*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	**Iron**[c]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8	27.8 g l^{-1}	10	
(b)	Chelating agent, Na_2EDTA	37.3	37.3 g l^{-1}	10	
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additives				
7	Peptone	1 g	No stock	No stock	Weigh
8	Coconut water	150 ml	No stock	No stock	Measure
	Vitamin				
9	Thiamine (vitamin B_1)	5	500 mg 100 ml^{-1} 95% ethanol[d]	1	
	Sugar				
10	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[e]	To 1000 ml			
	Solidifier				
12	Agar[e]	8 g	No stock	No stock	Weigh

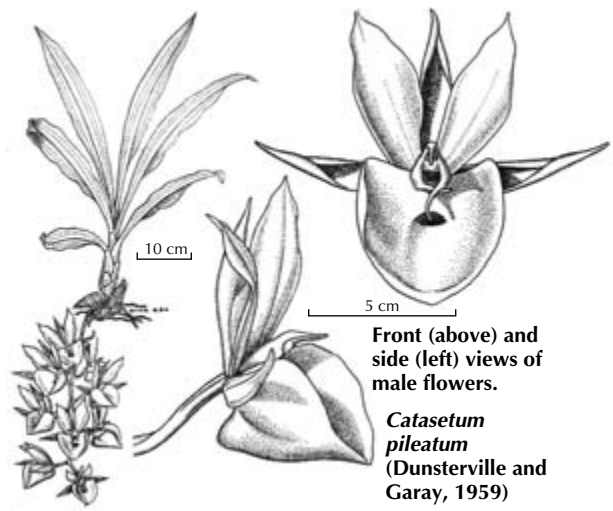
[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The iron in the original formulation is not chelated, but the form used here is preferable.

[d]Keep refrigerated between uses.

[e]Add items 1–8 to 900 ml distilled water (item 11), set pH to 5.5, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring solution to a gentle boil and add agar (item 12) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add vitamin (item 9) to the warm, still liquid solution. Mix well, and distribute into culture vessels. Do not add agar if liquid medium is desired. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added. especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower the pH if necessary.



Front (above) and side (left) views of male flowers.

***Catasetum pileatum* (Dunsterville and Garay, 1959)**

Production of Protocorm-like Bodies from *Catasetum fimbriatum* Root Tips

Professor Gilberto Barbante Kerbauy at the University of Sao Paulo in Brasil and his associates have studied hormone levels as well as carbohydrate and starch partition in excised root tips of *Catasetum fimbriatum* (Colli and Kerbauy, 1993; Vaz et al., 1998; Peres et al., 1999). Their culture method can be adapted to micropropagation.

Plant Material. Root tips, 5 mm in length, were taken from asymbiotic seedlings.

Surface Sterilization. There is no need to surface-sterilize root tips taken from asymbiotic seedlings in vitro. However, they must be washed to remove medium residues.

Culture Vessels. Erlenmeyer flasks, 125-ml capacity, were used as culture vessels. They should contain approximately 25 ml medium.

Culture Conditions. In the original research the cultures were maintained at 26 ± 2°C under 16-h photoperiods of 40–50 μmol m^{-2} s^{-1} provided by fluorescent lights (the type was not described in the original papers, but cool white tubes alone or in a mixture with incandescent bulbs will probably prove to be suitable). Standard culture room conditions are also appropriate.

Culture Media. The basal medium used in the original research (Colli and Kerbauy, 1993) was Linsmaier–Skoog solution (Linsmaier and Skoog, 1965). It can be supplemented with several growth regulators alone or in a number of combinations, but the medium suggested here is one with low mutation-inducing potential (Table Ctsm-5). Plantlets and/or callus can form on this medium. However, different media formulated in Professor Kerbauy's laboratory (Kerbauy, 1984*a*) may also be suitable for callus induction (Table Ctsm-3) and plantlet production (Table Ctsm-4).

Procedure. Excised root tips should be placed on the first medium (Table Ctsm-5). Callus (Fig. Ctsm-1B) and PLBs which develop on or from the root tips can be subcultured on the same (Table Ctsm-5) or different (Tables Ctsm-3 and Ctsm-4) media. When plantlets (Fig. Ctsm-1A) are large enough they should be taken out of culture and potted in a potting mix.

Developmental Sequence. The root tips may form PLBs or callus and eventually plantlets.

General Comments. This method does not allow for the selection of superior forms because it utilizes root tips from seedlings. It may prove to be suitable for root tips from mature plants. However, if root tips of mature plants are to be used they must be taken from roots which have not come into contact with potting mix or other objects or surfaces to ensure the absence of mycorrhizal fungi.

TABLE CTSM-5. **Linsmaier-Skoog (LS) medium (Linsmaier and Skoog, 1965) modified for the culture of *Catasetum fimbriatum* root tips (Colli and Kerbauy, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
9	Benzyladenine	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamin				
10	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
11	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[g]	To 1000 ml			
	Solidifier				
13	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1N HCl.

[g]Add items 1–8 to 900 ml of distilled water (item 12), adjust pH to 5.8, add sugar (item 11), and adjust volume to 1000 ml with distilled water (item 12). Bring the solution to a gentle boil and add the agar (item 13) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the cytokinin (item 9) and vitamin (item 10) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

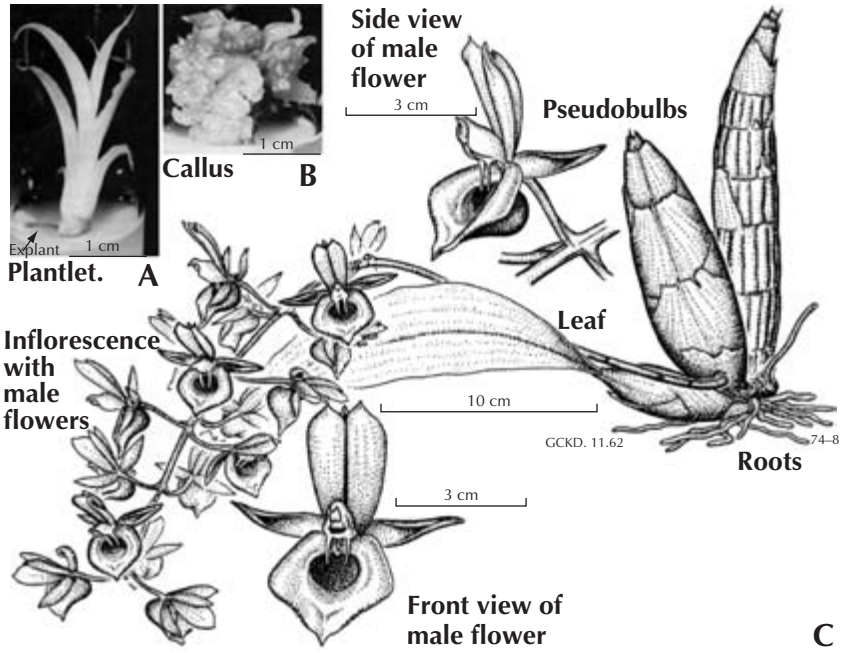


FIG. CTSM-1. ***Catasetum pileatum*. A. Plantlet in vitro. B. Callus. C. Flowering plant. (Dunsterville and Garay, 1966.)**

Formation of Protocorm-like Bodies from *Catasetum pileatum* Root Tips

When orchid root tips are cultured in vitro, PLB formation can be either direct or it may be preceded by callus development (Kraus and Monteiro, 1989; Kraus and Kerbauy, 1992). Research on the factors that affect or control these developmental patterns resulted in a method (Kraus and Monteiro, 1989; Kraus and Kerbauy, 1992) which can be used for micropropagation.

Plant Material. Root tips, 6 ± 1 mm long, were taken from asymbiotic seedlings of *C. pileatum*.

Surface Sterilization. There is no need to surface-sterilize root tips taken from seedlings growing in vitro. However, they should be rinsed thoroughly to remove medium residues.

Culture Vessels. Test tubes, Erlenmeyer flasks, and other common culture vessels can be used.

Culture Conditions. In the original research the culture was maintained at 26 ± 2°C under 16-h photoperiods of 9 W m^{-2} provided by fluorescent lamps (type is not specified in the original paper). Standard culture room conditions are also suitable.

Culture Media. MS medium (Murashige and Skoog, 1962) should be used to induce PLB formation (Table Ctsm-6). PLBs can be cultured on modified Knudson C medium (Table Ctsm-4) for plantlet production.

Procedure. Excised root tips should be cultured on the first medium (Table Ctsm-6) to induce PLB formation. The PLBs should be moved to the second medium for the production of plantlets.

Developmental Sequence. Root caps show some disorganization after 24–48 h of culture, and the promeristem and protoderm can no longer be discerned. After 48–72 h, not much remains of the root cap and cell division can be seen in the subapical region. A new cell group consisting of recently produced cells can be seen following 72–96 h of incubation. After 6 days there is evidence of active mitotic divisions in the apical region. This leads to the formation of PLB primordia in 10 days. Well-defined PLBs can be seen after 20 days of culture. Their apices become well defined after 30 days. Well-defined seedlings in the PLBs become evident 10 days after that. Seedlings become independent subsequently.

General Comments. This method cannot be used to propagate desirable forms because explants are taken from seedlings. However, it may perhaps be suitable for root tips of mature plants. Should an attempt be made to culture root tips of mature plants, care must be taken to ensure that they do not contain mycorrhiza.

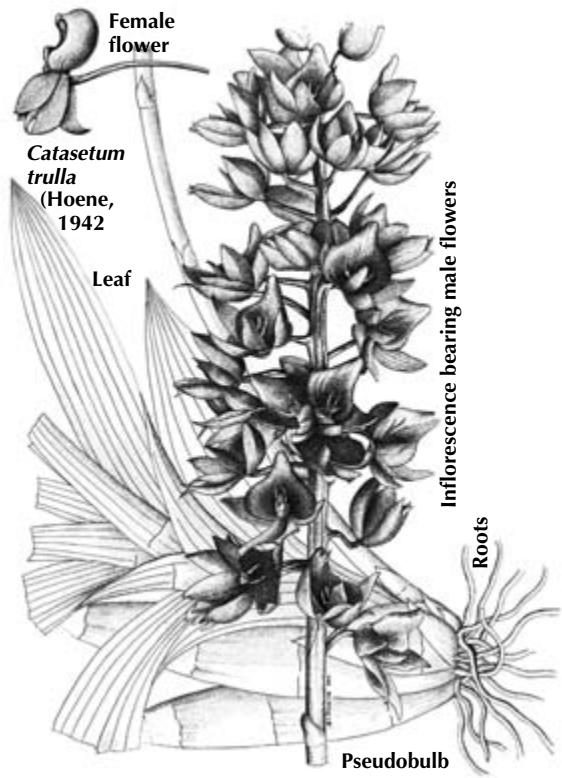


TABLE CTSM-6. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the culture of *Catasetum pileatum* root tips (Kraus and Monteiro, 1989; Kraus and Kerbauy, 1992)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[c]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[c]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[e]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[f]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[f]	1	
11	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[f]	1	
12	Thiamine (vitamin B_1)	5.0	500 mg 100 ml^{-1} 95% ethanol[f]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Complex additive				
14	Peptome[g]	1.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[h]	To 1000 ml			
	Solidifier				
16	Agar[h]	8.0 g	No stock	No stock	Weigh
	Darkening agent				
17	Activated charcoal	1.0 g	No stock	No stock	Weigh

[a]The original papers state that MS medium was used. This medium usually contains an auxin and a cytokinin, but the title of one of the original papers (Kraus and Kerbauy, 1992) indicates that these hormones were not included in the modified medium.

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect.

[f]Keep frozen between uses.

[g]The source and manufacturer were not listed in the original papers.

[h]Add items 1–7, 9, and 14 to 900 ml of distilled water (item 15), adjust pH to 5.5, add sugar (item 13), and adjust volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved add the charcoal (item 17) with vigorous stirring, pour the solution into a 2-l flask, and autoclave. Add the amino acid (item 8) and vitamins (items 10–12) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. In some cases the amino acid and vitamins can be autoclaved, but this should be tested before being adopted as standard practice.

Cattleya

In the minds of the general public, *Cattleya* is synonymous with orchids. For cut-flower growers, *Cattleya* is a very important crop. Clones of disease-free, photoperiodically controllable varieties, well suited as cut flowers, are very desirable and can be profitable. Many hobby growers would like to have in their possession plants of awarded varieties or those that appeal to them in particular. Hence it is not surprising that several techniques have been developed for the clonal propagation of *Cattleya* through tissue culture (Morel, 1964*a*, 1965*a*, 1965*b*, 1971*a*, 1971*b*; Lindemann, 1967*a*, 1967*b*; Marston and Vouraurai, 1967; Reinert and Mohr, 1967; Scully, 1967; Champagnat and Morel, 1969; Kukułczanka, 1969, 1970; Champagnat et al., 1970; Churchill et al., 1970, 1971*a*, 1971*c*, 1972*b*, 1973; Lindemann et al., 1970; Lis, 1970; Arditti et al., 1971, 1972; Ball et al., 1971; Bergman, 1971; Ichihashi and Kako, 1973; Arditti, 1974, 1977*b*). In addition to research reports the literature on *Cattleya* tissue culture also includes papers on morphology, organogenesis, and histology (Vanseveren, 1969); the isolation of protoplasts and nuclei (Capesius and Meyer, 1977); the elimination of virus (Ishii, 1974); and on phenolics produced by explants (Ishii et al., 1976). There are also reviews (Fast, 1973*a*; Morel, 1974; Alang, 1975; Tschauder, 1975; Arditti 1977*a*, 1977*b*; Rao, 1977), popular accounts in magazines (Williams, 1962; Weinstein, 1985), instruction sheets (Wilfret, no date), and privately published mimeographed manuals of lesser value (Jasper, no date). Since a number of techniques have been developed and each offers some advantages, we will outline several procedures here.

Clonal Propagation of *Cattleya* through Shoot-meristem Culture

The procedure is the same as that for *Brassocattleya* (Kako, 1973).

Meristem Culture of *Cattleya*

Solutions for orchid tissue culture contain four component groups: (1) mineral (macro and micro); (2) an energy source (always a sugar, usually sucrose); (3) substances like vitamins and hormones; and (4) undefined complex additives (coconut water, potato extract, banana homogenate, peptone, casein hydrolysate, etc.). The most appropriate combination can be expected to produce the best results. Hence the media used for this procedure include the most suitable components of a number of previously published solutions (Lindemann et al., 1970).

Plant Material. Use enlarging, non-dormant axillary shoots.

Surface Sterilization. Remove dead scales and swab the surface with 95% ethyl alcohol, rinse with distilled water, soak (i.e., sterilize) for 20–30 min in 0.4–0.5% calcium hypochlorite, and rinse in a large volume of sterile distilled water.

Culture Vessels. Use screw-cap vials or Erlenmeyer flasks containing a few milliliters of solution.

Culture Conditions. Details are not given. However, the conditions used in any of the other procedures for *Cattleya* would no doubt be appropriate. Liquid cultures are placed on a shaker (New Brunswick Scientific, Co. Roller Tube Apparatus was used in the original research).

Culture Media. Three media are used: (1) starting (Table C-1); (2) maintenance (Table C-2); and (3) rooting (Table C-3).

Procedure. Remove the bud, sterilize it, and make four longitudinal–tangential cuts with a sterile scalpel to expose the base of the leaf primordia. Next, make horizontal

TABLE C-1. **Starting medium for the culture of *Cattleya* explants (Lindemann et al., 1970)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	1000	100 g l^{-1}	10	Or weigh
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	500	50 g l^{-1}	10	Or weigh
3	Potassium chloride, KCl	1050	105 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	120	12 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	135	13.5 g l^{-1}	10	
6	Iron citrate, $FeC_6H_5O_7 \cdot 3H_2O$[c]	5.4	540 mg l^{-1}	10	
7	**Microelements**[d]				
(a)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.565	565 mg l^{-1}		
(b)	Boric acid, H_3BO_3	1.014	1014 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	0.068	68 mg l^{-1}		
(d)	Cupric sulfate, $CuSO_4 \cdot 5H_2O$	0.019	19 mg l^{-1}	1	One solution
(e)	Aluminium chloride, $AlCl_3$	0.031	31 mg l^{-1}		
(f)	Nickel chloride, $NiCl_2$	0.017	17 mg l^{-1}		
(g)	Potassium iodide, KI	0.099	99 mg l^{-1}		
	Auxin				
8	Naphthaleneacetic acid (NAA)[e]	1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin				
9	Kinetin[f]	0.2	20 mg 100 ml^{-1} 95% ethanol[f]	1	
	Complex additive				
10	Coconut water	150 ml	No stock	No stock	
	Sugar				
11	Sucrose[g]	0.500 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[h]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated with standing. Therefore, stock solutions should not be prepared. If made, keep frozen between uses.

[c]If the substance does not dissolve, add a pellet of KOH. Shake well before dispensing. Dissolve in hot water.

[d]Add all microelements to the same 1 l, stir or heat until all are dissolved, and add 1 ml per liter of culture medium.

[e]If auxin does not dissolve, add a drop or two of KOH. Keep solution refrigerated.

[f]If kinetin does not dissolve, add a few drops of dilute HCl. Keep refrigerated.

[g]The range is 0.5 to 20 g l^{-1}.

[h]Add all components to 600 ml of distilled water (item 12), adjust pH to 5.2–5.5, add the sugar (item 11), and bring volume to 1000 ml with more distilled water (item 12). Then dispense into culture vessels and autoclave.

TABLE C-2. **Standard maintenance medium for proliferating callus cultures of *Cattleya* (Lindemann et al., 1970)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	1000	100 g l^{-1}	10	Or weigh
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	500	50 g l^{-1}	10	Or weigh
3	Potassium chloride, KCl	1050	105 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	120	12 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	135	13.5 g l^{-1}	10	
6	Iron citrate, $FeC_6H_5O_7 \cdot 3H_2O$[c]	5.4	540 mg l^{-1}	10	
7	**Microelements**[d]				
(a)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.565	565 mg l^{-1}		
(b)	Boric acid, H_3BO_3	1.014	1014 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	0.068	68 mg l^{-1}		
(d)	Cupric sulfate, $CuSO_4 \cdot 5H_2O$	0.019	19 mg l^{-1}	1	One solution
(e)	Aluminium chloride, $AlCl_3$	0.031	31 mg l^{-1}		
(f)	Nickel chloride, $NiCl_2$	0.017	17 mg l^{-1}		
(g)	Potassium iodide, KI	0.099	99 mg l^{-1}		
8	**Vitamins**[e]				
(a)	Calcium-D-pantothenate	0.48	48 mg l^{-1}		
(b)	Pyridoxine·HCl (vitamin B_6)	0.21	21 mg l^{-1}		
(c)	Inositol (*myo-* or *iso-*)	18.0	1.8 g l^{-1}		
(d)	Folic acid	4.4	440 mg l^{-1}	10	One solution
(e)	Thiamine·HCl (vitamin B_1)	0.34	34 mg l^{-1}		
(f)	Niacin (nicotinic acid)	1.22	122 mg l^{-1}		
(g)	Biotin	0.024	24 mg l^{-1}		
9	**Amino acids**[f]				
(a)	Glutamic acid	15.0	1.5 g l^{-1}	10	One solution
(b)	Asparagine	13.0	1.3 g l^{-1}		
10	**Nucleotides**[g]				
(a)	Guanylic acid	182.0	18.2 g l^{-1}	10	One solution
(b)	Cytidylic acid	162.0	16.2 g l^{-1}		
	Hormones				
11	Naphthaleneacetic acid (NAA)[h]	0.18	20 mg 100 ml^{-1} 95% ethanol	0.9	
12	Gibberellic acid (GA_3)[i]	0.35	35 mg 100 ml^{-1} 95% ethanol	1	
13	Kinetin[j]	0.22	22 mg 100 ml^{-1} 95% ethanol	1	
14	**Complex additive**[k]				
(a)	Coconut water	50–150 ml			
(b)	Casein hydrolysate	100			
	Sugar				
15	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[l]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated with standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]If the substance does not dissolve, add a pellet of KOH. Dissolve in hot water. Shake well before dispensing.

[d]Add all microelements to the same 1 l, stir or heat until all are dissolved, and add 1 ml liter of culture medium.

[e]Dissolve in the same 1 l to save time in preparation. However, separate solutions to meet solubility requirements can also be made. Aqueous solutions must be kept frozen between uses.

[f]Add both to the same 1 l. Use 10 ml per liter of medium. Separate solutions can also be made. If solubility problems arise, add a few drops of HCl. Keep frozen between uses.

[g]Add both to the same 1 l. Use 10 ml per liter of medium. Although stock solutions can be made, it is better to weigh these substances before use. If stocks are made, keep them frozen.

[h]If auxin (NAA) fails to dissolve, add a few drops of KOH. Keep solution refrigerated.

[i]If GA_3 fails to dissolve, add a few drops of HCl. Keep solution refrigerated.

[j]If the kinetin fails to dissolve, add a few drops of dilute HCl. Keep refrigerated.

[k]Add either coconut water or casein hydrolysate, not both. This improves proliferation.

[l]Mix items 1–13 with 750 ml distilled water (item 16), adjust pH to 5.2, add sugar (item 15), bring the volume up to 1000 ml with more distilled water (item 16), distribute into culture vessels, and autoclave.

TABLE C-3. **Modified Knudson C (Knudson, 1946) medium plantlet formation and root induction in *Cattleya***

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	Or weigh
2	Monopotassium phosphate, KH_2PO_4[c]	250	25 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	Or Weigh
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
6	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.056	56 mg l^{-1}		
(b)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	7.5 g l^{-1}		
(c)	Molybdic acid, MoO_3	0.016	16 mg l^{-1}	1	One solution
(d)	Cupric sulfate (anhydrous), $CuSO_4$	0.040	40 mg l^{-1}		
(e)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.331	331 mg l^{-1}		
	Sugar				
7	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
8	Water, distilled[e]	To 1000 ml			
	Solidifier[e]				
9	Agar	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated with standing. Therefore stock solutions should not be prepared. If made, they must be kept frozen between uses.

[c]A phosphate buffer that will keep the pH steady may be substituted here. Prepare buffer by mixing 975 ml 0.1-*M* KH_2PO_4 (monopotassium phosphate) solution (13.6 g l^{-1}) with 25 ml 0.1-*M* K_2HPO_4 (dipotassium phosphate, 17.4 g l^{-1}); measure pH to be certain it is correct (pH 5.1–5.4), and use 18 ml l^{-1} culture medium.

[d]Add all microelements to the same 1 l of distilled water, stir and/or heat until all are dissolved, and add 1 ml per liter of culture medium.

[e]Add items 1–6 to 800 ml distilled water (item 8), and dissolve. Adjust pH to 5.3, add sugar (item 7), and bring volume up to 1000 ml with more distilled water. Add the agar (item 9), while stirring, to the gently boiling solution. When dissolved, distribute into culture vessels and autoclave.

cuts "above and below the meristem region to provide a 5-mm cube of tissue." This cube will probably contain some non-meristematic tissue as well. Then transfer the explant to a Petri dish containing sterile distilled water where all further operations are performed. Remove all remaining leaf primordia with sterile microscalpels using a dissecting microscope. Great care must be taken to maintain complete sterility of the working space.

Cut the tissue just below the youngest leaf primordium, discard the base, and place the explant into autoclaved starting medium (Table C-1). After about 2 months, or when the explant reaches 2 mm in diameter, it can be quartered and transferred to the second medium (Table C-2). Addition of 5–10% (v/v) coconut water or 100 mg casein hydrolysate per liter speeds up proliferation. Subdivisions of the tissue can be made at monthly intervals. Finally, transfer the PLBs to the solid rooting medium (Table C-3) for root development and plantlet formation. After that, treat the plantlets like seedlings.

Developmental Sequence. Explant enlargement is apparent within 3 days while cultures are maintained on a roller-tube apparatus. After 2 months, the explants reach

2 mm in diameter and are ready for subdivision. Proliferation occurs on the second medium, and callus masses are formed. Roots appear after about 10 days on the rooting (modified Knudson C) medium (Table C-3).

General Comments. Although a bit complicated due to the use of three media, this technique can produce unlimited numbers of plants of a desired clone, Excision of tissue may require a certain amount of practice.

Propagation of *Cattleya* by in Vitro Culture of Bud Meristems

Development of tissue culture methods for *Cattleya* was not as easy as doing the same for *Cymbidium*. More complex media and different culture conditions were required. Even with these media, the mortality rate of explants is high (Morel, 1970).

Plant Material. "Nur die Knospen, die an dem Ansatz junger Trieve sitzen [only the buds from the base of the younger shoots]" can be used (Morel, 1970).

Surface Sterilization. This was not given in the original paper (Morel, 1960). Use the sterilization procedures given in the section on meristem culture (Lindemann et al., 1970).

Culture Vessels. Test tubes (no other details are given) or Erlenmeyer flasks can be used.

Culture Conditions. The test tubes containing liquid medium and explants are placed on a klinostat that rotates at 2 rpm. Best results are obtained in the dark at 25–30°C.

Culture Medium. Several media are suggested, but few details are given about them. One solution that can be used is MS medium. Only the minerals for it are listed, however. In addition to them, the medium must contain auxin, 0.5–1 mg l^{-1} IAA or NAA, and coconut water (no concentration given). Growth is improved when glutamic acid (or glutamine), asparagine, peptone, or casein hydrolysate is added (no concentrations given). Cytokinins are not of much benefit. In addition to MS medium, the minerals of another substrate are also listed. Altogether it seems that the following combinations can be used:

1 Modified MS medium (Table C-4).
2 Another modification of MS medium (Table C-5).
3 Starting medium (Table C-1) followed by maintenance medium (Table C-2).
4 Modified Knudson C medium (Tables C-6 and C-7).

Whatever medium is used, it should be liquid. Once PLBs form, they should be transferred to one of the modifications of the Knudson C medium (Table C-3).

Procedure. Remove buds from the base of young shoots before the shoots reach 5–15 cm. Sterilize and excise the portion to the cultured. Take explants at least

TABLE C-4. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for the culture of *Cattleya* buds**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acids				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
9	Glutamic acid	15	No stock[f]		
10	Asparagine	13	No stock[f]		
	Polyol				
11	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
12	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[g,h]	1	
	Cytokinin				
13	Kinetin	0.2	20 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
14	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
15	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
16	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
17	Coconut water	150	No stock	No stock	Measure
	Sugar				
18	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[i]	To 1000 ml			
	Solidifier				
20	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Suspend the required amount in 1–2 ml 95% ethanol in a small, sterile screw-cap vial. Shake or swirl to ensure that amino acids are dissolved or thoroughly wetted by alcohol, let it stand for 5 min, then shake or swirl again. Repeat this process three times at 5-min intervals. Add suspension to culture medium, as indicated below. If substances remain in vial after it has been emptied into culture medium, wash them out with sterile distilled water.

[g]If auxin or cytokinin does not dissolve, add a few drops of dilute KOH or HCl, respectively.

[h]Keep refrigerated between uses.

[i]Add items 1–7, 11, and 17 to 750 ml distilled water (item 19). Adjust pH to 5.2–5.5, add sugar (item 18), and adjust volume to 1000 ml with distilled water (item 19). Bring solution to a gentle boil, and add agar (item 20) slowly while stirring. Agar can also be added to the cold solution which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acids, hormones, and vitamins (items 8–10 and 12–16) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

TABLE C-5. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for the culture of *Cattleya* buds**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acids				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
9	Glutamic acid[f]	15	No stock	No stock	
10	Asparagine[f]	13	No stock	No stock	
	Polyol				
11	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
12	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[g,h]	1	
	Cytokinin				
13	Kinetin	0.2	20 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
14	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
15	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
16	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
17	Casein hydrolysate	100	No stock	No stock	Weigh
	Sugar				
18	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[i]	To 1000 ml			
	Solidifier				
20	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. Murashige and Skoog (1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Suspend the required amount in 1–2 ml 95% ethanol in a small, sterile screw-cap vial. Shake or swirl to ensure that amino acids are dissolved or thoroughly wetted by alcohol, let it stand for 5 min, then shake or swirl again. Repeat this process three times at 5-min intervals. Add suspension to culture medium, as indicated below. If substances remain in vial after it has been emptied into culture medium, wash them out with sterile distilled water.

[g]If auxin or cytokinin does not dissolve, add a few drops of dilute KOH or HCl, respectively.

[h]Keep refrigerated between uses.

[i]Add items 1–7, 11, and 17 to 750 ml distilled water (item 19). Adjust pH to 5.2–5.5, add sugar (item 18), and adjust volume to 1000 ml with distilled water (item 19). Bring solution to a gentle boil, and add agar (item 20) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acids, hormones, and vitamins (items 8–10 and 12–16) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

TABLE C-6. **Modified Knudson C medium (Knudson, 1946) for the culture of *Cattleya* shoot meristems (Morel, 1965*a*, 1965*b*, 1970)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[c,d]	1 g	100 g l^{-1}	10	Or weigh
2	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Ammonium nitrate, NH_4NO_3[c,d]	500	50 g l^{-1}	10	Or weigh
5	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[d]	500	50 g l^{-1}	10	Or weigh
6	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
7	Potassium chloride, KCl	250	25 g l^{-1}	10	
	Microelement				
8	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Sugar[e]				
9	Sucrose	20 g	No stock	No stock	
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier[e]				
11	Agar	17.5 g	No stock	No stock	Weigh

[a]Only the inorganic salts are listed (Morel, 1965*a*, 1965*b*, 1970; see also Morel, 1974).

[b]Amounts are given in mg unless indicated otherwise.

[c]Either use 1 g ammonium sulfate and omit ammonium nitrate, or use 0.5 g of each.

[d]Solutions containing ammonium and/or nitrate may become contaminated with standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[e]Add items 1–8 to 700 ml distilled water (item 10), and dissolve. Adjust pH to 5.2–5.5, add sugar (item 9), dissolve, and bring volume up to 1000 ml with more distilled water. Distribute into culture vessels and autoclave. For solid culture medium, add agar (item 11) slowly while stirring to gently boiling solution. When fully dissolved, dispense into culture vessels and autoclave.

5 mm in size since the mortality rate of smaller ones is very high. In fact, the entire bud is taken for culture, "of which the two outer scales are removed; the piece still contains 3–6 scales besides the apical meristem" (translated by the author from Morel, 1970). Place explants in the liquid medium because, "it should be mentioned that the growth is accelerated." When the tissue proliferates, it will form PLBs, which should be subcultured onto a modified Knudson C medium (Table C-3) for differentiation and growth.

Developmental Sequence. As first reaction, callus formation is observed on the severed leaf rudiment (or connective tissue of the leaf) where large cells occur that form layers of palisade cells. Later, between these giant hypertrophic cells it is possible to note formation of small meristematic groups that may lie next to the necrotic tissue or near the shoot tip. At the same time the outermost scales become green, and their connective tissues increase in volume and become wavy. After 2 months, meristematic areas appear and eventually form PLBs. These develop into plants.

General Comments. This is a reliable method for the clonal propagation of *Cattleya* even if mortality of explants may be high at times.

TABLE C-7. **Modified Knudson C medium (Knudson, 1946) for the culture of *Cattleya* buds**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
	Iron[c]				
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Amino acids				
7	Glutamic acid	15	No stock	No stock	Weigh
8	Asparagine	13	No stock	No stock	Weigh
	Auxin				
9	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[d]	1	
	Cytokinin				
10	Kinetin	0.2	20 mg 100 ml^{-1} 95% ethanol[d]	1	
	Complex additive				
11	Coconut water	150	No stock	No stock	Measure
	Sugar				
12	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
13	Water, distilled[e]	To 1000 ml			
	Solidifier				
14	Agar[e]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g of chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[d]If auxin or cytokinin fails to dissolve, add a few drops of dilute KOH or HCl, respectively. Keep refrigerated between uses.

[e]Add items 1–6 and 11 to 900 ml distilled water (item 13). Adjust pH to 5.0–5.2, add sugar (item 12), and adjust volume to 1000 ml with distilled water (item 13). Bring solution to a gentle boil, and add agar (item 14) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acids and hormones (items 7–10) to hot, still liquid medium under sterile conditions with sterilized pipettes, and dispense into preautoclaved culture vessels. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary, adjust pH upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower the pH if necessary.

The genus *Cattleya* is named after Mr. William Cattley (d. 1832) of Barnet, England (Schules and Pease, 1963).

Propagation of *Cattleya* by in Vitro Culture of Lateral Bud Meristems

The difficulties encountered with the culture of *Cattleya* explants have spurred research by several groups. One method involves the culture of meristematic tissue from lateral buds (Reinert and Mohr, 1967).

Plant Material. Swollen lateral buds are obtained from rhizomes below new vegetative shoots of *Cattleya*.

Surface Sterilization. Rhizomes are placed in 5% (v/v) Clorox for 10 min, followed by 5 min in 70% ethanol, and concluding with 80 g l^{-1} calcium hypochlorite for 20 min. They are then allowed to dry in a sterilized (i.e., autoclaved) glass container.

Culture Vessels. Use 125-ml Erlenmeyer flasks containing 20 ml medium.

Culture Conditions. Explant-containing flasks are placed on a wheel that revolves at 1 rpm. Photoperiods are 16 h long at an intensity of 200 ft-c provided by four 6-ft cool white fluorescent tubes. The temperature should range between 24 and 28°C.

Culture Medium. A liquid medium (Table C-8) is used for the first 3 weeks. After that, the tissues are transferred to a solid medium (Table C-9).

Procedure. To excise meristems from swollen buds, make longitudinal cuts until the upper surface of the meristem is visible. This is evidenced by a change in color from creamy white to an almost translucent white. Block out the meristem by deep vertical cuts, removing an explant 1–3 mm^3 in size. These sections contain the meristem and small areas of leaf-sheath tissue. Place the explants into culture medium, and position the flasks on the revolving wheel. After 3 weeks, transfer the tissues to the solid medium.

Developmental Sequence. The meristems grow well in the liquid medium with a survival rate of at least 75%. Chlorophyll formation becomes evident fairly soon, and the tissues reach diameters of approximately 3 and 6 mm within 3 and 6 weeks, respectively. Following transfer onto solid medium, some browning may be noted, and growth is slow for the first 2 weeks. Proliferation is evident after 3 weeks. Most explants produce one to three PLBs that grow into plantlets. When the growing plants are cut in half, growth may be slow. Occasionally callus masses may develop; but in many cases each half develops into a new PLB, which eventually forms a plantlet.

General Comments. This method can produce multiplication up to 3000-fold with relative ease and should therefore prove useful.

TABLE C-8. **Reinert–Mohr medium (Reinert and Mohr, 1967) for the initial culture of *Cattleya* bud meristems**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400	40 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	400	40 g l^{-1}	10	
4	Potassium chloride, KCl	500	50 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	22.4	2.24 g l^{-1}	10	One solution
(b)	Ferric sulfate, $Fe_2(SO_4)_3$	10.7	1.07 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.03	3 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.001	0.1 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot H_2O$	7.5	750 mg l^{-1}		
(d)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.03	3 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Organic acid				
9	Citric acid[e]	150.1	No stock	No stock	Weigh
	Auxins				
10	Indole-3-butyric acid (IBA)	1.75	175 mg 100 ml^{-1} 95% ethanol[f,g]	1	
11	Napthaleneacetic acid	1.75	175 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose	15–30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved. If $Fe_2(SO_4)_3$ is not available, make a stock solution containing 3.73 g Na_2EDTA and 2.78 g $FeSO_4 \cdot 7H_2O$ per liter, and add 10 ml of that to the medium.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. Some recipes list $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Available as an anhydrous salt or a monohydrate. It probably matters little which one is used.

[f]If auxins do not dissolve, add a few drops of dilute KOH.

[g]Keep in a freezer or refrigerator between uses.

[h]Add items 1–7 and 9 to 850 ml distilled water (item 16), adjust pH to 5.0, and add sugar (item 15). Sugar is not listed in the original recipe, but this seems to be a typographical error, which is why the usual range is suggested here (a reasonable amount would be 20 g l^{-1}). Adjust volume to 1000 ml with distilled water (item 16). Pour solution into a 2-l flask, and autoclave. Add items 8 and 10–14 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

TABLE C-9. **Solid medium for subculture of *Cattleya* bud meristems (Reinert and Mohr, 1967)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	400	40 g l^{-1}	10	Or weigh
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1000	100 g l^{-1}	10	Or weigh
3	Potassium chloride, KCl	500	50 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	400	40 g l^{-1}	10	
6	**Microelements**[c]				
(a)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	7.5 g l^{-1}	1	One solution
(b)	Boric acid, H_3BO_3	0.03	30 mg l^{-1}		
(c)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.03	30 mg l^{-1}		
(d)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.001	1 mg l^{-1}		
	Organic acid				
7	Citric acid	150	No stock		Weigh
8	**Iron**				
(a)	Ferrous sulfate, $Fe_2(So_4)_3$	10.70	1.07 g l^{-1}	10	One solution
(b)	Na_2EDTA	22.40	2.24 g l^{-1}		
	Vitamins[d]				
9	Thiamine·HCl (vitamin B_1)[d]	0.1	10 mg 100 ml^{-1} 95% ethanol	1	
10	Niacin (nicotinic acid)[d]	0.5	50 mg 100 ml^{-1} 95% ethanol	1	
11	Pyridoxine·HCl (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol	1	
	Amino acid				
12	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Auxins				
13	Indole-3-butyric acid (IBA)	1.75	175 mg 100 ml^{-1} 95% ethanol[f]	1	
14	Naphthaleneacetic acid (NAA)	1.75	175 mg 100 ml^{-1} 95% ethanol[f]	1	
	Cytokinin				
15	Kinetin, 6-furfuryl amino purine[g]	1	100 mg 100 ml^{-1} 95% ethanol[g]	1	
	Polyol				
16	*myo*-inositol	100	10 g l^{-1}[h]	1	Or weigh
	Sugar				
17	Sucrose[i]	15–30 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[j]	To 1000 ml			
	Solidifier[k]				
19	Agar	6 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and nitrate may become contaminated with standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add all microelements to the same 1 l of distilled water; stir and/or heat until dissolved; add 1 ml per liter of culture medium.

[d]If desired, all three vitamins can be dissolved in the same 100 ml and 1 ml of this solution used per liter of culture medium. Keep frozen between uses.

[e]Keep refrigerated or frozen between uses.

[f]If auxins do not dissolve easily, add a few drops of dilute KOH. If desired, the two auxins can be combined in the same 100 ml 95% ethanol. If so, use 1 ml of the combined solution per liter of culture medium. Keep refrigerated.

[g]If cytokinin fails to dissolve, add a few drops of dilute HCl. Keep refrigerated.

[h]The stock solution must be kept frozen between uses.

[i]Sugar does not appear in the recipe (table 1 in Reinert and Mohr, 1967). Since this seems to be a typographical error, an amount in the usual range is suggested (item 17).

[j]For initial solution, mix items 1–14 with 750 ml distilled water (item 18), adjust pH to 5.0, add sugar (item 17), and bring volume up to 1000 ml with more distilled water (item 18). Dispense into culture vessels, and autoclave. To prepare second medium, mix items 1–16 with 750 ml distilled water (item 18), adjust pH to 5.0, add sugar (item 17), and bring volume to 1000 ml with more distilled water (item 18). Agar (item 19) is dissolved by adding it slowly, while stirring, to gently boiling solution. When it is fully dissolved, dispense into culture flasks and autoclave. In this instance all components are autoclaved.

Propagation of *Cattleya* by the Culture of Explants from Vegetative Shoots

When mortality of explants is high, a tissue culture method that does not carry with it the danger of losing an entire plant is very desirable. The utilization of explants from vegetative shoots is one such method. Failure may result in the loss of a shoot, but at least the plant is safe. Once the requirements of each genus are understood, at least partially suitable media can be developed. This, indeed, is the case with explants from vegetative shoots of *Cattleya* (Scully, 1967).

Plant Material. Use lateral meristems removed from vegetative shoots 1–8 cm long.

Surface Sterilization. Shoots are surface-sterilized for 5 min in 20% (v/v) Clorox. After exposure of the lateral buds, the shoots are again sterilized with the same solution, this time for 10 min.

Culture Vessels. Use 50-ml flasks containing 5 ml of liquid solution or 20 ml of solid medium.

Culture Conditions. Liquid cultures are placed on a rotary action shaker (a New Brunswick Model V was used in the original research) operating at 160 rpm under continuous illumination of 100–180 ft-c from fluorescent light (Sylvania Gro Lux or a mixture of cool or warm white plus incandescent bulbs) and a temperature of $26 \pm 4°C$.

Culture Medium. Liquid modified Vacin and Went medium (Vacin and Went, 1949) is used for the initial explants. Tissue masses are subcultured on solidified medium (Table C-10).

Procedure. Remove a vegetative shoot 1–8 cm long, and sterilize it for 5 min. Expose the lateral meristems by removing one to two young leaves, and sterilize for 5 min using the same Clorox solution as before. Make incisions just below the nodal origin and along the two sides of the bud. Separate the explant by making a cut behind the bud itself. Remove all but one of the remaining leaves, and make four vertical incisions and a horizontal one to remove the apical meristem as a 1-mm cube. The volume of explants should range between 1 and 2 mm^3. Collect the explants on sterile moist filter paper in sterilized Petri dishes.

Transfer explants to the liquid medium (Table C-10), and place the cultures on the shaker for 2–5 weeks. When tissue or callus masses form, section and subculture them. Once formation of PLBs starts, the proliferating masses can be divided and cultured on solid medium (Table C-10). If left undisturbed, the PLBs will form fully rooted plants within 6–8 weeks.

Development Sequence. The explant develops into a callus. PLBs are formed that give rise to plantlets.

TABLE C-10. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of explants from vegetative shoots of *Cattleya* (Scully, 1967)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Tricalcium phosphate, $Ca_3(PO_4)_2$[b]	200	20 g l^{-1}	10	Or weigh
2	Potassium nitrate, KNO_3[c]	525	52 g l^{-1}	10	Or weigh
3	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
4	Ammonium sulfate, $(NH_4)_2SO_4$[c]	500	50 g l^{-1}	10	Or weigh
5	Ferric tartrate, $Fe_2(C_4H_4O_6)_3$[d]	28	2.8 g l^{-1}	10	
6	Magnesium sulfate, $MgSO_4 \cdot 4H_2O$	250	25 g l^{-1}	10	Or weigh
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Complex additive				
9	Coconut water	250 ml	No stock	No stock	Fresh
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier[e]				
11	Agar	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]If all of the material does not dissolve, shake stock solution well before dispensing to insure an even suspension; better yet, weigh out each time.

[c]Solutions containing nitrate and/or ammonium may become contaminated with time. Therefore it is preferable not to make stock solutions. If prepared, keep frozen between uses.

[d]If the substance fails to dissolve, add a pellet of KOH and shake well. Also shake well before dispensing.

[e]Mix items 1–7 with 400 ml distilled water (item 10), adjust pH to 5.0–5.2, add the sugar (item 8), and bring volume up to 1000 ml with more distilled water (item 10). If a liquid medium is desired, distribute into culture vessels and autoclave for 10–12 min at 15 lb pressure. When solid medium is needed, bring solution to a gentle boil and add agar (item 11) slowly while stirring; distribute into culture vessels, and autoclave.

General Comments. This is a successful method, but Scully (1967) suggests that every worker may have to make a few modifications. An important point to keep in mind is that the initial culture medium must be liquid and requires shaking.

Clonal Propagation of *Cattleya* through the Culture of Dormant Buds from Backbulbs

The buds of backbulbs are generally dormant and do not seem to contribute much to the horticultural status of plants. For this reason and because when backbulbs are used as a source of explants there is no interference "with the production of shoots and flowers," a procedure for the culture of dormant buds was developed in Thailand (Vajrabhaya, 1978).

Plant Material. Backbulbs and portions of the rhizome are taken from mature plants. Most of the backbulb is removed leaving only 2–3 cm of tissue above the rhizome. Following initial surface sterilization, dissection of the buds is initiated by removing

several layers of leaf primordia. After additional sterilization, more leaf primordia are removed exposing the growing points, which are excised.

Surface Sterilization. Rhizomes and pseudobulbs should be washed several times with tap water and then submerged in a 1 : 10 dilution of Clorox (10 ml of Clorox diluted to 100 ml with distilled water) for 15–30 min. Following this step the rhizomes and pseudobulbs are moved to a 1 : 20 dilution (5 ml Clorox diluted to 100 ml with distilled water). They can remain for up to 1 h without any apparent damage to the tissues. After removal of the initial layers of leaf primordia, the tissues should be dipped for a few seconds in a 1 : 1000 dilution (0.1 ml Clorox diluted to 100 ml with distilled water).

Culture Vessels. Test tubes were used in the original research, but other containers may also be suitable.

Culture Conditions. Cultures should be maintained at 22–25°C under 16 h of diffuse light of 2000–4000 lx. In the original research the illumination was provided by Philips TLF 40W/33 fluorescent lamps, but other sources are also suitable.

Culture Medium. Modified Knudson C medium with the microelements of J. P. Nitsch is used (Table C-11).

Procedure. Place the excised tips on the medium, and allow them to remain until PLBs form. These can be subcultured on the same medium or other modifications of the Knudson C solution (see Tables Aranda-7 and Aranda-8) for plantlet production.

Developmental Sequence. Explants turn green after 2 weeks of culture. The tissue grows slowly during the first 2 months, and their diameters may increase only two to three times. Growth may be more rapid after that, with PLBs forming eventually. These form plantlets.

General Comments. Explants from dormant buds grow more slowly than those from actively growing young shoots. However, they develop in a similar manner. The use of dormant buds from backbulbs (i.e., buds that do not seem to be horticulturally important) more than makes up for the slower initial growth. According to Vajrabhaya (1978) this method is also suitable for "dormant buds of other orchid genera, including those of monopodial species" (but she did not list the other orchids).

TABLE C-11. **Modified Knudson C medium (Knudson, 1946) for the culture of dormant buds of *Cattleya* (Vajrabhaya, 1978)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
	Iron[c]				
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
6	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	1 g	100 g l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	20	2 g l^{-1}		
(c)	Copper chloride, $CuCl_2 \cdot 2H_2O$	10	1 g l^{-1}	10	One solution
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	2 g	200 g l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	20	2 g l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	100	10 g l^{-1}		
	Auxin				
7	2,4-Dichlorophenoxyacetic acid (2,4-D)	1	100 mg 100 ml^{-1} 95% ethanol	1	
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium. The original paper (Vajrabhaya, 1978) lists "Fe EDTA (chelated iron) . . . 0.005 gm."

[d]Add all microelements to the same 800 ml distilled water, adjust volume to 1000 ml with more distilled water, heat and/or stir until the salts are completely dissolved, and allow the solution to cool before dispensing.

[e]Add items 1–6 to 900 ml distilled water (item 9), adjust pH to 5.0–5.2, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour the solution into a 2-l flask and autoclave. Add hormone (item 7) to the hot, still liquid solution under sterile conditions with sterilized pipettes, swirl several times to mix well, and dispense into preautoclaved culture vessels. According to Knudson (1946), the pH of his solution without agar (i.e., liquid) can be close to 4.6–4.7. If necessary, adjust pH upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower the pH if necessary.

Cattleya Propagation by Tissue Culture

Considerable variability exists between media used for the propagation of even a single group of orchids. In addition, most procedures depend heavily on protocorm proliferation, a process that may lead to "significant incidences of genetically aberrant plants." A series of experiments designed to develop more uniform media of wider applicability and to enhance axillary branching rather than proliferation of PLBs was carried out at the University of California, Riverside, by a visitor from the Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan, with advice from

Toshio Murashige (one of the originators of the widely used MS medium; Huang, 1984).

Plant Material. Shoot apex explants taken from newly emerging shoots (2–5 cm in length with none of the leaves unfurled) of mature plants are preferable, but lateral and/or dormant buds can also be used. The outer leaves are removed to expose the terminal portion of the stem before washing the apices. After the washing, terminal portions of the stem (ca. 1 cm in length) and larger terminal buds are stored in a solution of 100 mg l^{-1} ascorbic acid and 150 mg l^{-1} citric acid until preliminary trimming of all shoots is completed. Following this step the apices are trimmed further until they are 4–5 mm long and wrapped in cheesecloth for sterilization. Additional emerging leaves (one or more) and the subjacent stem are removed from the sterilized apices under aseptic conditions with sterile scalpels. The small explants (2–3 mm long) are sterilized again before being placed in the starting medium.

Surface Sterilization. Explants are washed with tap water and a mild household detergent after removal from the plant. Following preliminary trimming, explants from the ascorbic acid–citric acid solution are immersed in a 1 : 10 dilution (10 ml diluted to 100 ml with distilled water) of Purex (or another household bleach containing 5–5.25% sodium hypochlorite) for 20 min under vacuum. After that the explants should be washed three times with sterile distilled water. The explants to be cultured (2–3 mm long) are placed for 1 min in the 1 : 10 Purex and then dipped in a 1 : 1000 dilution of the bleach (1 ml diluted to 1 l with sterile distilled water) before being placed in the medium.

Culture Vessels. Standard culture tubes, 25 × 150 mm, containing 5 ml of starting medium (Table C-12) or 25 ml multiplication medium (Table C-13) capped with propylene closures (Kaputs made by Belco Glass, Inc., Vineland, NJ) were used in the original experiments, but other vessels can also be employed. For root induction, plants were placed in autoclavable plastic Magenta GA7 culture containers (Magenta, Inc., Chicago, IL), containing 100 ml of rooting medium (Table C-14), but Erlenmeyer flasks and other culture vessels are also suitable.

Culture Conditions. Starting cultures are maintained under 1000 lx illumination for 16-h photoperiods. They should be rotated continuously at 1 rpm at 10° from the horizontal. Rooting-stage plants should be moved to 6000–7000 lx. No information is given regarding the light sources, but general experience with *Cattleya* suggests that fluorescent, incandescent, or a combination of the two is suitable. The temperature was a constant 27°C during the original experiments, but it is reasonable to assume that 22–29°C would also be appropriate.

Culture Media. Three media, all based on MS medium, are used: starting (Table C-12), shoot multiplication (Table C-13), and rooting (Table C-14).

Procedure. Allow explants to remain on the starting medium (Table C-12) for 1–4 months (length depending on the hybrid). They may have to be subcultured once or twice on the same solution until they reach a length of at least 1 cm and develop

TABLE C-12. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for use as the starting solution in the culture of *Cattleya* explants (Huang, 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	495	49.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	132	13.2 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	111	11.1 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	570	57 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	51	5.1 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	α-Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin and related substance				
11	Adenine sulfate$\cdot 2H_2O$	10	1 g 100 ml^{-1} 95% ethanol	1	
12	N^6-benzyladenine (BA)	1	100 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
15	Thiamine (vitamin B_1)	1.0	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Complex additive				
17	Coconut water from green nuts	150	No stock	No stock	Measure
	Solvent				
18	Water, distilled[h]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin does not dissolve, add a few drops of dilute KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–7, 9, and 17 to 750 ml of distilled water (item 18). Adjust pH to 5.7, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 18). Pour solution into a 2-l flask, and autoclave. Add amino acid, hormones, and vitamins (items 8 and 10–15) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

TABLE C-13. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for the culture of *Cattleya* shoots (Huang, 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	α-Napthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin and related substance				
11	Adenine sulfate·$2H_2O$	30	3 g 100 ml^{-1} 95% ethanol	1	
12	N^6-benzyladenine (BA)	1	100 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
16	Coconut water from green nuts	159	No stock	No stock	Measure
	Sugar				
17	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[h]	To 1000 ml			
	Solidifier				
19	Agar, TC[i]	8 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin does not dissolve, add a few drops of dilute KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–7, 9, and 16 to 750 ml distilled water (item 18). Adjust pH to 5.7, add sugar (item 17), and adjust volume to 1000 ml with distilled water (item 18). Bring solution to a gentle boil, and add agar (item 19) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid, hormones, and vitamins (items 8 and 10–15) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

[i]TC agar is of relatively high purity and available from KC Biological, Inc., Lenexa, KS. Other higher-purity agars are probably also suitable. Gelrite (Kelco Division, Merck & Co., San Diego, CA), 0.2%, can also be used.

TABLE C-14. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for the rooting of *Cattleya* shoots (Huang, 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Micropotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.3	30 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin-related substance				
11	Adenine sulfate·$2H_2O$	30	3.0 g 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	1.0	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, TC[i]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin does not dissolve, add a few drops of KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–7, and 9 to 900 ml distilled water (item 16), adjust pH to 5.7, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid, hormones, and vitamins (items 8 and 10–14) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

[i]TC agar is of relatively high purity and available from KC Biological, Inc., Lenexa, KS. Other higher-purity agars are probably also suitable. Gelrite (Kelco Division, Merck & Co., San Diego, CA) 0.2%, can also be used.

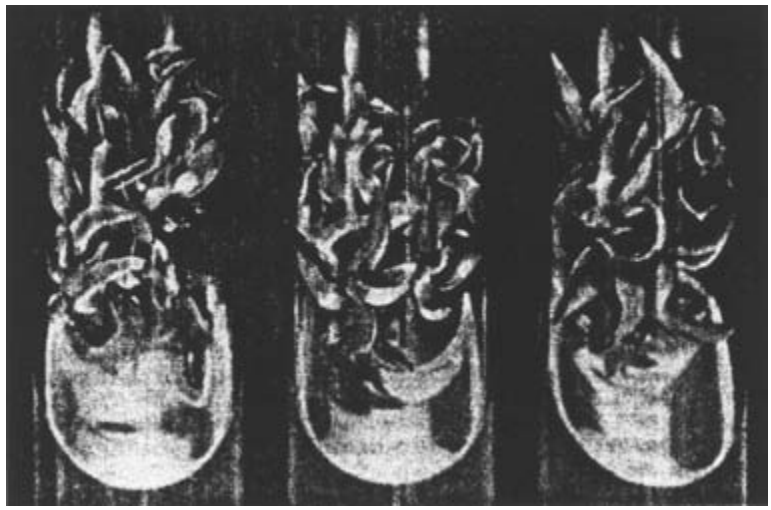


FIG. C-1 **Increase of *Cattleya* shoots by enhanced axillary branching.**

easily observable leaves. At this stage of development, transfer them to the shoot-multiplication medium (Table C-13), where axillary branching takes place and the number of shoots increases (Fig. C-1). A large number of shoots can be obtained after four to five subdivisions. Move individual shoots to the rooting medium (Table C-14) for root formation and plantlet development. Plantlets with several vigorous roots and large leaves can be moved to potting mix.

Developmental Sequence. Explants elongate and form leaves on the starting medium (Table C-12), branch on the multiplication solution (Table C-13), and form roots on the rooting formulation (Table C-14).

General Comments. This procedure may eliminate the undesirable variations that sometimes occur following excessive proliferation of PLBs. During the original research a minimum of 500 plantlets were obtained from each explant after 10 months.

Vegetative Multiplication of *Cattleya* Seedlings through the Culture of Leaf Bases

The ideal method for clonal propagation would utilize an organ or small mass of tissue that can be removed without damaging or endangering the plant. Leaves are one obvious source for such tissues. Meristems at the base of *Cattleya* leaves can be used to produce PLBs (Champagnat et al., 1970).

Plant Material. Leaves are removed from seedlings maintained in sterile culture.

Surface Sterilization. This is not necessary.

TABLE C-15. **Culture medium for *Cattleya* leaf bases (Champagnat et al., 1970)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements[b]				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	1 g	100 g l^{-1}	10	Or weigh
2	Potassium chloride, KCl	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	125	12.5 g l^{-1}	10	
4	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	500	50 g l^{-1}	10	Or weigh
5	Monopotassium phosphate, KH_2PO_4	125	12.5 g l^{-1}	10	
	Microelements of Heller				
6	*Solution A*[c]				
(a)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1	1 g l^{-1}	1	One solution
(b)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$[d]	0.010	10 g l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.030	30 mg l^{-1}		
(d)	Aluminium chloride, $AlCl_3$	0.030	30 mg l^{-1}		
(e)	Nickel chloride, $NiCl_2 \cdot 6H_2O$	0.030	30 mg l^{-1}		
(f)	Potassium iodide, KI	0.010	10 mg l^{-1}		
	Solution B				
7	Ferric chloride, $FeCl_3 \cdot 6H_2O$	1	1 g l^{-1}	1	
	Cytokinin				
8	Kinetin	1	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier[f]				
11	Agar	8 g	No stock	No stock	

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions are not advisable. If prepared, they must be kept frozen.

[c]Add all salts to the same 1 l. Stir or heat until dissolved and use 1 ml per liter of medium.

[d]As given in Champagnat et al. (1970), this solution contains magnesium, but no manganese. Since the medium already contains magnesium (item 3), a correction was made in the usual range.

[e]If cytokinin fails to dissolve, add a few drops of dilute HCl. Keep refrigerated.

[f]Mix items 1–8 with 700 ml distilled water (item 10), adjust pH to 5.3, add sugar (item 9), and bring volume to 1000 ml with more distilled water (item 10). To dissolve agar (item 11), bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Dispense the solution into the culture vessels, and autoclave.

Culture Vessels. Use 5-cm-diameter Petri dishes containing medium to a height of 5 mm; or test tubes and Erlenmeyer flasks filled with medium to one-fifth of their volume.

Culture Conditions. Use the same as for germinating seeds of *Cattleya* or its meristems (Morel, 1971*b*, Morel*c*, 1974).

Culture Medium. The usual salts (including the microelements of Heller's medium), plus 1 mg l^{-1} kinetin solidified with 8 g l^{-1} agar (Table C-15).

Procedure. Under aseptic conditions, remove leaves 6–10 mm long from seedlings growing in flasks. Excise and discard the axillary meristem from the base, but culture the rest. When PLBs form, subculture.

Developmental Sequence. When the axillary meristem at the base of a leaf is left in place, it starts to grow and inhibits the formation of adventitious buds. If it is removed, numerous PLBs form at the cut end and on the leaf blade.

General Comments. This is a simple, easy, and effective method. However, since the tissues to be cultured are obtained from seedlings, the procedure cannot be used for the propagation of established clones. Nevertheless, it should prove useful in cases where only very few seedlings can be obtained from a cross. It can also be used in cases where only a limited number of plantlets can be obtained from a meristem.

Clonal Propagation through the Culture of *Cattleya* Leaf Tips

In 1967 Ernest A. Ball, who first cultured meristems (Ball, 1946), gave a lecture at the University of California, Irvine, about the culture of leaf parenchyma cells. He had succeeded in producing whole plants (but not orchids) from them. This suggested to me (JA) that perhaps orchid leaf cells could be cultured. When Ernest Ball joined our department in 1968, we decided to try. The work was done by an undergraduate named Mary-Ellen Farrar (who, by the time her project was completed, had become Mrs. Churchill). We tried to cultivate all parts of a leaf but succeeded only with tips, and even those did not always grow. But this method barely damages the plant and therefore approaches an ideal procedure (Arditti et al., 1971; Ball et al., 1971; Churchill et al., 1971*a*, 1971*c*, 1972*a*, 1972*b*, 1973).

Plant Material. Leaf tips are obtained from young leaves on old plants before the appearance of the characteristic notch.

Surface Sterilization. Tips are sterilized for 10 min in saturated calcium hypochlorite solution. To prepare the solution, mix 10 g calcium hypochlorite with 140 ml water. Stir well, allow to stand for a few minutes, and stir again. Repeat three times, and filter or decant the supernatant, which is the sterilizing solution. Use within 6 h.

Culture Vessels. We use 125-ml Erlenmeyer flasks containing 25 ml medium for both liquid and solid media.

Culture Conditions. Liquid cultures are placed on a reciprocating shaker (60 oscillations per minute) at 22–25°C under 18-h photoperiods. The light intensity produced by a combination of two 40-W Sylvania Gro Lux tubes and two 50–100-W incandescent bulbs per fixture placed 50 cm above the cultures should be 150 ft-c. Solid cultures may be maintained on a table top or shelf under the same conditions. After transfer to Knudson C medium, the cultures are maintained under 12-h 22–25°C photoperiods, 36 cm from the same light sources.

Culture Media. Tips are cultured and callus formation occurs in liquid Heller's medium (Table C-16). These calli consist of PLBs. Proliferation continues very well, without much differentiation on solid MS (Table C-17) or Linsmaier–Skoog (Table C-18)

TABLE C-16. **Modified Heller's medium (Heller, 1953) for the culture of tips from young leaves of mature *Cattleya* plants (Churchill et al., 1971*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Potassium chloride, KCl	750	75 g l^{-1}	10	
2	Sodium nitrate, $NaNO_3$[b]	600	60 g l^{-1}	10	Weigh
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	125	12.5 g l^{-1}	10	
5	Calcium chloride, $CaCl_2 \cdot 2H_2O$	75	7.5 g l^{-1}	1	
	Iron				
6	Ferric chloride, $FeCl_3 \cdot 6H_2O$	1	1.0 g l^{-1}	1	
7	**Microelements**[c]				
(a)	Boric acid, H_3BO_3	1	1 g l^{-1}		
(b)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1	1 g l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	0.01	10 mg l^{-1}		
(d)	Cupric sulfate, $CuSO_4 \cdot 5H_2O$	0.03	30 mg l^{-1}	1	One solution
(e)	Aluminum chloride, $AlCl_3$	0.03	30 mg l^{-1}		
(f)	Nickel chloride, $NiCl_2 \cdot 6H_2O$	0.03	30 mg l^{-1}		
(g)	Potassium iodide, KI	0.01	10 mg l^{-1}		
	Auxin				
8	2,4-D	1	30 mg 30 ml^{-1} 95% ethanol[d]	1	
	Cytokinin				
9	6-Benzylaminopurine (benzyladenine)	500 µg	30 mg 30 ml^{-1} 95% ethanol[d]	0.5	
	Vitamin				
10	Thiamine (vitamin B_1)	1	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
11	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[f]	To 1000 ml			
	Solidifier[f]				
13	Agar	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing nitrate tend to become contaminated. Therefore, it is better to weigh the salt each time. If prepared, stock solutions must be kept frozen.

[c]Add all microelements to the same 1 l of distilled water. Stir or gently heat the solution until all salts are dissolved.

[d]If the auxin or cytokinin does not dissolve, add a few drops of dilute KOH or HCl, respectively.

[e]Keep refrigerated.

[f]Mix items 1–10 with 800 ml distilled water (item 12), adjust pH to 5.3, add sugar (item 11), and bring the volume to 1000 ml with more distilled water (item 12). If a liquid medium is required, distribute into culture vessels and autoclave. When a solid medium is needed, bring solution to a gentle boil and add agar (item 13) slowly while stirring. After agar has dissolved, distribute the medium into culture flasks and autoclave.

media. PLBs must be transferred to solid modified Knudson C medium (Tables C-3, C-6, and C-19) for plantlet production. Addition of banana homogenate (Arditti, 1967) can accelerate growth. Use of a flasking tool designed for seeds (Arditti, 1968) may facilitate transfers but is not required.

Procedure. Remove tips 15–25 mm long from young leaves with a sharp scalpel or razor blade and place on wet filter paper (or paper towels) in a closed container. Following surface sterilization and rinsing in sterile distilled water, remove the bottom part, leaving a tip 4–5 mm in length. Then place this tip in culture.

TABLE C-17. **Murashige–Skoog medium (Murashige and Skoog, 1962) as used for the maintenance of callus cultures derived from *Cattleya* leaf tips (Churchill et al., 1971)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc chloride, $ZnCl_2$	3.93	393 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Auxin				
9	Indoleacetic acid (IAA) or naphthaleneacetic acid (NAA)	1	1000 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
10	Kinetin	2.6	260 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamin				
11	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
12	Coconut water[e,h]	100–250	No stock	No stock	Measure
	Sugar				
13	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[i]	To 1000 ml			
	Solidifier				
15	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If auxin or cytokinin does not dissolve, add a few drops of KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Liquid from immature (green) nuts is preferable.

[i]Add items 1–7 and 12 to 700 ml distilled water (item 14), adjust pH to 5.2–5.5, add sugar (item 13), and adjust volume to 1000 ml with distilled water (item 14). Bring solution to a gentle boil, and add agar (item 15) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid, hormones, and vitamin (items 8–11) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

TABLE C-18. **Linsmaier–Skoog medium (Linsmaier and Skoog, 1965) as used for the maintenance of callus cultures derived from *Cattleya* leaf tips (Churchill et al., 1971*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1.65	82.5 g l^{-1}	20	Or weigh
2	Potassium nitrate, KNO_3[b]	1.9	95 g l^{-1}	20	Or weigh
3	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	18.5 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	8.5 g l^{-1}	20	
6	**Chelated iron**				
(a)	Na_2EDTA	37.3	3.70 g l^{-1}	10	One solution
(b)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.80 g l^{-1}		
7	**Microelements**[c]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(c)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	9	9 g l^{-1}		
(d)	Potassium iodide, KI	0.83	83 mg l^{-1}	10	One solution
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(f)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(g)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100	No stock	No stock	Weigh
	Cytokinin				
9	Kinetin	0.1	20 mg 100 ml^{-1} 95% ethanol[d]	0.5	
	Auxin				
10	Indoleacetic acid (IAA)	2	200 mg 100 ml^{-1} 95% ethanol[e]	1.0	
	Vitamin				
11	Thiamine·HCl (vitamin B_1)	0.4	80 mg 100 ml^{-1} 95% ethanol[f]	0.5	
	Sugar				
12	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
13	Water, distilled[g]	To 1000 ml			
	Solidifier[g]				
14	Agar	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing nitrate and/or ammonium tend to become contaminated. Therefore it is preferable not to make stock solutions. If prepared, they must be kept frozen.

[c]Add all microelements to the same 1 l, and stir and/or heat until dissolved. Add 10 ml per liter of culture medium.

[d]If there are difficulties in dissolving kinetin, add a few drops of dilute HCl. Keep stock solution refrigerated.

[e]If there are difficulties in dissolving IAA, add a few drops of KOH. Keep stock solution refrigerated.

[f]Keep the stock solution refrigerated.

[g]Mix items 1–11 with 700 ml distilled water (item 13), adjust pH to 5.5, and add sugar (item 12). Bring solution to a gentle boil, and add agar (item 14) slowly while stirring. When agar is completely dissolved, distribute medium into culture flasks and autoclave. However, it is preferable to mix items 1–8 with 700 ml distilled water (item 13), adjust pH to 5.5, add sugar (item 12), and bring volume to 1000 ml. Then add agar and autoclave. While solution is still hot, add a mixture of items 9–11 under sterile conditions. Swirl to mix, and distribute into preautoclaved culture vessels.

Developmental Sequence. Approximately 45 days after being placed in culture, the tip begins to proliferate. Soon thereafter, the original tissue dies and turns brown. Within 10–15 weeks, masses consisting of PLBs form. When these start to produce vegetative apices and leaves, transfer them to solid MS or Linsmaier–Skoog medium to increase proliferation. Plantlet formation occurs on Knudson C medium

TABLE C-19. **Modified Knudson C medium (Knudson, 1946) for the differentiation of plantlets from *Cattleya* leaf callus (Churchill et al., 1971*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	Or weigh
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
3	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	Or weigh
4	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$[c]	25	2.5 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4[d]	250	25 g l^{-1}	10	
6	**Microelements**[e]				
(a)	Boric acid, H_3BO_3	0.056	56 mg l^{-1}	1	One solution
(b)	Molybdic acid, MoO_3	0.016	16 mg l^{-1}		
(c)	Cupric sulfate, $CuSO_4$	0.040	40 mg l^{-1}		
(d)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.331	331 mg l^{-1}		
	Sugar				
7	Sucrose	20 g	No stock	No stock	Weigh
	Organic additive[f]				
8	Banana, ripe	150 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[f]	To 1000 ml			
	Solidifier[f]				
10	Agar	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate tend to become contaminated upon standing. Therefore it is better to weigh items 1 and 3 each time. If stock solutions are prepared, keep frozen.

[c]This solution tends to form a rust-colored precipitate on standing. Therefore shake before using.

[d]A phosphate buffer that will keep the pH steady may be substituted here. Prepare buffer by mixing 975 ml 0.1-*M* KH_2PO_4 (monopotassium phosphate; 13.6 g l^{-1}) with 25 ml 0.1-*M* K_2HPO_4 (dipotassium phosphate; 17.4 g l^{-1}). Measure the pH to be certain it is correct (pH 5.1–5.4), and use 18 ml per liter of culture medium.

[e]Add all microelements to the same 1 l of distilled water, stir and/or heat until dissolved, and add 1 ml per liter of culture medium.

[f]Add items 1–8 to 700 ml distilled water (item 9). Homogenize until banana (item 8) is completely broken down (about 2–3 min), adjust pH to 5.3, and bring volume to 1000 ml with more distilled water. To dissolve agar (item 10), add it slowly while stirring to gently boiling solution. When dissolved, distribute medium into culture vessels and autoclave.

(Table C-19). Once placed on this medium, treat the PLBs and the resulting plantlets like seedlings (Arditti et al., 1982).

General Comments. Tips of young leaves from mature plants can be cultured, but the rate of success is not always high. Formation of callus apparently depends on the presence of meristematic or undifferentiated tissue in the leaf tip. If such tissue is no longer present, there is no callus development. Appearance of the characteristic notch in *Cattleya* leaves indicates their tips will no longer form calli. Unfortunately, the potential to form a callus is lost even before the notch becomes evident, but the time at which this happens is not easily apparent. The low rate of success is due to this fact. Hence it is important to obtain tips from very young leaves. Also, instructions must be followed exactly.

This method was developed to eliminate the need to sacrifice entire plants or shoots and to provide a simple procedure for clonal propagation of *Cattleya*. In this we have succeeded since less skill is required than for shoot-tip ("meristem") cultures, and damage to the plant is minimal. It is worth noting that at least one orchid, *Malaxis paludosa*, produces foliar embryos (Taylor, 1967).

Clonal Propagation of *Cattleya* through the Culture of Leaf Tissues

Leaf tips and bases of mericlone plantlets of *Cattleya bowringiana* × *Cattleya forbesii* were cultured using the method employed for *Aranda* (Fu, 1978, 1979*b*). Only leaf bases proliferated, which suggests that this method can be used for clonal propagation. However, the need for a clonal propagation method for "mericlone plantlet[s]" is questionable.

Isolation of *Cattleya* Protoplasts

Protoplasts of "*Cattleya schombocattleya*" [sic] can be isolated by the method employed for *Brassia maculata* (Capesius and Meyer, 1977). The name used in the original paper presents a problem since it could refer to a *Cattleya* × *Schombocattleya* hybrid, a *Cattleya*, a *Schombocattleya*, or it could be an error. Those intending to use this procedure should proceed with caution.

Isolation of *Cattleya* Protoplasts from Protocorms, Leaves, and Roots

Isolated protoplasts of orchids could be used to produce interesting new hybrids through fusion and advanced plant bioengineering research. A method for the isolation of such protoplasts of several orchids including *Cattleya* was developed at Cornell University (Price and Earle, 1984).

Plant Material. Six-month-old seedlings of *Cattleya aurantiaca* were used in the original research as sources of protocorms, leaves, and roots.

Surface Sterilization. There is no need to sterilize seedlings since they are growing under aseptic conditions. It is important to keep in mind that all steps of the isolation and fusion of protoplasts must be carried out under sterile conditions.

Culture Vessels. The vessels needed for *Cattleya* are the same as those used for *Angraecum giryamae*.

Culture Conditions. See *Angraecum* entry, Isolation of Protoplasts from Green Sepals of *Angraecum giryamae* (p. 167, Vol. I).

Culture Media. For the isolation of protoplasts the tissues are incubated in an enzyme solution consisting of 2% Cellulysin (Cal Biochem, P.O. Box 12087, La Jolla, CA 92112), 0.5% Driselase (Fluka AG Bucks, Switzerland CH 9470, 980 South Second St., Ronkonkoma, New York), and 1% Macerozyme (Kanematsu-Geosho, Kinki Yalkut Mfg Co., Ltd., 8–21 Shingikau, Nishinomiya, Japan) in a solution of 36.4 g mannitol and 11.8 g calcium chloride ($CaCl_2 \cdot 2H_2O$) per liter. This medium must be sterilized through filtration (Millipore Filter Corp.; see Appendices 1 and 2). It may not be autoclaved. All other media and solutions are the same as those used for *A. giryamae*.

Procedure. Incubate protocorms and seedling roots in the enzyme solution for 2–4 days. Incubate leaves in the same solution for 2 days. All other procedures are the same as those used for *A. giryamae*.

Fusion experiments were not carried out with *Cattleya*, but a good starting point would be the method used for *Dendrobium*.

Developmental Sequence. Protoplast yield per gram of fresh weight of the source tissue was "very few" from protocorms and roots and "few" from leaves. There are no reports of callus formation and plantlet regeneration from *Cattleya* protoplasts.

General Comments. When refined and made practical this will be a useful procedure.

Isolation, Culture, and Fusion of *Cattleya* Protoplasts

A large number (5.5×10^5 to 3.1×10^6) of protoplasts can be isolated from leaf mesophyll of *Cattleya* Wine Festival, but seedling cultures were a better source (Gopalakrishnan and Seeni, 1987).

Clonal Propagation of *Cattleya* through Tissue Culture Methods

Several experiments were carried out to determine the effects of a number of factors on the survival of shoot-tip explants. According to the summary (which is in English; the paper is in Japanese) the following were observed (Ichihashi and Kako, 1973):

1. Either NAA or 2,4-D is essential for initiating growth of explants. The optimal concentration of each is 0.1 mg l^{-1}.
2. A culture medium described as RM-1962 in liquid form is better for survival and growth than Knudson C. The major reason for the effectiveness of the former medium may be its inorganic components. "RM" suggests that the medium may be the one formulated by Reinert and Mohr, but their paper was published in 1967. The MS medium was published in 1962. Both are cited in the paper.
3. The effects of coconut water (100 ml l^{-1}) varied depending on the hybrid.
4. The best survival rates and least browning occurred in liquid medium that was not agitated in any manner or in one that was rotated at 0.86 rpm.
5. Explants from middle or lower nodes survived better than those from upper parts of the shoot or apical buds.
6. Growth responses of several hybrids and species were different even when "cultured under the same conditions."

Effects of Growth Regulator and Organic Supplement Combinations on PLBs and Plantlets of *Cattleya*

Effective combinations of plant hormones for plantlet growth are 0.1–1.0 mg kinetin and 1.0–5.0 mm l^{-1} NAA or 0.1–0.5 mg kinetin and 0.1 mg 2,4-D per liter.

Shoot formation is enhanced by 1.0 mg BA and 0.5 mg NAA per liter or 0.1 mg kinetin and 0.1 mg 2,4-D per liter, or 0.1 mg BA and 0.1 mg 2,4-D per liter. Plantlet growth is best in the presence of 0.1–1.0 mg kinetin and 1.0–5.0 mm NAA per liter or 0.1–0.5 mg kinetin and 0.1 mg 2,4-D per liter. Proliferation of protocorms is enhanced by 5.0 mg BA and 0.1 mg NAA per liter.

Banana juice stimulated protocorm development and proliferation. Coconut water ("coconut milk") also enhanced proliferation of protocorms and stimulated shoot development. Yeast extract inhibited shoot formation (from the English summaries in Kusumoto 1979*a*, 1979*b*).

Protocorm and Plantlet Formation in *Cattleya*

Some *Cattleya* explants tend to turn brown after about 10 days in culture. Those that do not, form PLBs on a Hyponex medium. Plantlet formation is good on a Hyponex medium that contains apple or potato juice (from the English summaries in Yoneda et al., 1979, 1989).

Micropropagation of *Cattleya* Almakee through Root Explants

Professor S. P. Vij has carried out extensive research on the culture of orchid root explants. In one of his programs he attempted to culture root explants of 21 orchid species and hybrids. He was successful with only 12 of them. *Cattleya* Almakee was one of them (Fig. C-2), but only barely because just two of 25 explants responded (Vij, 1993). However, each of these produced 25 propagules.

Plant Material. Young and actively growing roots from 16–30-week-old axenic seedlings should be used. Roots from mature plants die after 8–10 weeks in culture.

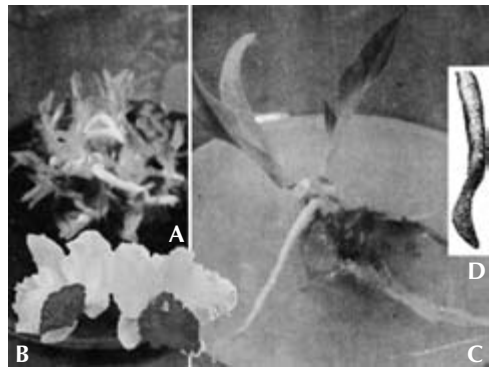


FIG. C-2. ***Cattleya*** **Almakee and the culture of its root tips. A. Proliferating plants. B. Flowers. C. Plantlet derived from the root tip. D. Root-tip explant. (Sources: A, C, D, Vij, 1993; B, www.solor.com.br/~bmdamiao/cattleyas.htm.)**

Surface Sterilization. No surface sterilization is needed because the roots are taken from axenically grown plants. However, they should be rinsed thoroughly with sterile distilled water to remove medium residues.

Culture Vessels. Culture vessels are not described in the original paper. Culture tubes 20 mm in diameter, about 125–150-ml capacity, and containing 30 ml of culture medium should be appropriate. Other vessels can also be used.

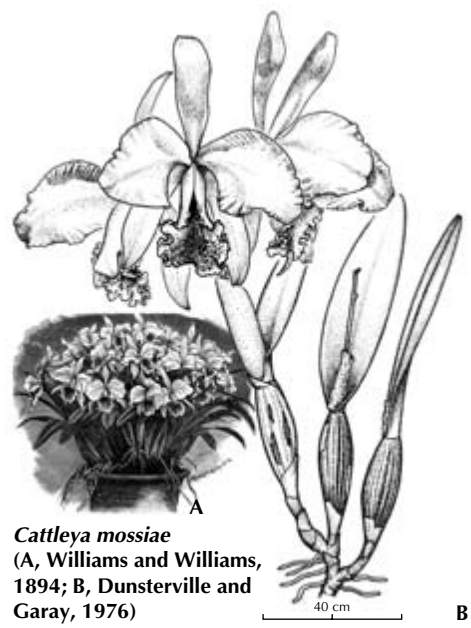
Culture Conditions. In the original experiments, cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx.

Culture Media. A modified Mitra, Prassad, and Roychowdhury medium (Mitra et al., 1976) containing kinetin (6-furfuryl, 1-aminopurine) and NAA induced the formation of PLBs and subsequently leaves, roots, and plantlets (see Table Aer-5).

Procedure. After removal from plants, the roots are placed on the medium (Table C-20). Well-developed PLBs or small plantlets should grow well on the Knudson C (see Tables Cym-2 and Cym-3), Tsuchiya (see Table Cym-4), Vacin and Went (see Table Cym-5) or basal MS (see Table Aer-2) media.

Developmental Sequence. The first leaves and roots are formed after 15 and 22 weeks of culture, respectively.

General Comments. When roots tip (or any) explants are taken from seedlings, clonal propagation of specific cultivars is not possible. However, this method can be employed to propagate orchids when only a few seeds are available and therefore not many seedlings can be obtained.



Cattleya mossiae
(A, Williams and Williams, 1894; B, Dunsterville and Garay, 1976)

TABLE C-20. **Mitra, Prassad, and Roychowdry medium (Mitra et al., 1976) as modified for the culture of root tips of *Cattleya* Almakee (Vij, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneleacetic acid (IAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
15	Kinetin	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[f]	To 1000 ml			
	Solidifier				
18	Agar[f]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original recipe calls for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.6, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule, media that contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976; Vij, 1993).

Micropropagation of *Cattleya mossiae*

Easily one of the most beautiful *Cattleya* species in existence, *Cattleya mossiae* is endemic to Venezuela where it is currently endangered. Therefore, research intended to develop a micropropagation method was undertaken at the Universidad Centroccidental "Lisandro Alvarado" in Barquisimento, Venezuela (Torres and Mogollón, 1998, 2000).

Plant Material. Apical and axillary buds can be used.

Surface Sterilization. Two surface sterilization steps were used in the original research. First, apical shoots, rhizomes, and pseudobulbs were submerged with agitation for 30 min in a mixture of antiseptic soap (4 ml l^{-1}) and 20% (20 ml made up to 100 ml with distilled water) Clorox (or other household bleach containing 5.25% sodium hypochlorite; 17.5 ml should be used if the bleach contains 6.0% sodium hypochlorite). Except for its action as a surfactant, the antiseptic soap seems superfluous in the presence of Clorox. Therefore any laboratory or household detergent can be added to the 20% Clorox. The second step was to submerge the plant material in 10% (10 ml of 5.25% or 8.8 ml of 6% product made up to 100 ml with distilled water) Clorox for 5 min. A sterile distilled water wash was not used in the original research, but several such washes are highly recommended.

Culture Vessels. Test tubes were used for the initial 48 h in the original research. Tubes or other vessels can be employed after that.

Culture Conditions. In the original research, the cultures were maintained at $25 \pm 2°C$ under 16-h photoperiods of 900 lx (the source of illumination was not described). Standard culture room conditions are also suitable. According the English summary in one of the original papers (Torres and Mogollón, 1998), the explants were placed "on filter paper bridges." The materials and methods section in the body of the paper states that during "*Las primeras 48 horas* [the tubes] *fueron agitadas en un tambor rotatorio a 1 rpm*" (were agitated on a 1-rpm drum for the first 48 h). The chances are that both of these steps serve no useful purpose, complicate the process, and can be skipped. The explants can be placed on an agar medium from the outset. Or, if explants are to be agitated, this should certainly be done for longer than 48 h. For rooting, shoots were placed on membrane rafts (Life Raft™, www.osmotek.com, osmotek@ATTglobal.Net) and floated on liquid medium (Fig. C-3). This also seems to be an unnecessary complication. Placing shoots on an agar medium will be just as appropriate. Other procedures for *Cattleya* and many additional orchids use agar media effectively.

Culture Media. In the original research, explants were first placed on a liquid modification (Huang, 1984) of the MS medium (Murashige and Skoog, 1962) either on filter paper bridges or in the liquid itself with agitation (see Table C-12). The second medium for "multiplication of shoots" is presumably the same medium, but supplemented with 3 mg BA l^{-1} and solidified with 0.7% agar (Table C-21). Shoots produced on the second medium were placed on membrane rafts and transferred to

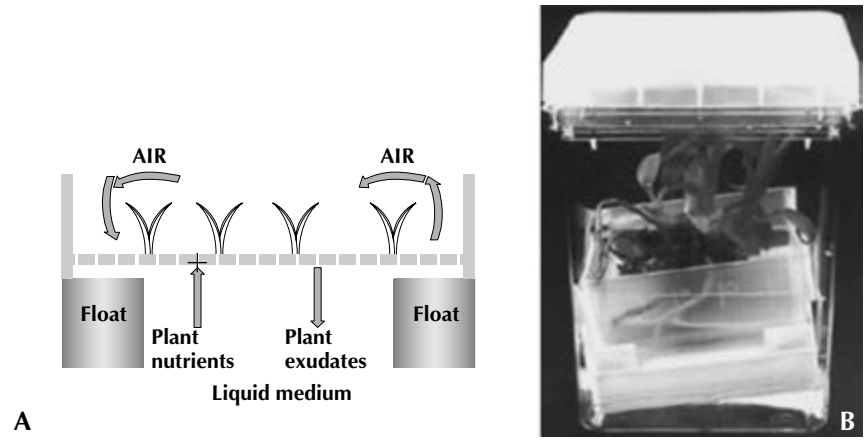


FIG. C-3. **Life Raft™. A. Diagram. B. Supporting plants inside a culture vessel. (Source: Osmotek web site, www.osmotek.com.)**

a third (liquid) modification of MS (modification of Table C-22) for rooting. The addition of TDZ, 3.3 μmol l^{-1}, to the culture medium can increase proliferation and production of PLBs. However, a side effect of TDZ is an increase in the number of fasciated shoots. This suggests that TDZ may disrupt normal development and could perhaps bring about undesirable mutations. Therefore it may be wise to not add TDZ to any of the culture media for *C. mossiae*.

Procedure. Details about excision are not provided in the original papers. The method employed in "Meristem culture of *Cattleya*" (Lindemann et al., 1970) can be used for shoot apices. Bud meristems can be excised as outlined in "Propagation of *Cattleya* by tissue culture of lateral bud meristems" (Reinert and Mohr, 1967). The procedure in "Aspects of meristem culture in the *Cattleya* alliance" (Scully, 1967) can also be used.

Placing explants on filter paper bridges affords aeration, but the advantages this provides are minimal and do not justify the extra work, expense, and complications. This is why such bridges are not used extensively in orchid micropropagation (Gandawijaja, 1980 in the section on *Dendrobium*).

When explants are cultured in liquid medium, agitation increases and accelerates proliferation. However, this requires 2–5 weeks (Reinert and Mohr, 1967; Scully, 1967), not 48 h as suggested for the current procedure (Torres and Mogollón, 1998). Therefore, if the initial explants of *C. mossiae* are placed in a liquid medium they should be agitated not for 48 h, but for 2–5 weeks or until the explants proliferate extensively and/or produce many PLBs. After that, parts of the proliferated mass or some PLBs should be transferred to fresh liquid medium and allowed to proliferate again (but this should not be done too many times to prevent mutations) or moved to solid medium and allowed to differentiate.

Floating shoots in membrane rafts like Life Raft™ (www.osmotek.com) on a liquid medium may provide additional aeration, but there is no evidence that the added

TABLE C-21. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the culture of proliferated *Cattleya mossiae* explants (Huang, 1984; Torres and Mogollón, 1998, 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	α-Napthaphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin and related substance				
11	N^6-benzyladenine (BA)	3.0	300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
12	Adenine sulfate$\cdot 2H_2O$	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
16	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 17), adjust pH to 5.7, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (items 10), and vitamins (items 13–15) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE C-22. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the culture of proliferated *Cattleya mossiae* explants (Huang, 1984; Torres and Mogollón, 1998, 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	α-Napthaphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1N KOH.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.7, add sugar (item 14), and adjust volume to 1000 ml with distilled water. Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10) and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

complication and expense are of much, if any, benefit. Therefore shoots can be placed on an agar medium for rooting as recommended here (i.e., not in rafts on liquid). Rooted plants should be potted in coconut peat, tree fern fiber, or a mixture of both (ratio not given in the original paper) and hardened under mist.

Developmental Sequence. The explants will proliferate and/or form callus and/or PLBs on the first medium (see Table C-12). Shoots multiply on the second medium (Table C-21). On transfer to the third medium (Table C-22), the shoots produce roots.

General Comments. On the whole this method does not seem well thought out or planned carefully. It is certainly not presented well. As originally proposed, this procedure is encumbered by unnecessary components (antiseptic soap used in conjunction with a Clorox dilution, for example), superfluous complications (as for instance culturing explants on bridges and placing shoots on rafts), and irrational steps (e.g., agitating explants for only 48 h). Despite all these complications, the procedure does not include the important step of washing the explants with sterile distilled water after the last sterilization step. On the whole this procedure seems effective and, if improved as suggested here, it should prove to be a productive method for propagating *C. mossiae* and perhaps even other orchids.

Formation of Protocorm-like Bodies by *Cattleya* Root Explants

Root tips are perhaps the most recalcitrant orchid explants, both in their incapacity to survive in vitro and in their ability to produce callus or PLBs. However, Professor Gilberto B. Kerbauy at the University of Sao Paulo has been successful in his attempt to induce PLB formation using root-tip explants taken from seedlings of a *Cattleya nobilior* × *Cattleya loddigesii* cross (Kerbauy, 1991).

Plant Material. Root tips, 10 mm long, were taken from 3–4-cm-tall asymbiotic seedlings growing on Vacin and Went medium (Vacin and Went, 1949).

Surface Sterilization. Explants taken from asymbiotic seedlings do not have to be surface-sterilized. However, they should be rinsed with sterile distilled water to remove medium residues.

Culture Vessels. Erlenmeyer flasks, 125-ml capacity containing approximately 25 ml medium, and other vessels can be used.

Culture Conditions. In the original research the cultures were maintained at 25°C under 16-h photoperiods of 9 W m^{-2} (the light source was not described). Standard culture room conditions are also suitable.

Culture Media. Modified Vacin and Went medium (Vacin and Went, 1949) is used for the culture of root-tip explants (Table C-23). The original paper (Kerbauy, 1991) makes no recommendations for a medium or media which can be used for plantlet development from PLBs. However, a medium (Table C-24) used to germinate the

TABLE C-23. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of root-tip explants from *Cattleya nobilior* × *Cattleya loddigesii* seedlings (Kerbauy, 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
9	Niacin	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
10	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Thiamine (vitamin B_1)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Auxins				
12	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
13	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
14	Benzyladenine	0.05	5 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
15	Ripe banana pulp	60.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	6.0 g	No stock	No stock	Weigh
	Darkening agent				
19	Activated charcoal[g]	1.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. This medium may be also suitable for the culture of protocorm-like bodies and plantlets.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve. Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 17) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_3(C_4H_4O_6) \cdot 2H_2O$, 28 ml l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible.

[e]Keep refrigerated between uses.

[f]If the auxins or cytokinin do not dissolve add a few drops of 0.1N KOH or 0.1N HCl, respectively.

[g]Add items 1–8 and 15 to 500–700 ml of distilled water (item 17), adjust pH to 5.7, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, add darkening agent (item 19) with vigorous stirring; pour the solution into a 2-l flask and autoclave. Add the vitamins (items 9–11) and hormones (items 12–14) to the hot solution under sterile conditions with sterile pipettes, mix well, and distribute to preautoclaved culture vessels. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949). It is not clear whether the addition of other nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

TABLE C-24. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the germination of *Cattleya nobilior* × *Cattleya loddigesii* seeds (Kerbauy, 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Ripe banana pulp	60.0 g	No stock	No stock	Weigh
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier				
11	Agar[e]	8.0	No stock	No stock	Weigh
	Darkening agent				
12	Activated charcoal	1.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. This medium may be also suitable for the culture of protocorm-like bodies and plantlets.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve. Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 ml l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible.

[e]Add items 1–8 to 500–700 ml of distilled water (item 10), adjust pH to 5.7 or as required, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, add darkening agent (item 12) with vigorous stirring; pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of other nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

Cattleya nobilior × *Cattleya loddigesii* (Fowlie, 1977)

seeds that produced the seedlings which were used as explant sources may prove to be suitable for this purpose.

Procedure. The explants should be placed on the first medium (Table C-23) and allowed to remain on it until callus and/or PLBs are formed. PLBs and sections of the callus should be transferred to the second medium for plantlet development.

Developmental Sequence. PLBs are produced by the root-tip explants only after 6–9 months of culture (Fig. C-4). Callus is produced by a region adjacent to the meristem of the root tips. The callus masses are friable and light green in color. They grow slowly and reach 1–2 cm in diameter after 6 months. Root formation starts after 4 months of culture (Fig. C-2B), but roots can be malformed (Fig. C-4A). Plantlets also start to form after 9 months of culture (Fig. C-4A).

General Comments. This procedure is interesting and could result in the production of plantlets, but it is probably more valuable as a research tool than as a method for micropropagation. It is certainly a very impressive pioneering effort.

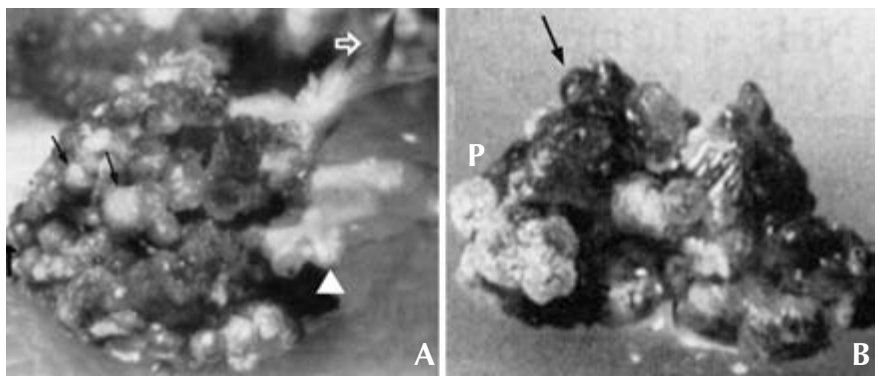
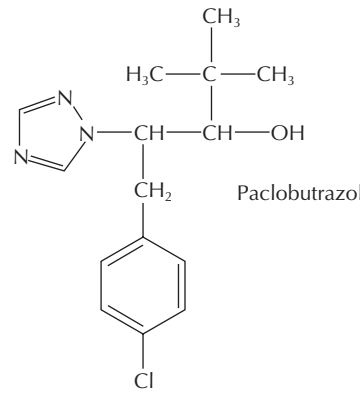


FIG. C-4. **Culture of *Cattleya nobilior* × *Cattleya loddigesii* root tips. A. Nine-month-old culture. Black arrows, malformed roots; white triangle, protocorm-like body; open white arrow, plantlet. B. Six-month-old culture. Black arrow, early stage of root formation; P, early stage of PLB formation.**

Paclobutrazol Effects on Leaf Development of *Cattleya mossiae* in Vitro

A herbicide, growth retardant, and an inhibitor of gibberellin biosynthesis, paclobutrazol (structural formula is shown below) was added to in vitro cultures of *Cattleya mossiae* at concentrations of 1, 2, and 4 mg l^{-1}. At levels of 2 mg l^{-1} and above it reduced root and shoot length, but not their number. It also affected the development of stomata (Torres and Mogollón, 2002). The addition of paclobutrazol and jasmonic acid increased tuber formation in seedlings of *Ptrerostylis sanguinea*, a terrestrial orchid from southwest Australia (Debeljak et al., 2002). These findings suggest that paclobutrazol may find some uses in micropropagation.



Paclobutrazol

Cattleya Protoplast Culture

Protoplasts of *Cattleya skinneri* were isolated using the method employed for *Dendrobium* (Yasugi, 1990).

Encapsulation of Protocorm-like Bodies of *Cattleya*

The method employed for *Dendrobium* Sonia (Saiprasad and Polisetty, 2003) is suitable.

Effects of Amino Acids on Protocorm-like Body Production by *Cattleya leopoldii*

Asparagine, glutamine, and phenylalanine reduced the number of PLBs produced by shoot-tip explants of *Cattleya leopoldii* (Saiprasad et al., 2002*b* based on abstract only).

Shipping of *Cattleya* Propagules in Sealed Membrane Vessels

Three-dimensional polypropylene vessels were fabricated for in vitro and ex vitro culture for *Cattleya* propagules. They consist of a microporous polypropylene membrane that allows nutrient transfer, molded side walls, and polypropylene membrane tops (Dellevigne et al., 1993 based on abstract only).

Micropropagation and Shipping of *Cattleya* in Sealed Semipermeable Membrane Vessels

Plantlets of a *Cattleya* hybrid shipped from Japan to the USA in vessels of the type described above arrived in good order after 7 days in transit at 14–30°C in the dark (Adelberg et al., 1997*b* based on abstract only).

Effects of Four Different Culture Media on Protocorm-like Body and Shoot Production by *Cattleya*

In a comparative study involving four culture media – Knudson C (KC), Vacin and Went (VW), Murashige and Skoog (MS), and Rosa and Laneri (RL) – *Cattleya leopoldii* showed that the greatest production of PLBs, multiple shoots, and most extensive differentiation occurred on MS (Saiprasad et al., 2002*c* based on abstract only).

Size of Explant and Virus Transmission in *Cattleya*

"In [cymbidium mosaic virus] CyMV infected *Cattleya*, 4 of 16 plantlets derived from 1.5 mm apical meristems and 1 of 5 plantlets from 0.2–10 mm apical meristems were virus free. However no [odontoglossum ringspot virus] ORSV plant was obtained." This paper also states that viruses could be transmitted through virus particles that adhere to razor blades used to excise leaf primordia (Fujiwara, 2001). Virus particles probably adhere to any tool that comes in contact with infected plants and can be transmitted in this fashion.

Micropropagation of *Cattleya leopoldii*

A very interesting study on the effects of growth regulators on the production of PLBs and multiple shoots during orchid micropropagation was carried out at the Division of Plant Physiology, Indian Agricultural Research Institute in New Delhi (Saiprasad et al., 2002*a*). The findings of this study are beyond the scope of this book, but a method for the micropropagation of *Cattleya leopoldii* will be described here.

Plant Material. Greenhouse-grown small plants and pseudobulbs of *C. leopoldii* were used as explant sources.

Surface Sterilization. Older leaves and dried parts should be removed from the explant sources before washing them thoroughly with running tap water. Shoot-tip sections, 5 cm in length, should be cut after the washing. These sections should be washed with a solution of 10 drops of Tween 20 (www.sigmaaldrich.com, www.fishersci.com, http://search.wako-chem.com), baby shampoo, or a mild household detergent per 100 ml of sterile distilled water, and taken to a sterile working

area where they should be placed in sterile distilled water. After that, the sections should be surface-sterilized by submerging them in a solution of 0.1 mg mercuric chloride (HgCl) plus a few drops of Tween 20, baby shampoo, or a mild household detergent per 100 ml of distilled water for 3 min (HgCl is very toxic and should be handled with care). This step should be followed by three washes with sterile distilled water.

Culture Vessels. Culture tubes or bottles, Erlenmeyer flasks, and other containers can be used.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 16-h photoperiods of 50–60 μE m^{-2} s^{-1} (the light sources used in the original research were not described). Standard culture room conditions are also suitable.

Culture Media. Explants should be cultured initially on MS medium (Murashige and Skoog, 1962) containing 1 mg BA l^{-1} (Table C-25). PLBs that form on this medium should be transferred to MS supplemented with 1 mg NAA l^{-1} (Table C-26). For further growth, the plantlets can be moved to one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, and V-4) with or without banana homogenate (BH) or activated charcoal (AC). If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). AC should be added to the medium selected for use as suggested for KC that does contain this darkening agent (see Table Pln-1, footnote *f*).

Procedure. Apices are excised from the sterilized 5-cm-long shoot tips and placed on the first medium (Table C-25). PLBs that form on this medium are moved to the second solution (Table C-26). Plantlets that develop from the PLBs should be cultured on one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, and V-4) until they are ready for community pots.

Developmental Sequence. PLBs form on the BA-containing medium (Table C-25). They produce plantlets on the NAA-containing substrate (Table C-26). These plantlets develop further on one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, and V-4).

General Comments. This procedure is a byproduct of an excellent comparative research project on the effects of plant hormones on explants.

TABLE C-25. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of shoot-tip explants of *Cattleya leopoldii* (Saiprasad et al., 2002*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1N HCl.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.2–5.7, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium into culture vessels and autoclave. Agar is not added to liquid media.

TABLE C-26. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for plantlet production of shoot-tip explants derived from protocorm-like bodies (PLB) of *Cattleya leopoldii* (Saiprasad et al., 2002*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1N KOH.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.2–5.7, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium into culture vessels and autoclave. Agar is not added to liquid media.

Browning of *Cattleya* Explants

Cattleya shoot-tip explants may turn brown and die following excision. This is due to the production of phenolics (Kako, 1973; Ichihashi and Kako, 1977; Ishii, 1980). Eucomic acid and tyramine were isolated from *Cattleya trianaei* "Mooreana" (Ishii et al., 1976). Five phenols were identified on another *Cattleya*. One of them has a molecular weight of 238 and a molecular formula of $C_{11}H_{10}O_6$; it is a *para*-hydroxyphenolic dicarboxylic acid (Kako, 1973). Polyphenol oxidase isolated from *Cattleya* has a pH optimum of 6.5 (Kako, 1973; Ichihashi and Kako, 1977).

Production of phenolics is lower when explants are cultured on a medium with a pH of 5.5. Incubation of the tissues in sterile water at 15–20°C also reduced browning (Ishii et al., 1979).

In Vitro Organogenesis of *Cattleya* Plantlets

Shoot apices that initiate foliar primordia are produced by PLBs. The phyllotaxy is always distichous. Roots arise endogenously in leaf axils (from illustrations and one summary in English in Fréson and Vanseveren, 1968; Vanseveren, 1969; Vanseveren and Fréson, 1969).

Cattleya mossiae was brought into cultivation by George Green of Liverpool, England. He received it from Venezuela in 1836. It flowered for the first time outside its native habitation in the collection of a Mrs. Moss for whom it was named by Sir William Hooker in 1838 (Bechtel et al., 1992).

Cleisostoma

See *Sarcanthus*.

Clowesia

Clowesia is a relatively small genus established by John Lindley in 1843 and named in honor of the Rev. John Clowes (1777–1846), a dedicated orchid grower in Manchester, UK, who was the first to flower the type species. Both the plants and flowers resemble *Catasetum*, but its blossoms are bisexual.

Production of Protocorm-like Bodies by Root Apices of *Clowesia warscewiczii*

Several *Clowesia* species were shuttled between that genus and *Catasetum*. One of these species, known at different times as *Catasetum warczewitzii* Lindl & Paxt. and *Catasetum scurra* Rchb. *f.* (Dunsterville and Garay, 1965), is now *Clowesia warscewiczii* (Lindl & Paxton) Dodson (Dunsterville and Garay, 1976). A procedure for PLB formation from root tips of this species was developed in Brazil (Kerbauy and Estelita, 1996).

Plant Material. Root apices, 4–6 mm long, were taken from asymbiotic seedlings growing on modified (Colli and Kerbauy, 1993) Vacin and Went medium (Vacin and Went, 1949).

Surface Sterilization. There is no need to surface-sterilize explants taken from asymbiotic seedlings in vitro. However, the explants should be washed with sterile distilled water to remove medium residues.

Culture Vessels. Erlenmeyer flasks, 125-ml capacity, were used in the original research. Other culture vessels are also suitable. They should contain medium equivalent to 20–30% of their capacity.

Culture Conditions. Cultures should be maintained at $25 \pm 1°C$. In the original research "the frequency of root tip conversion was substantially higher in the dark . . . than under illumination." However, "illumination seems to maintain more efficiently the functional and structural patterns of apical root meristem." Therefore (especially since plenty of root tips are usually available in seedling cultures) the cultures should be illuminated. In the original research the cultures were given 16-h photoperiods of 40 $\mu mol\ m^{-2}\ s^{-1}$ provided by fluorescent lights (the type is not given). Standard culture room illumination is also suitable.

The original paper (Kerbauy and Estelita, 1996) also states: "A similar inhibitory effect of light was also observed in protocorm-like bodies formation from excised root apexes of *Catasetum* (Colli & Kerbauy, 1993)."

TABLE CLOWESIA-1. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of root-tip explants from *Clowesia warscewiczii* seedlings (Kerbauy, 1996)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier				
11	Agar[e]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. This medium may be also suitable for the culture of protocorm-like bodies and plantlets.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve. Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 ml l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible.

[e]Add items 1–8 to 500–700 ml of distilled water (item 10), adjust pH to 5.8, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, dispense the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949). It is not clear whether the addition of other nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

Culture Media. A modification of the Vacin and Went medium (Vacin and Went, 1949) is used for the culture of root-tip explants (Table Clowesia-1). The original paper (Kerbauy and Estelita, 1996) does not recommend a medium or media for plantlet development from PLBs. A medium (see Table C-24) used to germinate seeds which produced *Cattleya* seedlings that were used as explant sources (Kerbauy, 1991) may prove to be suitable for this purpose.

Procedure. The root-tip explants should be placed in culture on the first medium (Table Clowesia-1) and allowed to develop PLBs. After approximately 25 days the PLBs produce bud primordia. At that point they should be moved to the second medium for plantlet production. When the plantlets are large enough they should be moved to pots.

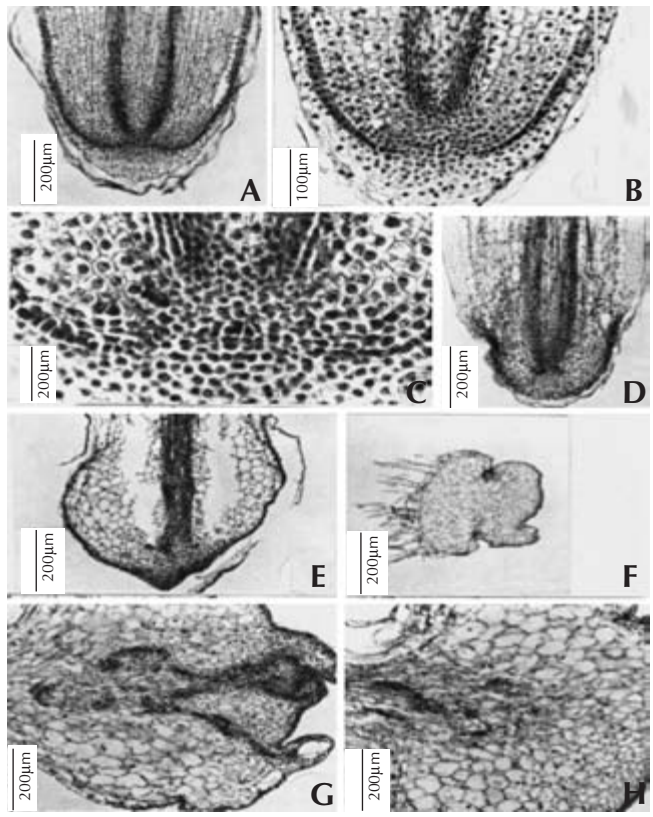


FIG. CLOWESIA-1. **Root tips in culture. A. Longitudinal section showing lysis of the root-cap layers. B. Cellular expansion. C. Cell division. D. Formation of a PLB primordium. E. Bud primordium on a PLB. F. Hairs at the base of a PLB. G. Procambial strands after 31 days in culture. H. Peripheral meristematic tissue in a PLB.**

Developmental Sequence. Starch grains and raphides cannot be seen and there is marked lysis in root cap cells after 6 days (Fig. Clowesia-1A). Cellular extensions (Figure Clowesia-1B) and cell divisions (Figure Clowesia-1C) become apparent in the apical meristem after 9 days. This leads to the production of PLB primordia (Fig. Clowesia-1D). The PLBs become larger than the original explants after 25 days and produce a bud primordium (Fig. Clowesia-1E). They also have rhizomes (Fig. Clowesia-1F). The buds become conspicuous in 31 days, have apical meristems, two to three leaf primordials, and tracheary elements which are connected to the vascular system of the roots (Fig. Clowesia-1G, H).

General Comments. This research provides interesting insights into the developmental anatomy of PLBs from root tips. The method can also be used for micropropagation, but its value is limited because the use of seedling root tips does not makes possible the multiplication of desirable clones. Additional research is needed to determine whether the method can be used to culture root tips taken from mature plants.

Cymbidium

Phalaenopsis was the first orchid genus to be propagated in vitro (Rotor, 1949). *Cymbidium* was the first to be propagated by shoot-tip ("meristem") culture (Morel, 1960, 1963, 1964*a*, 1964*b*, 1965*a*, 1965*b*, 1971*c*; Wimber, 1963) following the development of tissue-culture methods for excised meristems of other plants (Ball, 1964). Since *Cymbidium* is one of the more popular orchid genera in cultivation, a number of workers have developed tissue-culture procedures, and the subject has been discussed in many publications (Champagnat et al., 1966, 1968; Gripp, 1966; Sagawa et al., 1966; Vacherot, 1966; Wilfret, 1966, no date; Anonymous, 1967; Bertsch, 1967; Marston and Vouraurai, 1967; Borriss and Hiibel, 1968; Fréson and Vanseveren, 1968; Leffring, 1968; Penningsfeld and Fast, 1968; Ueda and Torikata, 1968, 1969*a*, 1969*b*, 1972; Alpi and Garibaldi, 1969; Bivins and Hackett, 1969; Vanseveren and Fréson, 1969; Matsui et al., 1970; Werkmeister, 1970*a*, 1970*b*, 1971; Rutkowski, 1971; Steward and Mapes, 1971; Thompson, 1971; Fonnesbech, 1972*a*, 1972*b*; Koch, 1973; Penningsfeld, 1973; Zákrejs, 1974, 1976; Dalla Rosa, 1975; Maróti, 1975; Tschauder, 1975; Pieper and Zimmer, 1976*a*; Khaw et al., 1978*a*, 1978*b*; Subasinghe, 1978; Sagawa and Kunisaki, 1982; and others). Details of several procedures and summaries of others will be presented here.

In Vitro Culture of *Cymbidium* Shoot Meristems

Work with potatoes, dahlias, carnations, and hyacinths led to the culture of *Cymbidium* shoot tips at the National Institute for Agronomic Research in Versailles, France, and the subsequent development of procedures for mass rapid clonal propagation (Morel, 1960, 1963, 1964*a*, 1964*b*, 1970, 1974; Champagnat et al., 1966; Morel and Champagnat, 1969).

Plant Material. Shoot tips from buds on pseudobulbs are used.

Surface Sterilization. To surface-sterilize buds, dip them for a few seconds in 75% ethyl alcohol (ethanol), place in saturated calcium hypochlorite for 20 min, and rinse with sterile distilled water. (The calcium hypochlorite solution should be freshly prepared with 10 g per 140 ml; stir, allow to stand, stir again, and let the precipitate settle before decanting and using.)

Culture Vessels. Use Khan test tubes or Erlenmeyer flasks.

Culture Conditions. The cultures are kept under 12-h photoperiods provided by Sylvania Gro Lux fluorescent tubes at 22°C.

Culture Media. Several modifications of the Knudson C medium can be used (Tables Cym-1 to Cym-3).

TABLE CYM-1. **Modified Knudson C medium (Knudson, 1946) for plantlet formation from *Cymbidium* shoot-tip meristems (Morel, 1965*a*, 1965*b*, 1970)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	Or weigh
2	Monopotassium phosphate, KH_2PO_4[c]	250	25 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	Or weigh
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
6	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.056	56 mg l^{-1}		
(b)	Molybdic acid, MoO_3	0.016	16 mg l^{-1}		
(c)	Cupric sulfate (anhydrous), $CuSO_4$	0.040	40 mg l^{-1}	1	One solution
(d)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.331	331 mg l^{-1}		
(e)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	7.5 g l^{-1}		
	Sugar				
7	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
8	Water, distilled[e]	To 1000 ml			
	Solidifier[e]				
9	Agar	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and nitrate may become contaminated with standing. Therefore, stock solutions should not be prepared. If made, they should be kept frozen between uses.

[c]A phosphate buffer that will keep the pH steady may be substituted here. Prepare buffer by mixing 975 ml 0.1-*M* KH_2PO_4 (monopotassium phosphate; 13.6 g l^{-1}) with 25 ml 0.1-*M* K_2HPO_4 (dipotassium phosphate; 17.4 g l^{-1}). Measure the pH to be certain it is correct (pH 5.1–5.4), and use 18 ml per liter of culture medium.

[d]Add all microelements to the same 1 l of distilled water, stir and/or heat until they are dissolved, and add 1 ml per liter of culture medium.

[e]Add items 1–6 to 800 ml distilled water (item 8) and dissolve. Adjust pH to 5.3, add sugar (item 7), and bring volume to 1000 ml with more distilled water. Add agar (item 9) while stirring to the gently boiling solution. When dissolved, distribute into culture vessels, and autoclave.

Procedure. Remove all adult leaves from the pseudobulbs. Cut down to half size other leaves around the growing buds. Then sterilize and dissect the buds under aseptic conditions. The exposed apex appears as a brilliant hemisphere, usually surrounded by one or two leaf primordia. Excise it by making four cuts at right angles and delimiting a small cube, which is placed on the culture medium.

Developmental Sequence. Within 2 months the explant develops into a PLB, 1–2 mm in size. This body is colorless at first but soon turns green and produces rhizoids and a leaf primordium. At this point it may be sectioned into quarters and subcultured. Within a month each section will produce a new PLB, which can be divided again.

General Comments. This process provides a means of propagation that can reach 4,000,000 plants per year from a single bud.

TABLE CYM-2. **Modified Knudson C medium (Knudson, 1946) for plantlet production from *Cymbidium* shoot-tip meristems (Morel, 1965*a*, 1965*b*, 1970)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[c,d]	1 g	100 g l^{-1}	10	Or weigh
2	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Ammonium nitrate, NH_4NO_3[c,d]	500	50 g l^{-1}	10	Or weigh
5	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[d]	500	50 g l^{-1}	10	Or weigh
6	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
7	Potassium chloride, KCl	250	25 g l^{-1}	10	
	Microelement				
8	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Sugar				
9	Sucrose	20 g	No stock	No stock	
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier[e]				
11	Agar	17.5 g	No stock	No stock	Weigh

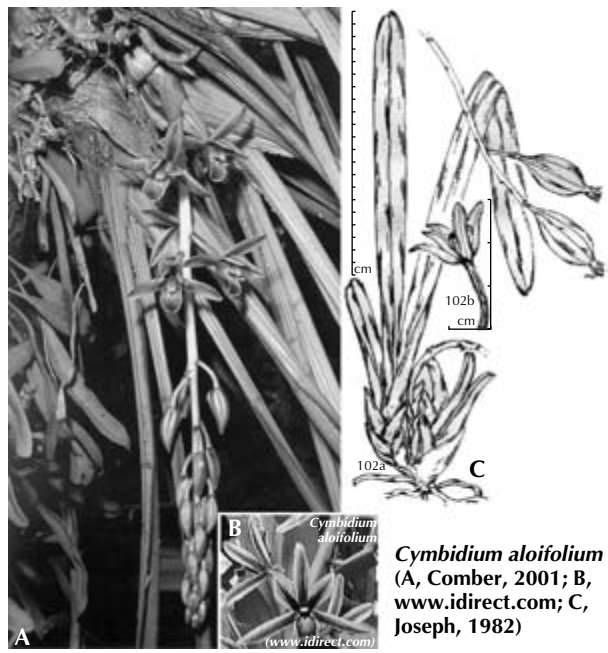
[a]Only the inorganic salts are listed (Morel, 1965*a*, 1965*b*, 1970; also see Morel, 1974).

[b]Amounts are given in mg unless indicated otherwise.

[c]Either use 1 g ammonium sulfate and omit the ammonium nitrate, or use 0.5 g of each.

[d]Solutions containing ammonium and/or nitrate may become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[e]Add items 1–8 to 700 ml distilled water (item 10) and dissolve. Adjust pH to 5.2–5.5, add sugar (item 9) and dissolve, and bring volume to 1000 ml with more distilled water. Distribute into culture vessels and autoclave. For solid culture medium, add agar (item 11) slowly while stirring to the gently boiling solution. When fully dissolved, dispense into culture vessels and autoclave.



Cymbidium aloifolium **(A, Comber, 2001; B, www.idirect.com; C, Joseph, 1982)**

TABLE CYM-3. **Modified Knudson C medium (Knudson, 1946) for the differentiation of plantlets from leaf-tip callus (Arditti et al., 1971, 1972, Ball et al., 1971; Churchill et al., 1971*a*, 1971*b*, 1971*c*, 1972*b*, 1973) which is suitable for *Cymbidium* shoot-tip explants**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	Or weigh
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
3	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	Or weigh
4	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$[c]	25	2.5 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4[d]	250	25 g l^{-1}	10	
6	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.056	56 mg l^{-1}	1	One solution
(b)	Molybdic acid, MoO_3	0.016	16 mg l^{-1}		
(c)	Cupric sulfate, $CuSO_4$	0.040	40 mg l^{-1}		
(d)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.331	331 mg l^{-1}		
	Sugar				
7	Sucrose	20 g	No stock	No stock	Weigh
	Organic additive[f]				
8	Banana, ripe	150 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[f]	To 1000 ml			
	Solidifier[f]				
10	Agar	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate tend to become contaminated on standing. Therefore it is better to weigh items 1 and 3 each time. If prepared, keep frozen between uses.

[c]This solution tends to form a rust-colored precipitate on standing. Therefore shake before using.

[d]A phosphate buffer that will keep the pH steady may be substituted here. Prepare buffer by mixing 975 ml 0.1-*M* KH_2PO_4 (monopotassium phosphate; 13.6 g l^{-1}) with 25 ml 0.1-*M* K_2HPO_4 (dipotassium phosphate; 17.4 g l^{-1}). Measure pH to be certain it is correct (pH 5.1–5.4), and use 18 ml per liter of culture medium.

[e]Add all microelements to the same 1 l of distilled water, stir and/or heat until dissolved, and add 1 ml per liter of culture medium.

[f]Add items 1–8 to 700 ml distilled water (item 9), homogenize until banana (item 8) is completely broken down (about 2–3 min), adjust pH to 5.3, and bring volume to 1000 ml with more distilled water. To dissolve agar (item 10), add it slowly while stirring to gently boiling solution. When dissolved, distribute into culture vessels and autoclave.

Clonal Multiplication of *Cymbidium* through in Vitro Culture of Shoot Tips

Morel's *Cymbidium* shoot-tip culture method (Morel, 1960) may have been the first to be published, but it was relatively slow, and details were lacking in the early papers. A faster method employing a liquid medium and described in more detail from the outset was developed in the United States as a result of work at the Dos Pueblos Orchid Co. (Santa Barbara, CA), the Biology Department, Brookhaven National Laboratory (Upton, NY), and the Biology Department, University of Oregon (Wimber, 1963, 1965).

Plant Material. Shoots approximately 3 cm long are used.

Surface Sterilization. Use a 2% (w/v) solution of calcium hypochlorite, which must be freshly prepared.

TABLE CYM-4. **Culture medium for *Cymbidium* shoot tips (Wimber, 1963)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	Or weigh
2	Monocalcium phosphate, dibasic $CaHPO_4$[c]	200	20.0 g l^{-1}	10	Or weigh
3	Monopotassium phosphate, KH_2PO_4	250	25.0 g l^{-1}	10	
4	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50.0 g l^{-1}	10	Or weigh
5	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25.0 g l^{-1}	10	
6	**Iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Complex additive				
7	Tryptone	2 g	No stock	No stock	Weigh
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated with standing. Therefore it is preferable to weigh the salts every time. If prepared, keep frozen between uses.

[c]If some of the compound fails to dissolve, shake stock solution well before dispensing to insure an even suspension; better yet, weigh out each time.

[d]The original solution uses ferric tartrate, $Fe_2(C_4H_4O_6)_3$; chelated iron is preferable.

[e]Mix items 1–7 with 700 ml distilled water (item 9), adjust pH to 5.2–5.5, add sugar (item 8), and bring volume to 1000 ml with more distilled water (item 9). Distribute into culture vessels, and autoclave.

Culture Vessels. Use 125-ml Erlenmeyer flasks containing 25 ml medium.

Culture Conditions. Constant light of 100 ft-c or less (the light source is not listed, but Sylvania Gro Lux tubes would prove satisfactory) at 22°C on a rotary shaker.

Culture Medium. A modified Tsuchiya medium is used (Table Cym-4).

Procedure. Remove the outermost leaves and sterilize for 10 min in calcium hypochlorite. Under sterile conditions using sterilized tools, remove the rest of the leaves, exposing the apical meristem, which should be "immediately cut off" and placed into culture.

Developmental Sequence. The explants turn green and show noticeable growth within 1 week. In 1 month PLBs form. Shoots appear and differentiation follows when the PLBs reach approximately 4 mm. If the flasks are agitated, proliferation occurs instead of shoot formation. Masses of tissue are formed and can be subcultured after 2 months. On a rotary shaker the subdivisions reach a diameter of 1 cm within 30 days. When tissue fragments are placed on a solid medium, they form plantlets within 2.5 months.

General Comments. This is a simple, fast technique requiring inexpensive tools, a shaker, and skills that can be acquired easily.

Seed and Tissue Culture of *Cymbidium iridioides* × *Cymbidium longifolium*

Modified Knudson C medium with 100 mg l^{-1} banana enhanced proliferation of PLBs. Rapid differentiation is obtained on a medium containing 6% ripe banana. PLBs can be subcultured on Knudson C medium containing 100 ml pineapple juice l^{-1} (Hedge et al., no date).

Carbohydrate Metabolism during Protocorm Differentiation in a *Cymbidium* Hybrid

PLBs were produced from leaf bases of *Cymbidium* Burgundian Chateau. Accumulation of endogenous free sugar reached peaks after 3–5 and 25 days in culture. Starch synthesis reached a maximum 20 days after the start of culture (Gopalan et al., 1988).

Clonal Propagation of *Cymbidium* through Shoot-tip Meristem Culture

One of the early methods for the culture of *Cymbidium* shoot-tip explants was developed in the laboratory of Yoneo Sagawa (Sagawa et al., 1966).

Plant Material. Explants are obtained from shoots 5–7.5 cm long growing on pseudobulbs.

Surface Sterilization. Sterilization is with 1, 5, and 10% (v/v) Clorox (see Procedure below).

Culture Vessels. Use 50-ml Erlenmeyer flasks containing 15 ml medium.

Culture Conditions. Explants are maintained under 120 ft-c of continuous illumination (General Electric Power Groove White) at 22–25°C on a shaker for 4 weeks, after which they are kept under the same conditions on solid medium.

Culture Media. Modified Knudson C (Tables Cym-1 to Cym-3) and Vacin and Went (Table Cym-5) media can be used.

Procedure. Remove new growths or leads 5–7.5 cm long from a pseudobulb, and cut away three to four leaves until an axillary bud is exposed. Sterilize in 5% (v/v) Clorox for 5–8 min. Now remove the remaining leaves (except perhaps one leaf). If axillary buds are used, remove a 2–3-mm cube, and sterilize in 1% (v/v) Clorox for 3 min. Place this cube in liquid culture. Tools should be dipped in 10% (v/v) Clorox often and washed in sterile distilled water to insure sterility.

TABLE CYM-5. **Modified Vacin and Went medium for the culture of *Cymbidium* shoot-tip explants (Sagawa et al., 1966)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Tricalcium phosphate, $Ca_3(PO_4)_2$[b]	200.0	20.0 g l^{-1}	10	Or weigh
2	Potassium nitrate, KNO_3[c]	525.0	52.5 g l^{-1}	10	Or weigh
3	Monopotassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
5	Ammonium sulfate, $(NH_4)_2SO_4$[c]	500.0	50.0 g l^{-1}	10	Or weigh
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3$[d]	28.0	2.8 g l^{-1}	10	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier[e]				
10	Agar	16.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]If the material does not completely dissolve, shake stock solution well before dispensing to insure an even suspension; better yet, weigh out each time.

[c]Solutions containing nitrate and/or ammonium may become contaminated with standing. Therefore it is preferable not to make stock solutions. If prepared, keep frozen between uses.

[d]Add dilute KOH (ca. 0.1 *M*) dropwise until the solution clears. Shake well before dispensing.

[e]Mix items 1–7 with 800 ml distilled water (item 9), adjust pH to 5.2–5.5, add sugar (item 8), and adjust volume to 1000 ml with more distilled water. To add agar (item 10), bring solution to a slow boil and add slowly while stirring. When fully dissolved, dispense into culture vessels and autoclave. For liquid medium, omit agar (item 10).

Developmental Sequence. PLBs form after 6 weeks (4 weeks on liquid medium and 2 weeks on solid medium) of culture. These bodies, measuring 2–3 mm in diameter, are quartered and subcultured. The process can be repeated every 10 days. If left undisturbed, the PLBs grow into plantlets.

General Comments. This is a well-conceived procedure that can produce many plantlets quickly.

Transformation of Isoprenoids by Orchids in Tissue Culture

Tissue cultures of *Cymbidium* Saint Pierre maintained in vitro on media used for other orchids (Kukułczanka, 1985) transformed some isoprenoids (Mironowicz et al., 1987).

Rhizome Formation from Shoot Tips of *Cymbidium goeringii* and *Cymbidium kanran* in Vitro

Rhizome formation from shoot tips of *Cymbidium goeringii* can occur throughout the year. However, maximal formation occurs in April on MS and Linsmaier–

Skoog media containing 1 ppm NAA and 0.1 ppm kinetin, in the dark. The Linsmaier–Skoog medium containing one-fifth or one-tenth the usual level of inorganics and 10% coconut water is most suitable for the culture of *C. kanran* shoot tips. Rhizome length was reduced with increased dilution, suggesting that, unless the explants are transferred following rhizome formation, one-fifth dilution may be a more suitable medium. Plant-growth regulators had no effect on shoot-tip cultures of the two species (Hasegawa and Goi, 1987).

Propagation of *Cymbidium* in Vitro

To determine the precise tissue culture requirements of a species, it is necessary to perform careful comparative experiments in which one factor at a time is added, removed, or varied in concentration. The volumes of data that accumulate from these experiments must be analyzed statistically before their full meaning can be appreciated. Such experiments are tedious, often complicated, and always time-consuming. As a result investigators tend to shy away from them, which is a pity. Occasionally, however, researchers carry out experiments of this type and use them as a basis for articles that are full of information and a pleasure to read (Fonnesbech, 1972*a*, 1972*b*). Propagation methods based on such experiments tend to produce a higher degree of success and better plants.

Plant Material. The explants are similar to those used in the previous procedure (Sagawa et al., 1966).

Surface Sterilization. The procedure is the same as that used in the previous section on in vitro shoot-tip culture (Sagawa et al., 1966).

Culture Vessels. Use 150-ml Erlenmeyer flasks containing 30 ml liquid medium, or 25×100 mm test tubes with 10 ml medium. For solid cultures, use 100-ml flasks containing 40 ml medium.

Culture Conditions. Tissues are maintained 90 cm under a light source consisting of three 40-W Atlas Super-Gro and three Osram L-Fluor 77 fluorescent tubes producing radiation of 3.3 W m^{-2}. Illumination may be continuous (Sagawa et al., 1966). When liquid medium in 150-ml flasks is used, the tissues are placed on a reciprocal shaker at 80 rpm. Test tubes are rotated on a vertical wheel at 0.66 rpm. The temperature should be 22–25°C.

Culture Media. Explants can be first cultured in the media used in the previous procedure (Tables Cym-1 to Cym-3, and Cym-5). Proliferation is induced in a different formulation (Table Cym-6) that can also be used as a starting medium. Plantlets are formed on a solid version of the latter (Table Cym-6).

Procedure. The procedure is the same as that in the previous section (Sagawa et al., 1966) using the new medium (Table Cym-6).

TABLE CYM-6. **Medium for the in vitro propagation of *Cymbidium* (Fonnesbech, 1972*a*, 1972*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	400	40 g l^{-1}	10	Or weigh
2	Ammonium sulfate, $(NH_4)_2SO_4$[b]	300	30 g l^{-1}	10	Or weigh
3	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
4	Dipotassium phosphate, K_2HPO_4	212	21.2 g l^{-1}	10	
5	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
6	**Chelated iron**				
(a)	Na_2EDTA	37.8	7.5 g l^{-1}	5	One solution
(b)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.9	5.57 g l^{-1}		
7	**Microelements**[c]				
(a)	Manganous sulfate, $MnSO_4 \cdot 4H_2O$	25	25 g l^{-1}		
(b)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	10	10 g l^{-1}		
(c)	Boric acid, H_3BO_3	10	10 g l^{-1}	1	One solution
(d)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	250 mg l^{-1}		
(e)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	25 mg l^{-1}		
	Amino acid[d]				
8	Glycine	2	2 g l^{-1} 95% ethanol	1	
	Vitamins[e]				
9	Niacin (nicotinic acid)	1	100 mg 100 ml^{-1} 95% ethanol	1	
10	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol	1	
11	Thiamine (vitamin B_1)	0.5	50 mg 100 ml^{-1} 95% ethanol	1	
	Auxin				
12	Naphthaleneacetic acid (NAA)	1.86	186 mg 100 ml^{-1} 95% ethanol[f]	1	
	Polyol				
13	*myo*-inositol	100	No stock	No stock	Weigh
	Cytokinin				
14	Kinetin	0.215	21.5 mg 100 ml^{-1} 95% ethanol[f]	1	
15	**Complex additives**				
(a)	Casamino acids[g]	2–3 g	No stock	No stock	Weigh
(b)	Tryptone[g]	3–4 g	No stock	No stock	Weigh
16	Coconut water[h]	100–150 ml	No stock	No stock	
	Sugar				
17	Sucrose	30–40 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[i]	To 1000 ml			
	Solidifier[j]				
19	Agar	8 g			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add all microelements to the same 1 l, and stir and/or heat until dissolved. Add 1 ml to culture medium.

[d]By using 95% ethanol, the stock solution is made sterile. Add 1 ml stock solution to the autoclaved medium before it has solidified. Keep stock solution refrigerated.

[e]The vitamins may be combined into one stock solution. Add 100 mg niacin and 50 mg each of pyridoxine and thiamine to the same 100 ml of 95% ethanol, and use 1 ml of this per liter of medium. Refrigerate between uses.

[f]If the auxin or cytokinin fails to dissolve, add a few drops of dilute KOH or HCl, respectively, until the solution clears.

[g]Add either casamino acid or tryptone; not both.

[h]Coconut water increases growth 10–15%, but its addition is not essential.

[i]Add items 1–7, 13, 15, and 16 to 750 ml distilled water (item 18), adjust pH to 5.5–5.8, add sucrose (item 17), and bring volume to 1000 ml with more distilled water (item 18). Bring solution to a gentle boil, and add agar (item 19) slowly with stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour medium into a 2-l flask and autoclave. Add items 8–12 and 14 to the warm, still liquid medium, swirl to mix well, and distribute into preautoclaved vessels. For liquid media omit agar.

Developmental Sequence. Tissues and plantlets develop like those in the previous procedure (Sagawa et al., 1966), but there are a few minor differences.

General Comments. The medium formulated for this procedure (Table Cym-6) is based on comparisons between several media.

Heterogeneity of Acid Phosphatase and Ribonuclease in PLBs of *Cymbidium*

Streptomycin affected the enzyme activity and electrophoretic patterns of acid phosphatase and ribonuclease from PLBs of *Cymbidium* (Morawiecka et al., 1973).

Effects of Biostimin on the Growth of Meristematic Tissue and the Formation of PLBs by *Cymbidium* in Vitro

The addition of Biostimin FB, a medicinal preparation from the tissue of an *Aloe* species from the former Soviet Union, improved the growth, color, fresh weight, and dry weight of *Cymbidium* in vitro and increased the number of PLBs (Kukułczanka, 1985).

Tissue Culture and Flowering in Vitro of *Cymbidium ensifolium*

Orchids in general and *Cymbidium* in particular have been cultivated in China for a much longer time period than in the West. Some species are regarded as "king of the fragrant flowers." In recent years a number of Chinese investigators have initiated tissue culture research with *Cymbidium dayanum*, *Cymbidium ensifolium*, *Cymbidium faberi*, *Cymbidium goeringii*, and *Cymbidium tracyanum* (Wang et al., 1981; Wang, 1984, 1988*a*, 1988*b*, no date *a*, no date *b*).

Plant Material. Shoot meristem and axillary buds of *C. ensifolium* and *C. goeringii* Qiulan are used.

Surface Sterilization. Shoots, 5–6 cm long, from mature plants must be scrubbed with a very soft toothbrush under running water before the roots and outer leaves are stripped and the remaining tissues are sprayed with 70% ethanol. The axillary buds and shoot tips should be excised after that and (1) dipped in 70% ethanol for 30 s, (2) immersed in 5% sodium hypochlorite for 30 min, and (3) rinsed six times with sterile distilled water.

Culture Vessels. T-shaped tubes were used for liquid cultures in the original research, but other containers are also suitable. Bottles, test tubes, and Erlenmeyer flasks can be used to culture plantlets.

Culture Conditions. In the original research some explants of *C. goeringii* were cultured initially in the dark at 25°C for 3 months. (PLBs did not form under these conditions.) After that they were moved for 3 months to 12-h photoperiods (provided by fluorescent lights) at the same temperature (with the result that explants turned green). Explants of *C. ensifolium* (Wang, 1988*b*) were placed on agar and cultured under 9-h photoperiods of 2000–4000 lx (fluorescent lights) at 25°C. PLBs proliferate in a liquid medium on a shaker at 1 rpm.

Culture Media. Modified and basic White's medium (Tables Cym-7 to Cym-10) and MS (Tables Cym-11, Cym-12) are used for initial culture, protocorm formation and proliferation, plantlet development, and flower induction. Liquid unmodified MS medium (Table Cym-11) or solid modified Vacin and Went medium (Table Cym-5) can also be used.

Procedure. Place the explants on modified solid White's medium containing 5 mg l^{-1} NAA and 10% coconut water (Table Cym-7). Transfer PLBs or green explants that form after approximately 2 months to another solid or liquid modification of White's medium (Table Cym-8). If placed on solid medium (Table Cym-8), move the PLBs later to liquid medium (Table Cym-8) for further growth. After about a month transfer the PLBs to yet another modification of White's medium (Table Cym-9) for proliferation. Rosettes of bright green PLBs form in this medium. Liquid and similarly modified MS (Table Cym-11) or Vacin and Went (Table Cym-5) media can also be used for this step. Then transfer the PLBs to unmodified White's medium (Table Cym-10), where they form plantlets. Leaves are formed on solid MS medium (Table Cym-11). The plantlets flower when moved to another modified MS medium (Table Cym-12).

Developmental Sequence. Explants turn green and/or form PLBs 2–3 months after being placed in culture. Sections of these form new PLBs, which can be divided again. Rosettes of PLBs form in a medium that does not contain NAA. When moved to liquid medium these rosette PLBs develop plantlets. Complete plantlets with three to four leaves form following the final transfer onto an unmodified medium. The plantlets flower in vitro on an appropriate medium.

General Comments. Some Chinese *Cymbidium* plants are very desirable for their beautiful leaves, fragrant blossoms, flower shape, and other characteristics. Until now the only means of clonally propagating these plants was to divide them. This is a slow, inefficient method. The tissue culture method described here is much faster and may be suitable for other species and hybrids of *Cymbidium*.

Flower production in vitro from young seedlings of several orchid species and hybrids has been reported a number of times in the literature. There is also a report of "rarely but repeatedly observed . . . well structured inflorescence of *Cymbidium* [and] flowers produced *in vitro* . . ." (Tran Thanh Van, 1974). The report leaves open the question of whether these inflorescences were induced in vitro or before the buds were placed in culture. Consequently the flowering of *C. ensifolium* "mericlones" at the Shanghai Institute of Plant Physiology is the first instance of reproducible flower induction in orchids in vitro (Fig. Cym-1). Plants could flower in vitro for several months (Wang, 1988*a*).

TABLE CYM-7. **White's medium (Singh and Krikorian, 1981)[a] modified for the culture of *Cymbidium ensifolium* shoot tips (Wang et al., 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[c]	288	28.8 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	737	73.7 g l^{-1}	10	
3	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	19	1.9 g l^{-1}	10	
4	Potassium chloride, KCl	65	6.5 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	80	8 g l^{-1}	10	
6	Sodium sulfate, Na_2SO_4	200	20 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	3.4	344 mg l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	4.66	466 mg l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3[f]	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$[f]	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$[f]	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	6.65	665 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.75	75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$[f]	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.67	267 g l^{-1}		
	Amino acid				
9	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[g]	1	
	Auxin				
10	Naphthaleneacetic acid (NAA)	5	500 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
14	Coconut water	100 ml	No stock	No stock	Measure
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]There are many inconsistencies and contradictions in the literature regarding White's medium, some of them in his own papers. This table is based on an excellent recent study, reevaluation, and modernization of the medium (Singh and Krikorian, 1981).

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]To prepare chelated iron add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[f]Not part of White's recipe but advisable to use.

[g]Keep refrigerated or frozen between uses.

[h]Add items 1–8 and 14 to 850 ml distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add items 9–13 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. For liquid medium omit the agar.

TABLE CYM-8. **White's medium (Singh and Krikorian, 1981)[a] modified for the culture of *Cymbidium ensifolium* shoot tips (Wang et al., 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[c]	288	28.8 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	737	73.7 g l^{-1}	10	
3	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	19	1.9 g l^{-1}	10	
4	Potassium chloride, KCl	65	6.5 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	80	8 g l^{-1}	10	
6	Sodium sulfate, Na_2SO_4	200	20 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	3.4	344 mg l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	4.66	466 mg l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3[f]	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$[f]	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$[f]	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	6.65	665 mg l^{-1}		
(e)	Potassium iodide, KI	0.75	75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$[f]	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.67	267 g l^{-1}		
	Amino acid				
9	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[g]	1	
	Auxin				
10	Naphthaleneacetic acid (NAA)	2.5	250 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
14	Coconut water	100 ml	No stock	No stock	Measure
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]There are many inconsistencies and contradictions in the literature regarding White's medium, some of them in his own papers. This table is based on an excellent recent study, reevaluation and modernization of the medium (Singh and Krikorian, 1981).

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]To prepare chelated iron, add chelating agent (item 7a) and iron salt (item 7b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[f]Not part of White's recipe but advisable to include.

[g]Keep refrigerated or frozen between uses.

[h]Add items 1–8 and 14 to 850 ml distilled water (item 16). Adjust pH to 5.5, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add items 9–13 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE CYM-9. **White's medium (Singh and Krikorian, 1981)[a] modified for the culture of *Cymbidium ensifolium* shoot tips (Wang et al., 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[c]	288	28.8 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	737	73.7 g l^{-1}	10	
3	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	19	1.9 g l^{-1}	10	
4	Potassium chloride, KCl	65	6.5 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	80	8 g l^{-1}	10	
6	Sodium sulfate, Na_2SO_4	200	20 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	3.4	344 mg l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	4.66	466 mg l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3[f]	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$[f]	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$[f]	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	6.65	665 mg l^{-1}		
(e)	Potassium iodide, KI	0.75	75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$[f]	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.67	267 g l^{-1}		
	Amino acid				
9	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
13	Coconut water	100 ml	No stock	No stock	Measure
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[h]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]There are many inconsistencies and contradictions in the literature regarding White's medium, some of them in his own papers. This table is based on an excellent recent study, reevaluation and modernization of the medium (Singh and Krikorian, 1981).

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]To prepare chelated iron add chelating agent (item 7a) and iron salt (item 7b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[f]Not part of White's recipe but advisable to include.

[g]Keep refrigerated or frozen between uses.

[h]Add items 1–8 and 13 to 850 ml distilled water (item 15), adjust pH to 5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add items 9–12 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE CYM-10. **White's medium (Singh and Krikorian, 1981)[a] for the culture of *Cymbidium ensifolium* (Wang et al., 1981; Wang, 1984, 1988*a*, 1988*b*, no date *a*, no date *b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[c]	288	28.8 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	737	73.7 g l^{-1}	10	
3	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	19	1.9 g l^{-1}	10	
4	Potassium chloride, KCl	65	6.5 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	80	8 g l^{-1}	10	
6	Sodium sulfate, Na_2SO_4	200	20 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	3.4	344 mg l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	4.66	466 mg l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3[f]	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$[f]	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$[f]	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	6.65	665 mg l^{-1}		
(e)	Potassium iodide, KI	0.75	75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$[f]	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.67	267 g l^{-1}		
	Amino acid				
9	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
13	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[h]	To 1000 ml			
	Solidifier				
15	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]There are many inconsistencies and contradictions in the literature regarding White's medium, some of them in his own papers. This table is based on an excellent recent study, reevaluation and modernization of the medium (Singh and Krikorian, 1981).

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]To prepare chelated iron add chelating agent (item 7a) and iron salt (item 7b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved. Use of chelated iron is a modern modification.

[e]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[f]Not part of White's recipe but advisable to include.

[g]Keep refrigerated or frozen between uses.

[h]Add items 1–8 to 900 ml distilled water (item 14), adjust pH to 5.5, add sugar (item 13), and adjust volume to 1000 ml with distilled water (item 14). Bring solution to a gentle boil, and add agar (item 15) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add items 9–12 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE CYM-11. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for the culture of protocorm-like bodies derived from *Cymbidium ensifolium***

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol[f]	100	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive[h]				
13	Coconut water	100 ml	No stock	No stock	Measure
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[i]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Also known as *meso*- or *i*-inositol.

[g]Keep refrigerated between uses.

[h]To prepare unmodified medium, omit coconut water. Unmodified Murashige–Skoog medium may contain auxin(s) and/or cytokinin(s). The original paper does not mention these hormones, so it is therefore reasonable to assume they are not added.

[i]Add items 1–7, 9, and 13 to 850 ml distilled water (item 15). Adjust pH to 5.2–5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid and vitamins (items 8 and 10–12) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE CYM-12. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for flower induction in *Cymbidium ensifolium* in vitro (Wang, no date *a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
11	Benzyladenine (BA)	10	100 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin does not dissolve, use a few drops of KOH or HCl, respectively, to solubilize them.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 16), adjust pH to 5.2–5.5, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid, hormones, and vitamins (items 8 and 10–14) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

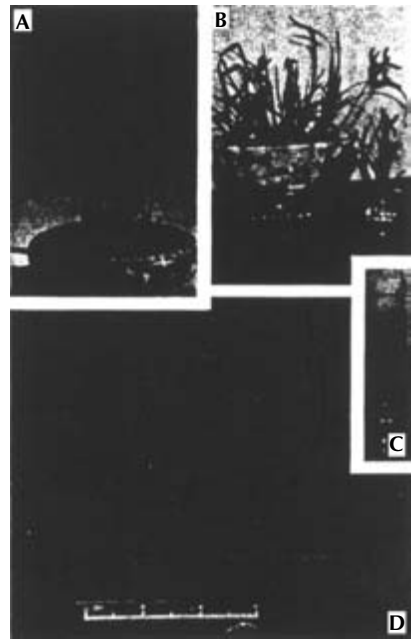


FIG. CYM-1 ***Cymbidium ensifolum*. A, B, D. Tissue culture derived from plants in flower following transfer to horticultural containers. C. Plant flowering in test tube. (Courtesy Dr. X. Wang, Shanhai Institute of Plant Physiology, Shanghai, People's Republic of China.)**

Tissue Culture Propagation of *Cymbidium goeringii*

Another beautiful Chinese orchid, *Cymbidium goeringii* (*Cymbidium virescens*), was also cultured at the Shanghai Institute of Plant Physiology (Wang et al., 1981; Wang, 1984, no date *a*, no date *b*).

Plant Material. Shoot meristems and axillary buds of *C. goeringii* are used.

Surface Sterilization. Shoots, 5–6 cm long, from mature plants must be scrubbed with a very soft toothbrush under running water before the roots and outer leaves are stripped and the remaining tissues are sprayed with 70% ethanol. The axillary buds and shoot tips should be excised after that and (1) dipped in 70% ethanol for 30 s, (2) immersed in 5% sodium hydrochlorite for 30 min, and (3) rinsed six times with sterile distilled water.

Culture Vessels. T-shaped tubes were used for liquid cultures in the original research, but other containers are also suitable. Bottles, test tubes, and Erlenmeyer flasks can be used to culture plantlets.

Culture Conditions. Explants are cultured initially for 3 months in the dark at 25°C. After that they are moved to 12-h photoperiods provided by fluorescent tubes.

Culture Media. Modified and unmodified MS medium (Tables Cym-11, Cym-13 to Cym-15) are used. A cytokinin-containing medium (Table Cym-16) is used to induce buds. Root formation occurs in a medium that contains only NAA (Table Cym-17).

Procedure. Initially culture the explants on modified MS medium with 10% coconut water and 5 mg l^{-1} NAA (Table Cym-13) in the dark at 25°C. After 3 months move the cultures to 12-h photoperiods at the same temperature. After the explants turn green (under these conditions for 3 months), transfer them to a solid medium with less NAA (Table Cym-14). Section a green PLB that forms on this medium within 1–2 months, and culture the pieces in the same medium (Table Cym-14) in liquid form. The sections form new protocorms within a month, and these too can be divided and subcultured. Rosettes of PLBs formed in this solution are cultured on a liquid medium without NAA (Table Cym-15) and rotated at 1 rpm. Plantlets form from the PLBs when the latter are moved to unmodified White's medium (Table Cym-10).

An alternate procedure is to culture the PLBs on a medium that contains BA and auxin (Table Cym-16). Culture the shoots formed on this medium on a modification of MS medium that contains only NAA (Table Cym-17) to induce roots. Culture plantlets produced by this procedure on unmodified MS medium (Table Cym-11) for further growth.

Developmental Sequence. Development is similar to that of *C. ensifolium* except that there are no reports of flowering in vitro.

General Comments. This is an effective procedure for the clonal propagation of yet another Chinese *Cymbidium* species.

TABLE CYM-13. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for the culture of *Cymbidium goeringii* in vitro (Wang, no date *a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	5.0	500 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Complex additive				
15	Coconut water	100 ml	No stock	No stock	Measure
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h,i]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin does not dissolve, use a few drops of KOH to solubilize it.

[g]Keep refrigerated between uses.

[h]Add items 1–7, 9, and 13 to 850 ml distilled water (item 16). Adjust pH to 5.2–5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. The agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid, hormone, and vitamins (items 8, 10–13) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

[i]Agar is not added to liquid media.

TABLE CYM-14. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for the culture of *Cymbidium goeringii* in vitro (Wang, no date *a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	2.5	250 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Complex additive				
15	Coconut water	100 ml	No stock	No stock	Measure
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin does not dissolve, use a few drops of KOH to solubilize it.

[g]Keep refrigerated between uses.

[h]Add items 1–7, 9, and 15 to 850 ml distilled water (item 16). Adjust pH to 5.2–5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid, hormone, and vitamins (items 8 and 10–13) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE CYM-15. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for the culture of *Cymbidium goeringii* in vitro (Wang, no date *a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol[f]	100	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
13	Sucrose	30 g	No stock	No stock	Weigh
	Complex additive				
14	Coconut water	100 ml	No stock	No stock	Measure
	Solvent				
15	Water, distilled[h]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Also known as *i*-inositol or *meso*-inositol.

[g]Keep refrigerated between uses.

[h]Add items 1–7, 9, and 14 to 850 ml distilled water (item 15), adjust pH to 5.2–5.5, add sugar (item 13), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid and vitamins (items 8 and 10–12) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE CYM-16. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for the culture of *Cymbidium goeringii* in vitro (Wang, no date *a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.2	20 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
11	Benzyladenine (BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin does not dissolve, add a few drops of KOH or HCl, respectively, to solubilize them.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 850 ml distilled water (item 16), adjust pH to 5.2–5.5, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid, hormones, and vitamins (items 8 and 10–14) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE CYM-17. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for the culture of *Cymbidium goeringii* in vitro (Wang, no date *a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	2.5	250 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[h]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin does not dissolve, add a few drops of KOH to solubilize it.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 850 ml distilled water (item 15), adjust pH to 5.2–5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid, hormone, and vitamins (items 8 and 10–13) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

Effects of Morphactin on the Initiation and Development of PLBs of *Cymbidium*

When applied in the range of 0.01 to 10 ppm, morphactin may increase the number of PLBs that form from excised meristematic tissues in vitro. Morphactin may also affect the shape and proliferation of the PLBs. Depending on the dose, morphactin can retard or inhibit the development of rhizoids, shoots, and roots and can also cause deformities (Kukułczanka and Twarda-Predota, 1973). This suggests that morphactin should not be used or that it must be employed very cautiously in attempts to increase proliferation.

Root Induction in Aseptically Produced Shoots of *Cymbidium goeringii*

Shoot tips of terrestrial *Cymbidium* species [*Cymbidium ensifolium, Cymbidium goeringii* (*Cymbidium virescens*), and *Cymbidium kanran* among others] form PLBs in vitro. These PLBs develop into a rhizome, but root and shoot formation occur with difficulty. A method that overcomes these difficulties was developed at the University of Nagoya (Ueda and Toikata, 1972).

Plant Material. Shoots formed on PLBs or rhizome tips of plants produced in vitro are excised.

Surface Sterilization. Shoots taken from aseptic cultures need not be sterilized.

Culture Vessels. Erlenmeyer flasks, of 100-ml capacity and containing 20 ml solid medium, were used in the original research, but other containers are also suitable.

Culture Conditions. Cultures should be maintained under fluorescent tubes, either white (Toshiba, FL20 SW was used in the original research, but other tubes can also be used) or Vitalux (NEC, FL 20 BR in the original research, but Sylvania Gro Lux are also appropriate) at an intensity of 8200 and 7300 erg cm^{-1} s^{-1} respectively. Roots may not be produced under lower intensities. The original paper (Ueda and Torikata, 1972) does not mention photoperiod length, but it is likely that 12- or 24-h illumination will be satisfactory. Temperature should be 22°C.

Culture Medium. Knudson C medium containing kinetin, arginine, and the microelements of Nitsch (Table Cym-18) is used.

Procedure. The shoots are taken from existing cultures and placed on the medium surface.

Developmental Sequence. The shoots form roots.

General Comments. This procedure is designed to induce root formation and development on shoots produced from PLBs in vitro. Its purpose is not to initiate

TABLE CYM-18. **Knudson C medium (Knudson, 1946) modified for root induction in *Cymbidium goeringii***

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
	Iron[c]				
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
6	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	500 mg l^{-1}	1	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	25 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	3.0	3 g l^{-1}		
(d)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	25 mg l^{-1}		
(e)	Sulfuric acid (concentrated), H_2SO_4	0.5	No stock		Measure carefully
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	500 mg l^{-1}		One solution
	Amino acid				
7	Arginine·HCl	211	No stock	No stock	Weigh
	Cytokinin				
8	Kinetin	10	1000 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[d]Add all microelements to the same 1000 ml distilled water, stir and/or heat to dissolve, and add 1 ml per liter of medium.

[e]Keep refrigerated or frozen between uses. If the kinetin fails to dissolve, add a few drops of dilute HCL.

[f]Add items 1–6 to 900 ml distilled water (item 10), adjust pH to 5.0, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Weigh the arginine (item 7), add it to the kinetin (item 8), mix well, and pour the mixture into the warm and still liquid medium. Mix well, and distribute the solution into preautoclaved culture vessels. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower pH if necessary.

the formation of PLBs, shoots, or plantlets from explants. Although formulated for *C. goeringii*, this method may prove suitable for additional *Cymbidium* species and other orchid shoots that are difficult to root in vitro.

Rooting of *Cymbidium insigne* Shoots in Vitro

This method is the same as the one used for *Cymbidium goeringii* (Ueda and Torikata, 1972).

Growth of *Cymbidium* in Vitro

The bleeding sap of birch trees seems to enhance growth of *Brassia*, *Cattleya*, and *Cymbidium* explants (Zimmer and Pieper, 1976, 1977).

Effects of Substrate on Cymbidium Cultures

The development of *Cymbidium* shoots and roots was better on a medium of pearl barley than on one solidified with agar (Kukułczanka and Paluch, 1971; Kukułczanka, 1976; Kukułczanka et al., 1987).

Effects of Microelements and Magnesium on *Cymbidium* Development in Vitro

The addition of microelements and magnesium enhanced the growth of PLBs of *Cymbidium* (Kukułczanka and Jastrzebska-Kolodynska, 1976/77).

Clonal Propagation of *Cymbidium* through the Culture of Dormant Buds taken from Pseudobulbs

Clonal propagation of *Cymbidium* through the culture of shoot tips (erroneously called meristems) requires the excision under sterile conditions of a small explant. In some instances removal of the explant may jeopardize the existence of the donor plant. On the other hand, dormant buds on a pseudobulb seem to serve no horticulturally useful purpose, they are easy to remove, and their excision does not endanger the plant (Tran Thanh Van, 1974).

Plant Material. Buds are excised from the upper two-thirds ("aerial") portion of backbulbs (pseudobulbs). The buds are removed following surface sterilization under aseptic conditions by using a sterile scalpel to make an incision 1–2 mm long 1–2 mm wide, and 5–7 mm deep (Fig. Cym-2). This explant (approximately 12.4 mm^3) is placed in culture.

Surface Sterilization. The upper two-thirds ("aerial") portion is removed, and the top parts of the leaves are cut away before washing with water and a mild detergent (Teepol, a liquid dishwashing formulation, was used in the original research). After the wash the pseudobulbs are dipped in 70–80% ethanol. The leaf bases are removed after that, and the pseudobulb is washed a second time with water and detergent. This is followed by a second dip for a few seconds in 70–80% ethanol. The final surface sterilization step consists of: (1) soaking the pseudobulb for 15 min in a 5 or 7% solution of calcium hypochlorite (5–7 g calcium hypochlorite in 100 ml distilled water stirred several times at 5-min intervals and decanted or filtered), and (2) washing it several times with sterile distilled water. The buds are excised after the final wash.

FIG. CYM-2. ***Cymbidium*** **bud explant at the start of culture (Tran Thanh Van, 1974).**

Culture Vessels. Small glass or plastic Petri dishes or plastic bags containing medium were used in the original research. The plastic bags (which must be sterile before introduction of the medium) can simply be hung on a wall or laid on top of each other. Other containers can also be used.

Culture Conditions. The cultures should be maintained under 12-h photoperiods of 3300 lx and a temperature of 22–24°C.

Culture Media. The explants should be placed initially on a modified MS medium (Table Cym-19). Plantlet production occurs on a different modification of this medium (Table Cym-20).

Procedure. Place the explants on the first medium (Table Cym-19), and culture until PLBs form. These can be cut and subcultured on the same medium to bring about further proliferation. To produce plantlets, place the PLBs on the second medium (Table Cym-20).

Developmental Sequence. Buds from near the base of a pseudobulb that are more developed may form a single plant when cultured on Knudson C medium (see Tables Aranda-7, Aranda-8, Cym-2, and Cym-3). On the first medium (Table Cym-19) the buds may form one or more PLBs. In some cases meristems situated at the axils of floral initials may fasciate and thus can result in the formation of multiple plantlets. Floral shoots and flowers may develop from buds excised from the upper portions of pseudobulbs.

General Comments. This procedure is relatively simple and makes good use of buds that normally remain dormant for the life of a pseudobulb. A possible improvement would be a method that could allow excision of the buds without having to cut away part of the pseudobulb.

TABLE CYM-19. **Murashige-Skoog medium (Murashige and Skoog, 1962) for the culture of dormant buds of *Cymbidium* (Tran Thanh Van, 1974)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Polyol				
8	*myo*-inositol[e]	100	No stock	No stock	Weigh
	Auxin				
9	Indoleacetic acid (IAA)	1.9	190 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
10	Kinetin	2.2	220 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamin				
11	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
12	Glucose	30 g	No stock	No stock	Weigh
	Solvent				
13	Water, distilled[h]	To 1000 ml			
	Solidifier				
14	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Sometimes listed as *meso*-inositol.

[f]If auxin or cytokinin does not dissolve, add a few drops of dilute KOH or HCl, respectively, to solubilize them.

[g]Keep refrigerated frozen between uses.

[h]Add items 1–8 to 900 ml distilled water (item 13), adjust pH to 5.2–5.5, add sugar (item 12), and adjust volume to 1000 ml with distilled water (item 13). Bring solution to a gentle boil, and add agar (item 14) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add hormones and vitamin (items 9–11) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

TABLE CYM-20. **Murashige–Skoog medium (Murashige and Skoog, 1962) for plantlet production from protocorm-like bodies derived from dormant buds of *Cymbidium* (Tran Thanh Van, 1974)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Polyol				
8	*myo*-inositol[e]	100	No stock	No stock	Weigh
	Auxin				
9	Indoleacetic acid (IAA)	1.9	190 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
10	N^6-benzyladenine (BA)	2.3	230 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamin				
11	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
12	Glucose	30 g	No stock	No stock	Weigh
	Solvent				
13	Water, distilled[h]	To 1000 ml			
	Solidifier				
14	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add the chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Sometimes listed as *meso*-inositol.

[f]If auxin or cytokinin does not dissolve, add a few drops of dilute KOH or HCl, respectively, to solubilize them.

[g]Keep refrigerated between uses.

[h]Add items 1–8 to 900 ml distilled water (item 13), adjust pH to 5.2–5.5, add sugar (item 12), and adjust volume to 1000 ml with distilled water (item 13). Bring solution to a gentle boil, and add agar (item 14) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add hormones and vitamin (items 9–11) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

Vegetative Propagation of *Cymbidium* on Modified Knudson C Medium

Since allied species or even related cultivars do not always respond similarly to a specific medium, an attempt was made to develop "media . . . versatile enough to be used for seed germination and meristem culture . . ." (Dalla Rosa and Laneri, 1977).

Plant Material. Meristematic tissues should be excised from the lateral buds on young shoots that are approximately 10 cm long. Explants from lateral buds (on cubes approximately 2 mm thick, 2 mm wide, and 1 mm thick) and apices (slightly larger squares but not thicker than 1 mm) are cut under sterile conditions while the tissues are submerged in sterile distilled water to prevent browning.

Surface Sterilization. Remove outer leaves, bud scales, and necrotic tissues carefully, and cut yellowish internal leaves approximately 1 cm above the apex. After that the shoots are dipped in 70% ethanol for a few seconds, submerged in freshly prepared 10% calcium hypochlorite solution for 20 min, and rinsed with sterile distilled water three times. (The hypochlorite solution is made by placing 10 g calcium hypochlorite in 80 ml distilled water, adjusting to 100 ml, stirring a few times at 5–10-min intervals, and filtering or decanting.)

Culture Vessels. Erlenmeyer flasks and culture tubes were used in the original experiment, but other containers are equally suitable. Culture vessels should contain medium equal in volume to one-fifth of their capacity (e.g., a 125-ml Erlenmeyer flask should contain 25 ml medium).

Culture Conditions. Cultures should be maintained under 18-h photoperiods; in the original research illumination was provided by "solar spectrum fluorescent tubes," but other light sources should prove to be equally suitable. Maintain temperatures at 25 ± 2°C during the light period and 22 ± 2°C in the dark, although a constant temperature in the 22–25°C range would also be appropriate.

Culture Medium. A modification of the Knudson C medium is used (Table Cym-21).

Procedure. Place explants on the medium, and subculture as necessary.

Developmental Sequence. Differentiation starts 10–15 days after the explants are placed in culture; PLBs appear within 1.5 months.

General Comments. This appears to be a relatively simple and useful procedure.

TABLE CYM-21. **Knudson C medium (Knudson, 1946) modified for the culture of meristematic tissues from *Cymbidium* buds (Dalla Rosa and Laneri, 1977)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	250	25 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
6	**Microelements**[d]				
(a)	Aluminum chloride, $AlCl_3$	0.03	3 mg l^{-1}		
(b)	Boric acid, H_3BO_3	1	100 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.03	3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	0.1	10 mg l^{-1}	10	One solution
(e)	Nickel chloride, $NiCl_2 \cdot 6H_2O$	0.03	3 mg l^{-1}		
(f)	Potassium iodide, KI	0.01	1 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1	100 mg l^{-1}		
(h)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	Separate solution
	Complex additive				
7	Coconut water[e]	100 ml	No stock	No stock	Measure
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Solvent[f]				
9	Distilled water	To 1000 ml			
	Solidifier				
10	Agar[f]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original version of the Knudson C medium calls for 25 mg $FeSO_4 \cdot 7H_2O$. Chelated iron is preferable.

[d]These are the microelements of Heller's medium (Heller, 1953). They contain manganese, but the recipe for this modification (Dalla Rosa and Laneri, 1977) also lists the quality of manganese included as part of the Knudson C medium. Either would suffice, but both are given here to conform with the modified recipe. Add items 6a–6g to the same 1 l of water, stir and/or heat to mix, and dispense as indicated. Prepare a separate solution of item 6h, and add it to the medium as shown. If toxicity symptoms develop, omit item 6h.

[e]Like many other publications the original paper (Dalla Rosa and Laneri, 1977) lists this item as "coconut milk." What is actually added is the liquid endosperm of green (i.e., unripe) coconuts, so a more appropriate term would be "coconut water." Store excess coconut water in a freezer.

[f]Add items 1–7 to 800 ml distilled water (item 9), adjust pH to 6.0, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When the agar is completely dissolved pour the medium into culture vessels and autoclave. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower the pH if necessary.

Effects of Extracts from Symbiotic Rhizomes of *Cymbidium goeringii*

Extract from fungus-containing rhizomes seems to enhance growth and development of some *Cymbidium* explants (Ueda and Torikata, 1974).

Shoot-tip Culture of Oriental Orchids

Researchers at the Institute of Utilization of Atomic Energy in Agriculture, Sichuan Academy of Agricultural Science, People's Republic of China, have attempted to culture 45 cultivars of oriental orchids. They succeeded with 20 of them. Techniques were perfected for the culture of *Cymbidium goeringii* and *C. goeringii* var. *longibracteatum* (Wu et al., 1987).

Multiplication of PLBs of *Cymbidium*

In some instances PLBs die after being sectioned and mashed (Yoneda et al., 1980).

Clonal Propagation of *Cymbidium* through Shoot-tip Cultures

Plant hormones can enhance the usefulness of many culture media, but at high concentrations they may induce undesirable mutations (Haas-von Schmude, 1985). For this reason a micropropagation procedure for *Cymbidium* was developed that uses low hormone concentrations (Gu et al., 1987*a*).

Plant Material. Shoot tips from lateral buds on *Cymbidium* pseudobulbs or shoots are excised and cultured. To expose the shoot tips, adult leaves are removed and the younger leaves cut in half. The tips are excised following surface sterilization under aseptic conditions as cubes measuring $2 \times 1.5 \times 1.5$ mm.

Surface Sterilization. Shoots (ca. 4–6 cm long) or pseudobulbs should be scrubbed with a soft (i.e., used) toothbrush under running water and then dipped for a few seconds in 70% ethanol. After that they are immersed in 50% Clorox (50 ml Clorox diluted to 100 ml with distilled water) for 20 min and rinsed three times with sterile distilled water. Shoot tips approximately 5 mm long are removed and immersed in 10% Clorox (10 ml Clorox diluted to 100 ml with sterile distilled water) and rinsed as before. The tips are trimmed to $2 \times 1.5 \times 1.5$ mm (4.5 mm^3) following the last rinse.

Culture Conditions. Liquid cultures should be placed on a reciprocal shaker at 55 oscillations per minute. Both liquid and solid cultures are maintained at $22 \pm 3°C$ under 16-h photoperiods and a light intensity of 0.8 W cm^{-2} provided by Sylvania Gro Lux fluorescent tubes.

Culture Media. A modification of Vacin and Went medium (Table Cym-22) is used for initial culture. Plantlet formation occurs on a second modification of the same medium (Table Cym-23).

Procedure. First place shoot tips on liquid medium (Table Cym-22). When PLBs form, section and subculture them. For plantlet formation, place the PLBs on the second medium (Table Cym-23).

TABLE CYM-22. **Vacin and Went medium (Vacin and Went, 1949) modified for *Cymbidium* shoot tip cultures (Gu et al., 1987*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
7	**Microelement**				
	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Polyol				
8	*myo*-inositol	100	No stock	No stock	Weigh
	Cytokinin				
9	N^6-benzyladenine (BA)	1	100 mg 100 ml^{-1} 95% ethanol[e]		
	Sugar				
10	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Agar[f]	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, stir, and/or heat until it dissolves. Add the other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 37.3 mg chelating agent (Na_2EDTA) and 27.8 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Keep refrigerated or frozen between uses.

[f]Add items 1 and 3–8 to the 500 ml distilled water (item 11) which contain the calcium phosphate (item 2), adjust pH to 5.5, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring solution to a gentle boil, and add agar (item 12) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When the agar is completely dissolved pour the medium into a 2-l flask, and autoclave. Add hormone (item 9) to the hot, still liquid medium, mix well by swirling, and dispense into preautoclaved culture vessels. Omit agar if preparing liquid medium.

Developmental Sequence. Approximately 75% of the shoot-tip explants can be expected to form PLBs within 2 months (Fig. Cym-3A, B). They are white at first but turn green 10 days after becoming apparent. Sections form new PLBs after 4 weeks. The growth rate of PLBs is 3.8 g in 2 weeks. Shoot production (Fig. Cym-3C, D) occurs on nearly 50% of the PLBs. Complete plantlets (Fig. Cym-3E, F) are produced in 5 months.

General Comments. This is an easy, rapid method for the clonal propagation of *Cymbidium*.

TABLE CYM-23. **Vacin and Went medium (Vacin and Went, 1949) modified for plantlet production from *Cymbidium* protocorm-like bodies (Gu et al., 1987*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Polyol				
8	*myo*-inositol	100	No stock	No stock	Weigh
	Cytokinin				
9	N^6-benzyladenine (BA)	1	100 mg 100 ml^{-1} 95% ethanol[e]		
	Sugar				
10	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Agar[f]	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve and it is not possible to prepare a stock solution. To facilitate the preparation of medium place 200 mg of the salt in 500 ml water, stir, and/or heat until it dissolves. Add the other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 37.3 mg chelating agent (Na_2EDTA) and 27.8 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Keep refrigerated or frozen between uses.

[f]Add items 1 and 3–8 to the 500 ml distilled water (item 11) that contain the calcium phosphate (item 2). Adjust pH to 5.5, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring solution to a gentle boil, and add agar (item 12) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When the agar is completely dissolved pour the medium into a 2-l flask, and autoclave. Add hormone (item 9) to the hot, still liquid medium, mix well by swirling, and dispense into preautoclaved culture vessels.

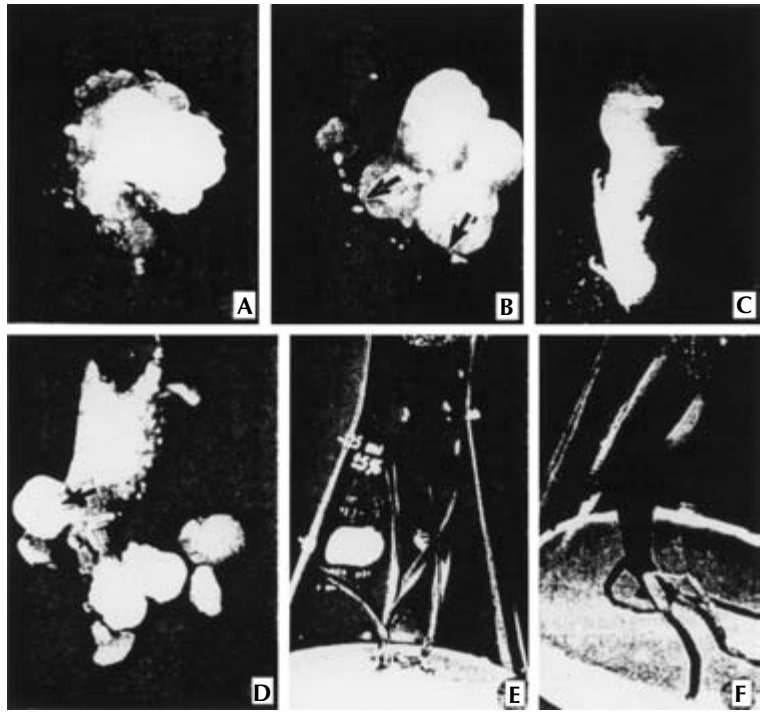


FIG. CYM-3. **Shoot-tip culture and plantlet differentiation in vitro of *Cymbidium*. A. PLBs formed from a shoot tip after 2 months of culture on Vacin and Went medium containing 1 mg l^{-1} BA (VW1BA; × 25). B. Multiple PLBs following a 4-week culture period on VW1BA (× 10). C. Shoot differentiation from a PLB within 4 weeks in culture on Vacin and Went medium containing 0.4 mg l^{-1} BA (VW0.4BA; × 5). D. Several PLBs on the base of a differentiated shoot after 4 weeks on VW1BA (× 5). E. A plant that developed after 5 months of culture on unmodified Vacin and Went medium (see Table Cym-5; × 0.7). F. Plantlet that formed after 5 months on VW0.4BA. (Gu et al., 1987*b*.)**

Effects of N^6-benzylaminopurine and NAA on Organogenesis in *Cymbidium*

Addition of NAA alone to Knudson C medium with the microelements of Nitsch had no effect on the formation of PLBs. BA at 0.1 mg l^{-1} increased production of PLBs considerably. A combination of BA (10 mg l^{-1}) and NAA (concentration not given, but it could have been 1–10 mg l^{-1}; 10 mg l^{-1} seems high) greatly enhanced shoot production during the first 90 days of culture. NAA at 0.1 mg l^{-1} increased root production slightly. BA had an inhibitory effect on root production (Matsui et al., 1970).

Rapid Propagation of *Cymbidium* through the Culture of Apical and Axillary Buds

Tissue culture as a means of rapid propagation of orchids has received "more and more attention from South African growers," one reason for the development of a method for *Cymbidium* propagation at the Horticultural Research Institute in Pretoria (van Rensburg and Vcelar, 1984).

Plant Material. Apical and axillary buds from actively growing pseudobulbs with six to seven leaves are used. Explants should be 1–5 mm in size. The excision steps described for dormant buds (Tran Thanh Van, 1974) can be used.

Surface Sterilization. All excess and dead tissue should be removed from the pseudobulbs before washing them thoroughly with water. The original paper does not mention a detergent, but using one may be a good idea (Tran Thanh Van, 1974; Gu et al., 1987*b*). The pseudobulbs are sterilized by soaking them in 1% sodium hypochlorite (20 ml Clorox diluted to 100 ml with distilled water) for 20 min. Further washing and sterilization are not described in the original paper, but if needed the appropriate steps of other procedures (Tran Thanh Van, 1974; Gu et al., 1987*b*) can be used.

Culture Vessels. No details are given regarding culture vessels, but it is reasonable to assume that standard containers (Erlenmeyer flasks, Petri dishes, test tubes, etc.) would be suitable.

Culture Conditions. The cultures should be maintained under 16-h photoperiods provided by standard light sources at 24°C.

Culture Medium. A modified Knudson C medium containing banana homogenate from "bananas [that are] . . . ripe but still firm" (Table Cym-24) is used.

Procedure. Place explants on the medium. Subdivide the PLBs that form, and transfer them to fresh medium every 6 weeks. (PLBs not subdivided and transferred to new medium will form plantlets.)

Developmental Sequence. Approximately 4 weeks after the start of culture, the explants form one to five PLBs. On being subdivided their sections develop several PLBs, which can be subcultured again. Plantlets differentiate when PLBs are not subdivided and allowed to remain on the culture medium for a while.

General Comments. This procedure can be used to free *Cymbidium* cultivars from virus, but care must be taken, and the new plants must be screened for infection. If this is not done, this (or any other) tissue culture method can "result in thousands of infected daughter plants." Even when plants are screened and found to be free of virus, the findings are valid for only a short period. Cultivated plants can be infected easily.

TABLE CYM-24. **Knudson C medium (Knudson, 1946) modified for tissue culture of *Cymbidium* (van Rensburg and Vcelar, 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
	Iron[c]				
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 g l^{-1}	10	
	Complex additive				
7	Banana homogenate[d]	100 g	No stock	No stock	Weigh
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[d]The bananas should be "ripe but still firm." Cut the banana into slices, and place them in 200 ml water in a homogenizer. Homogenize for 1–2 min, pause for 3–5 min and homogenize for another minute. Pour homogenate into a 2-l beaker. Rinse homogenizer three times with 100 ml distilled water, pour the washings into homogenate, and stir well to prepare item 7.

[e]Add items 1–7 to 800 ml distilled water (item 9), adjust pH to 5.5, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. The agar can also be added to the cold solution; then bring to a boil and stir. When agar is completely dissolved, pour medium into culture vessels and autoclave. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. The addition of K_2HPO_4 raises the pH to about 5.3. Use a dilute solution of hydrochloric acid (HCl) to lower the pH if necessary.

Effects of Phytohormones and Peptone on Meristem Culture of *Cymbidium*

Studies in Poland (Kukułczanka and Paluch, 1971; Kukułczanka and Sarosiek, 1971; Morawiecka et al., 1973; Kukułczanka, 1976) have shown the following:

1. Peptobak-Bacutil peptone from Poland enhances shoot-tip cultures of *Cymbidium* when added to a modified Tsuchiya medium at a concentration of 2 g l^{-1}.
2. Addition of auxin after 4 weeks of culture is advisable since it enhances the growth of PLBs and plantlets.
3. Kinetin increases the number of PLBs.
4. Gibberellic acid accelerates shoot growth but inhibits root development.

5 Morphactin can increase the number of PLBs, but it could also induce developmental anomalies.
6 Additions of streptomycin to culture media brought about changes in the activity and electrophoretic patterns of acid phosphatase and ribonuclease.

Clonal Propagation of *Cymbidium* through the Culture of Floral Organs in Vitro

Gynostemia, ovaries, and flower stalks were better than sepals, petals, and labella for the production of buds when cultured on solid or liquid MS medium supplemented with 0.1 ppm NAA and 1 ppm BA. Production of buds was enhanced by liquid medium (Kim and Kako, 1984).

Clonal Propagation of *Cymbidium* through the Culture of Shoot Apices in Vitro

A series of investigations regarding the effects of media components on PLBs of *Cymbidium* have resulted in the formulation of a micropropagation method (Kusumoto and Furukawa, 1977; Kusumoto, 1978, 1980*a,* 1980*b*, 1981*a*).

Plant Material. New shoots approximately 5 cm in length are excised and their outer leaves are removed. After sterilization their shoot apices are removed and cultured.

Surface Sterilization. The shoots are soaked in Wilson's calcium hypochlorite solution (7 g calcium hypochlorite in 100 ml of water stirred several times at 5-min intervals, filtered or decanted, and used within a few hours). There is no mention of subsequent washing of the explants, but two to three rinses with sterile distilled water are advisable.

Culture Vessels. During the original research the explants were cultured in 200-ml Erlenmeyer flasks. These are very suitable containers, but other culture vessels can also be used.

Culture Conditions. Cultures are maintained under an illumination of 5000 lx at 25–30°C.

Culture Medium. Several media with additives were screened in the original research; the best growth occurred on a modification of Knudson C medium (Table Cym-25).

Procedure. The explants are cultured until PLBs form. These are divided and cultured on the same medium for proliferation and plantlet formation.

Developmental Sequence. PLBs form on the explants. On being divided, the PLBs proliferate and/or produce plantlets.

TABLE CYM-25. **Knudson C medium (Knudson, 1946) modified for *Cymbidium* culture in vitro (Kusumoto and Furukawa, 1977; Kusumoto, 1978, 1980*a*, 1980*b*, 1981*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
	Iron[c]				
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
	Microelements				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additives				
7	Banana juice[d]	150 ml	No stock	No stock	Measure
8	Apple juice[e]	150 ml	No stock	No stock	Measure
9	Peptone, Difco Bacto[f]	2 g	No stock	No stock	Weigh
	Sugar				
10	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium

[d]If banana juice is not available, prepare the homogenate given in Table Cym-24. If so, recalculate the volumes in footnote *g* below. Commercial juices often contain preservatives that can inhibit growth. Even so-called natural juices are not always free of preservatives. Therefore exercise great care when juices are used and read the label carefully. If information is not available, it is best to prepare a homogenate.

[e]The same comments in footnote *d* above apply to apple juice. If preparing an apple homogenate in the laboratory, remove the seeds before homogenizing the tissues like those of banana (Table Cym-24). Alternately apple juice may be prepared by squeezing the sections. Filter apple juice or homogenate through cheesecloth, nylon stockings, or similar fabric.

[f]The composition of peptones from various manufacturers may vary. Make substitutions with caution, and test carefully before using them with valuable plants.

[g]Add items 1–9 to 500 ml distilled water (item 11), adjust pH to 5.4, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring solution to a gentle boil, and add agar (item 12) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into culture vessels, and autoclave. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower the pH if necessary.

General Comments. Related findings from the same laboratory are as follows:

1. Bud formation on protocorms is enhanced by 0.01 mg l^{-1} 2,4-D and 0.1 mg l^{-1} gibberellic acid (GA_3). A combination of 0.1–1.0 mg l^{-1} kinetin and 0.01–0.1 mg l^{-1} NAA, or 0.1–1.0 mg l^{-1} GA_3 and 0.01–0.1 mg l^{-1} NAA has similar effects.
2. GA_3 (1.0 mg l^{-1}) and 2,4-D (0.1 mg l^{-1}) enhance shoot development.
3. Root formation is best in the presence of 1.0 mg l^{-1} GA_3 and 0.1 mg l^{-1} NAA.
4. Tryptone promotes organogenesis.
5. Coconut water (10%) promotes proliferation of PLBs.
6. Growth is enhanced when cultures are placed on 16 ml of the appropriate medium in a 200-ml Erlenmeyer flask.

Protoplasts Isolation from Leaves of *Cymbidium aloifolium*

Protoplasts can be isolated from leaves of *Cymbidium aloifolium* by the method used for *Acampe praemorsa*. The yield from leaves was 18.9×10^4 g^{-1} fresh weight per tissue. A much higher yield, 89.5×10^4 g^{-1} fresh weight per tissue, was obtained from "the tender leaf bases" (Seeni and Abraham, 1986).

Morphogenesis and Clonal Propagation of *Cymbidium* through Aseptic Cell Cultures

Research on the growth and development in aseptic cell and tissue cultures of *Cymbidium* has shown that cultured cells can be used for clonal propagation (Steward and Mapes, 1971*a*).

Plant Material. Callus cultures obtained by culturing *Cymbidium* shoot tips on White's medium (Table Cym-26) are used.

Surface Sterilization. The original paper (Steward and Mapes, 1971*a*) does not provide information on the surface sterilization of the shoot tips used to produce the callus, but the steps employed in other procedures (Tran Thanh Van, 1974; Gu et al., 1987*b*) should prove suitable. Callus sections need not be sterilized since they are taken from aseptic cultures.

Culture Vessels. Erlenmeyer flasks or test tubes can be used for cultures on solid medium. T-shaped tubes were used for liquid cultures in the original research. Plastic containers and 30-ml sample bottles were also used. Other containers should also prove suitable.

Culture Conditions. General information about Steward's laboratory suggests that the liquid cultures were placed on a horizontal rotating shaker. Solid cultures were probably maintained on a table, shelf, or bench. "Moderate light" and 21°C are suitable culture conditions, but "there was some evidence of better greening of the cultures at 18 hours of day and at 24°C."

Culture Media. White's medium with coconut water and auxin (Table Cym-26) is used for shoot-tip cultures. A different modification of this medium (Table Cym-27) is used for the production and multiplication of free cells. The first medium is best for the production of PLBs. When grown on a medium devoid of auxin (Table Cym-28), the PLBs multiply to form rosettes. It is not clear from the original paper which medium was used for plantlet production, but it is reasonable to assume that Knudson C medium (see Tables Aranda-7, Aranda-8, Cym-2, and Cym-3), Vacin and Went medium (see Table Cym-5), or some other combination used for *Cymbidium* would be suitable.

Procedure. The first step of this procedure is to produce callus from a shoot-tip explant. Any one of the procedures described earlier for *Cymbidium* should prove

TABLE CYM-26. **White's medium (Singh and Krikorian, 1981)[a] for the culture of *Cymbidium* shoot tips (Steward and Mapes, 1971*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[c]	288	28.8 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	737	73.7 g l^{-1}	10	
3	Monsodium phosphate, $NaH_2PO_4 \cdot H_2O$	19	1.9 g l^{-1}	10	
4	Potassium chloride, KCl	65	6.5 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	80	8 g l^{-1}	10	
6	Sodium sulfate, Na_2SO_4	200	20 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	3.4	344 mg l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	4.66	466 mg l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3[f]	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$[f]	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$[f]	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	6.65	665 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.75	75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$[f]	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.67	267 mg l^{-1}		
	Amino acid				
9	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Auxin				
13	α-Naphthaleneacetic acid (NAA)	5	500 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
14	Coconut water[h]	100 ml	No stock	No stock	Measure
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[i]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]There are many inconsistencies and contradictions in the literature regarding White's medium, some of them in his own papers. This table is based on an excellent recent study, reevaluation, and modernization of the medium (Singh and Krikorian, 1981). $ZnSO_4 \cdot 4H_2O$ is very hard to find, if at all. Use an equal amount of $ZnSO_4 \cdot 7H_2O$ instead.

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]To prepare chelated iron add chelating agent (item 7a) and iron salt (item 7b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium.

[f]Not part of White's recipe but advisable to include.

[g]Keep refrigerated or frozen between uses.

[h]Like many other publications, Steward and Mapes (1971*a*) lists this item as "coconut milk." What is actually added is the liquid endosperm of green (i.e., unripe) coconuts, so a more appropriate term is "coconut water." Store excess coconut water in a freezer.

[i]Add items 1–8 and 14 to 800 ml distilled water (item 16), adjust pH to 5.2–5.5, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid, vitamins, and hormone (items 9–13) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE CYM-27. **White's medium (Singh and Krikorian, 1981)[a] for the production of free cells from callus cultures derived from *Cymbidium* shoot tips (Steward and Mapes, 1971*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[c]	288	28.8 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	737	73.7 g l^{-1}	10	
3	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	19	1.9 g l^{-1}	10	
4	Potassium chloride, KCl	65	6.5 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	80	8 g l^{-1}	10	
6	Sodium sulfate, Na_2SO_4	200	20 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	3.4	344 mg l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	4.66	466 mg l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3[f]	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$[f]	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$[f]	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	6.65	665 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.75	75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$[f]	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.67	267 g l^{-1}		
	Amino acid				
9	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Auxin				
13	2,4-Dichlorophenoxyacetic acid (2,4-D)	5	500 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
14	Coconut water[h]	100 ml	No stock	No stock	Measure
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[i]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]There are many inconsistencies and contradictions in the literature regarding White's medium, some of them in his own papers. This table is based on an excellent recent study, reevaluation, and modernization of the medium (Singh and Krikorian, 1981). $ZnSO_4 \cdot 4H_2O$ is very hard to find, if at all. Use an equal amount of $ZnSO_4 \cdot 7H_2O$ instead.

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]To prepare chelated iron add chelating agent (item 7a) and iron salt (item 7b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[f]Not part of White's recipe but advisable to include.

[g]Keep refrigerated or frozen between uses.

[h]Like many other publications, Steward and Mapes (1971*a*) lists this item as "coconut milk." What is actually added is the liquid endosperm of green (i.e., unripe) coconuts, so a more appropriate term is "coconut water." Store excess coconut water in a freezer.

[i]Add items 1–8 and 14 to 800 ml distilled water (item 16), adjust pH to 5.2–5.5, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid, vitamins, and hormone (items 9–13) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE CYM-28. **White's medium (Singh and Krikorian, 1981)[a] for the production of free cells from callus cultures derived from *Cymbidium* shoot tips (Steward and Mapes, 1971*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[c]	288	28.8 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	737	73.7 g l^{-1}	10	
3	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	19	1.9 g l^{-1}	10	
4	Potassium chloride, KCl	65	6.5 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	80	8 g l^{-1}	10	
6	Sodium sulfate, Na_2SO_4	200	20 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	3.4	344 mg l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 4H_2O$	4.66	466 mg l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3[f]	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$[f]	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$[f]	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	6.65	665 mg l^{-1}		
(e)	Potassium iodide, KI	0.75	75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$[f]	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.67	267 g l^{-1}		
	Amino acid				
9	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
13	Coconut water[h]	100 ml	No stock	No stock	Measure
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[i]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]There are many inconsistencies and contradictions in the literature regarding White's medium, some of them in his own papers. This table is based on an excellent recent study, reevaluation, and modernization of the medium (Singh and Krikorian, 1981). $ZnSO_4 \cdot 4H_2O$ is very hard to find, if at all. Use an equal amount of $ZnSO_4 \cdot 7H_2O$ instead.

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]To prepare chelated iron add chelating agent (item 7a) and iron salt (item 7b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[f]Not part of White's recipe but advisable to include.

[g]Keep refrigerated or frozen between uses.

[h]Like many other publications, Steward and Mapes (1971*a*) lists this item as "coconut milk." What is actually added is the liquid endosperm of green (i.e., unripe) coconuts, so a more appropriate term is "coconut water." Store excess coconut water in a freezer.

[i]Add items 1–8 and 13 to 800 ml distilled water (item 15), adjust pH to 5.2–5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid and vitamins (items 9–12) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

suitable, but it is advisable to grow the explants on the modification of White's medium (Table Cym-26). To release cells, place the callus produced in the first step in a liquid medium (Table Cym-27) under agitation. Move the cells (in aliquots of medium) to the first medium (Table Cym-26) to induce the production of PLBs. These are moved to another medium for greening and proliferation (Table Cym-28). Plantlets can be produced from the PLBs on several media.

Developmental Sequence. Cells that slough off from the callus mass produce somatic embryos that give rise to PLBs. These proliferate and produce plantlets.

General Comments. Since many cells can slough off from each callus mass, this procedure can produce a very large number of plants very rapidly. Plants produced by this method have flowered and their flowers are normal.

Shoot Formation from Rhizomes of *Cymbidium faberi*

Propagation of terrestrial *Cymbidium* species from East Asia is difficult. Several attempts were made to develop tissue culture propagation methods for these orchids. The procedure described here is based on experiments designed to "clarify the effects of N^6-benzyladenine (BA), rhizome length, application of mechanical treatment and liquid shaking culture on shoot formation from rhizome[s] cultured *in vitro* . . ." (Hasegawa et al., 1985).

Plant Material. Rhizomes for the original research were taken from seedlings of *Cymbidium faberi* Ro-Shanghai-bai × *Cymbidium faberi* Kin-o-so cultured in vitro. Rhizome-tip sections 2–3 mm thick were removed and placed on solid medium.

Surface Sterilization. Since rhizomes for the original research were taken from seedlings, they were not sterilized. The procedures used for *Cymbidium ensifolium* and *Cymbidium goeringii* should be suitable for surface sterilization of rhizomes taken from horticulturally grown plants.

Culture Vessels. Test tubes 10 × 180 cm were used in the original research. Other containers may also prove suitable.

Culture Conditions. Cultures should be maintained at 25°C under 16-h photoperiods of 500 lx provided by fluorescent lamps.

Culture Media. Modified Kyoto medium is used for shoot formation and growth (Table Cym-29). The shoots are moved to a BA-free medium (Table Cym-30) for rhizome formation.

Procedure. Place the explants on the first medium (Table Cym-29), maintain them on it until shoots are formed, and then move them to the second medium (Table Cym-30).

TABLE CYM-29. **Modified Kyoto medium as used for initial culture of rhizome tips of *Cymbidium faberi* (Hasegawa et al., 1985)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Minerals				
1	Hyponex (nitrogen : phosphorus : potassium in the ratio 7 : 6 : 19)[b]	3 g	No stock	No stock	Weigh
	Amino acid				
2	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[c]	1	
	Vitamins				
3	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[c]	1	
4	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[c]	1	
5	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[c]	1	
	Auxin				
6	α-Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[c,d]	1	
	Cytokinin				
7	N^6-benzylaminopurine (benzyladenine; BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[c,d]	1	
	Complex additive				
8	Tryptone, Difco Bacto	3.0 g	No stock	No stock	Weigh
	Darkening agent				
9	Activated charcoal	2.0 g	No stock	No stock	Weigh
	Sugar				
10	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[e]	To 1000 ml			
	Solidifier				
12	Agar, Difco Bacto	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Available from retail nurseries and garden shops.

[c]Keep refrigerated or frozen between uses.

[d]If the hormones fail to dissolve, add a few drops of dilute KOH (for the auxin) or HCl (cytokinin).

[e]Add items 1 and 8 to 700 ml distilled water (item 11); set pH to 5.0–5.1, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring solution to a gentle boil, and add agar (item 12) slowly while stirring. The agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, add charcoal (item 9) and stir well. After charcoal is dispersed evenly, pour solution into a 2-l flask, and autoclave. Add items 2–7 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

Developmental Sequence. Shoot formation can occur on both 1 and 10 mg l^{-1} BA, but subsequent growth is inhibited by the higher concentration. In fact, a hormone-free medium is most suitable for shoot growth.

General Comments. East Asian terrestrial *Cymbidium* species are difficult to culture. Therefore this procedure is an important advance. However, it is not known whether it is suitable for rhizome sections taken from mature plants.

TABLE CYM-30. **Modified Kyoto medium as used for the growth of shoots derived from rhizome tips of *Cymbidium faberi* (Hasegawa et al., 1985)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Minerals				
1	Hyponex (nitrogen : phosphorus : potassium in the ratio 7 : 6 : 19)[b]	3 g	No stock	No stock	Weigh
	Amino acid				
2	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[c]	1	
	Vitamins				
3	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[c]	1	
4	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[c]	1	
5	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[c]	1	
	Auxin				
6	α-Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[c,d]	1	
	Complex additive				
7	Tryptone, Difco Bacto	3.0 g	No stock	No stock	Weigh
	Darkening agent				
8	Activated charcoal	2.0 g	No stock	No stock	Weigh
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier				
11	Agar, Difco Bacto	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Available from retail nurseries and garden shops.

[c]Keep refrigerated or frozen between uses.

[d]If the hormone fails to dissolve, add a few drops of dilute KOH to solubilize it.

[e]Add items 1 and 7 to 700 ml distilled water (item 10), set pH to 5.0–5.1, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. The agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, add charcoal (item 8) and stir well. After charcoal is dispersed evenly, pour solution into a 2-l flask, and autoclave. Add items 2–7 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

Propagation of Oriental *Cymbidium* Species (*Cymbidium faberi, Cymbidium forestii, Cymbidium goeringii, Cymbidium hakuran, Cymbidium insigne, Cymbidium kanran,* and *Cymbidium sinense*) through the Culture of Various Explants

Shoot tips of *Cymbidium goeringii* that were cultured in the dark on MS or Linsmaier–Skoog media containing 1 ppm NAA and 1 ppm kinetin or hormone-free Hyponex medium formed rhizomes (Hasegawa, 1987).

Shoot tips of *Cymbidium kanran* were cultured on the inorganic constituents of Linsmaier–Skoog medium diluted to one-fifth of the original concentration. Hormones were not required. Liquid media, static or agitated, inhibited rhizome formation (Hasegawa, 1987).

Explants from rhizomes of *Cymbidium kanran, Cymbidium faberi*, *C. goeringii,* and *Cymbidium sinense*, as well as variegated forms were also cultured with varying success (Hasegawa, 1987). The methods seem to be similar to those used for *C. faberi* as described above (Hasegawa et al., 1985).

The publication from which this was taken (Hasegawa, 1987) is impressive and seems to contain many interesting details regarding the culture of these orchids. Unfortunately it is in Japanese (104 pages) with only a 3.5-page English summary. This is yet another example why investigators should make every effort to publish their work in English.

Shoot-tip Culture of *Cymbidium grandiflorum*

Shoot tips of *Cymbidium grandiflorum* cultured on liquid or solid Vacin and Went medium containing BA formed PLBs. These PLBs were sectioned and subcultured on the same medium, where they proliferated. PLBs cultured on solid Knudson C medium produced plantlets. Production of PLBs is also enhanced by 1.0 mg l^{-1} BA. Development and differentiation are promoted by 0.4 mg l^{-1} BA (Gu and Yan, 1989).

Isolation of *Cymbidium* Protoplasts from Leaves

Protoplasts of *Cymbidium* were isolated as a step in the isolation of nuclei (Capesius and Meyer, 1977).

Plant Material. Leaves of *Cymbidium* Ceres were used in the original research. The leaves of other cultivars or species could also be used.

Surface Sterilization. No information is given regarding the surface sterilization procedure, but the procedures used for *Aranda* (Fu, 1978, 1979*b*) or other *Cymbidium* tissues should prove suitable.

Culture Vessels. The vessels used for protoplast isolation from protocorms of *Brassia maculata* and *Cattleya* are used (Capesius and Meyer, 1977).

Culture Conditions. The conditions employed for protoplast isolation from protocorms of *Brassia maculata* and *Cattleya* are suitable (Capesius and Meyer, 1977).

Culture Media. Protoplasts were not cultured during the original research and culture media were not formulated. The plasmolysis solution consists (per liter) of 10 g magnesium sulfate ($MgSO_4 \cdot 7H_2O$) and 25 g potassium chloride (KCl). For premaceration 100 mg Pectinol fest (Fa Röhm, Darmstadt, Germany) is added to 100 ml of this solution. The digestion medium is prepared by adding to 100 ml of plasmolysis solution, 1 g cellulase and 0.3 g Pectinol fest (for sources see *Brassia* and *Cattleya* entries, procedures for the isolation of protoplasts; Capesius and Meyer, 1977) and adjusting the pH to 5.8.

Procedure. After sterilization strip the epidermis from the leaves and plasmolyse the tissues in the plasmolysis solution for 30 min. After that move them to the pre-maceration mixture for 1 h without shaking. At the end of the hour replace the pre-maceration mixture with digestion medium and allow the tissues to remain in it for 3–4 h with occasional manual shaking. Then filter the suspension through a nylon screen (for sources see *Cattleya* entry, sections on protoplast isolation procedures) and centrifuge at $50 \times g$. A pellet formed as a result of the centrifugation contains the protoplasts. This pellet should be washed three times with the plasmolysis solution.

Developmental Sequence. The protoplasts were not cultured.

General Comments. This procedure could be a useful starting point for the isolation and culture of *Cymbidium* protoplasts.

Isolation of *Cymbidium* Protoplasts from Protocorms

The procedures are the same as those for *Brassia maculata* and *Cattleya* (Capesius and Meyer, 1977). Protocorms of *Cymbidium pumilum* were used in the original research.

Isolation of Mesophyll Protoplasts of *Cymbidium*

Protoplasts can be isolated from leaves of *Cymbidium* Miracle × *Cymbidium* Alexanderi 'Godlen Hill' by the method used for *Calanthe discolor*.

Use of Charcoal in Culture Media for *Cymbidium*

Inclusion of charcoal (0.2–3 g l^{-1}) enhances the growth and development of seedlings and plantlets of *Cymbidium* (Werkmeister, 1970*a,* 1970*b,* 1971; Ernst, 1974, 1975; Arditti, 1982*b*; Wang and Huang, no date).

Inclusion of Anticontaminants in Culture Media for *Cymbidium* Shoot Tips

Experiments in our laboratory have led to the following recommendations (Arditti, 1982*b*; Cvitanic and Arditti, 1984):

1. It is preferable not to use anticontaminants in shoot-tip culture media without previous screening.
2. Should contamination occur, it is best to use 10 mg l^{-1} amphotericin B, 25 mg l^{-1} nystatin, or 5 mg l^{-1} sodium omadine (i.e., these compounds should be used singly). Mixtures like those used for seedlings (Thurston et al., 1979, 1980; Spencer et al., 1979/1980; Arditti, 1982*b*; Brown et al., 1982) should be used with great caution and only following the formation of PLBs and/or plantlets.

Effects of Plant Hormones on Organogenesis of *Cymbidium* Shoot Apices in Vitro

Research at the Faculty of Agriculture, Nagoya University, Japan (Kim and Kako, 1982, 1983), has shown the following:

1 Auxins induce roots on shoot apex explants but inhibit shoots.
2 Fresh weight of plantlets increases in the presence of high levels of 2,4-D, but roots are abnormal.
3 NAA at 1 mg l^{-1} or 2,4-D at 0.1 mg l^{-1} can enhance plantlet formation and development.
4 BA can enhance the formation of PLBs and shoots, but at high concentrations it inhibits root initiation.
5 Gibberellic acid (GA) and ABA are without effect.
6 Explants with three leaf primordia do not require an exogenous supply of hormones for development.
7 Production of PLBs varies with the season. It starts to increase in April and reaches a maximum in June.
8 PLBs form in both the axillary and basal regions.

Method for the Clonal Propagation of *Cymbidium*

Orchid research is being carried out at what was the Central Republic Botanical Garden of the Academy of Sciences of the Ukrainian Soviet Socialist Republic in the USSR and is now the Botanical Garden in Kiev, Ukraine (Lavrentyeva, 1980, 1986). These studies have shown the following:

1 When the cytokinin : auxin ratio is 1 : 1 or 2 : 1, PLBs are larger.
2 The optimal size of explants is about 0.5 mm including three to four leaf primordia.
3 PLBs arise at the base of leaf primordia.
4 A suitable medium is modified Knudson C, which contains (per liter) 400 ml potato extract (no details are given regarding its preparation), 10 mg niacin, 7 mg adenine (no indication is given whether the sulfate was used), 40 g sucrose, and 7 g agar. The pH should be 5.5–5.8.

System for Vegetative Propagation of *Cymbidium* in Vitro

Studies of *Cymbidium* explant development in vitro at the V. L. Komarov Botanical Institute in St. Petersburg are concerned with differentiation and organogenesis (Shevtsova and Batygina, 1986).

Micropropagation of Terrestrial *Cymbidium* Species from Rhizome Explants

Axillary buds of *Cymbidium kanran* and *Cymbidium goeringii* develop vigorous rhizomes in vitro when treated with "higher concentration[s] of NAA and 2,4-D . . . [in] topical application[s] . . ." Apices of these rhizomes are removed and cultured on "MS [Murashige–Skoog] medium supplemented with auxin and cytokinin . . . at 25°C and 24" hour illumination. Shoot formation is accelerated by lower auxin : cytokinin ratios following "several passages in . . . higher" ratios. Root formation is inhibited when "both auxin and cytokinin were added," but occurred when the concentrations of potassium nitrate and ammonium nitrate were reduced. "Half strength of potassium nitrate and quarter strength of ammonium nitrate in MS medium were optimal for the formation of roots in rhizome cultures" (Shimasaki and Uemoto, 1987*a*, 1987*b*, 1990).

Clonal Propagation of *Cymbidium aloifolium* through Shoot-tip Culture

Cymbidium aloifolium is a commercially important orchid in northeast India. Propagation through conventional methods is relatively slow. A shoot-tip culture method was developed to accelerate its propagation (Devi et al., 1997).

Plant Material. Shoot tips, 2–5 mm in length, were taken from 8-month-old seedlings growing in vitro.

Surface Sterilization. Explants taken from seedlings growing in vitro do not require surface sterilization.

Culture Vessels. The original paper does not describe the culture vessels. However, a photograph shows a test tube. Other culture vessels are also suitable.

Culture Conditions. The research cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3000 lx (the source of illumination was not described). Standard culture room conditions are also suitable.

Culture Media. The Nitsch and Nitsch (NN) medium (Nitsch and Nitsch, 1969) (Table Cym-31) was used for the formation and proliferation of PLBs. When shoots and roots were initiated, the PLBs were transferred to fresh medium of the same composition (Table Cym-31) for plantlet development. Plantlets were hardened by sequential transfers to full-, half- and quarter-strength NN medium free of sucrose, vitamins, and hormones (Tables Cym-32 to Cym-34). These media and steps are listed here because they are included in the original research but they seem superfluous and appear to add steps, work, complications, and expense and minimal – if any – benefits. If hardening is needed (a questionable supposition because it is not used in other procedures), a period of culture on full-strength basal medium (Table Cym-32) should be sufficient. Plantlets, 6–8 cm tall, should be removed from the culture and

TABLE CYM-31. **Nitsch and Nitsch (NN) medium (Nitsch and Nitsch, 1969) as used for the culture of *Cymbidium aloifolium* shoot tips (Devi et al., 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	720.0	72.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2$	166.0	16.6 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	68.0	6.8 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	10.0	620.0 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	25.0	2.23 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Biotin	0.05	5 mg 100 ml^{-1} 95% ethanol[e,f]	1	
11	Folic acid	5.0	500 mg 100 ml^{-1} 95% ethanol[e,f]	1	
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]This medium is one of a few media to contain biotin and/or folic acid. Since the explants grew better on it than on any of the other media tested it is possible that one or both of these vitamins may be: required by, needed by, or of benefit to the explants.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust the pH to 5.4, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8) and vitamins (items 10–12) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE CYM-32. **Basal Nitsch and Nitsch (NN) medium (Nitsch and Nitsch, 1969) as used for hardening plantlets produced through the culture of *Cymbidium aloifolium* shoot tips (Devi et al., 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	720.0	72.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2$	166.0	16.6 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, $KH_2PO_4 \cdot H_2O$	68.0	6.8 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	10.0	620.0 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	25.0	2.23 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Solvent				
8	Water, distilled[e]	To 1000 ml			
	Solidifier				
9	Agar[e]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Add items 1–7 to 900 ml of distilled water (item 8), adjust the pH to 5.4 and adjust volume to 1000 ml with distilled water (item 8). Bring the solution to a gentle boil and add the agar (item 9) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media.

TABLE CYM-33. **Half-strength basal Nitsch and Nitsch ($^1/_2$NN) medium (Nitsch and Nitsch, 1969) as used for hardening plantlets produced through the culture of *Cymbidium aloifolium* shoot tips (Devi et al., 1997)**

Step 1. Make 1 l of basal NN medium (Table Cym-32) but do not add the agar

Step 2. Take 500 ml of the NN medium and dilute it to 1000 ml with distilled water to make $^1/_2$NN

Step 3. Bring the solution to a gentle boil and add the agar (item 9 in Table Cym-32) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media

potted in a mixture of charcoal chunks, brick pieces, and coconut husks (proportions not given), watered regularly, and sprayed periodically with a dilute fertilizer solution. The original paper states "micro-nutrient solution" without: (1) indicating why only micronutrients, (2) describing the solution and its concentration, or (3) listing the frequency of applications. Established mature plants can be grown on fern blocks.

TABLE CYM-34. **Quarter-strength basal Nitsch and Nitsch (1/4NN) medium (Nitsch and Nitsch, 1969) as used for hardening plantlets produced through the culture of *Cymbidium aloifolium* shoot tips (Devi et al., 1997)**

Method A

Step 1. Make 1 l of basal NN medium (Table Cym-32) but do not add the agar

Step 2. Take 250 ml of the basal NN medium (Table Cym-32) and dilute it to 1000 ml with distilled water to make 1/4NN

Step 3. Bring the solution to a gentle boil and add the agar (item 9 in Table Cym-32) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media

Method B

Step 1. Make 1 l of half-strength basal NN (1/2NN) medium (Table Cym-33) but do not add the agar

Step 2. Take 500 ml of the half-strength basal NN (1/2NN) medium (Table Cym-33) and dilute it to 1000 ml with distilled water to make 1/4NN

Step 3. Bring the solution to a gentle boil and add the agar (item 9 in Table Cym-32) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media

Procedure. The explants should be placed on the first medium (Table Cym-31). PLBs with visible shoots and roots should be subcultured on the same medium (Table Cym-31). Once plantlets are formed they should be hardened through passage (several weeks each) on sequential dilutions of the NN medium (Tables Cym-32 to Cym-34). After the plants become large enough, they should be potted in a potting mix in pots. Since the sequential dilution steps (Tables Cym-32 to Cym-34) may not be necessary, users of this procedure should utilize a few plants in attempts to bypass them. If the attempts are successful, these steps should be eliminated.

Developmental Sequence. Explants remain green and enlarge slowly. After 6 weeks the explants form PLBs which range from 2 to 3 mm in diameter and multiply rapidly. The first leaves appear after 8 weeks. Roots form following 10 weeks of culture. Plantlets reach a height of 6–8 cm after 120–140 days.

General Comments. Like other procedures in which seedling explants are cultured, this method cannot be used to propagate desirable forms because the quality of seedlings is not known. Also, this method seems to include steps and media which may not be necessary. Best results were obtained on NN medium; however MS medium (Murashige and Skoog, 1962) was also effective. The late Jean Paul Nitsch (who died in an accident in 1971) and his wife Colette Nitsch formulated and published several culture media. These media differ from each other. Therefore, another Nitsch medium should not be substituted for NN (Nitsch and Nitsch, 1969) without careful prior testing.

Plantlet Production from Root Tips of *Cymbidium mastersii*

Root tips of a large number of orchids were cultured successfully by Professor Suraj P. Vij at the Orchid Laboratory, Botany Department, Panjab University, Chandigarh, India. One of these orchids is *Cymbidium mastersii* (Vij, 1993).

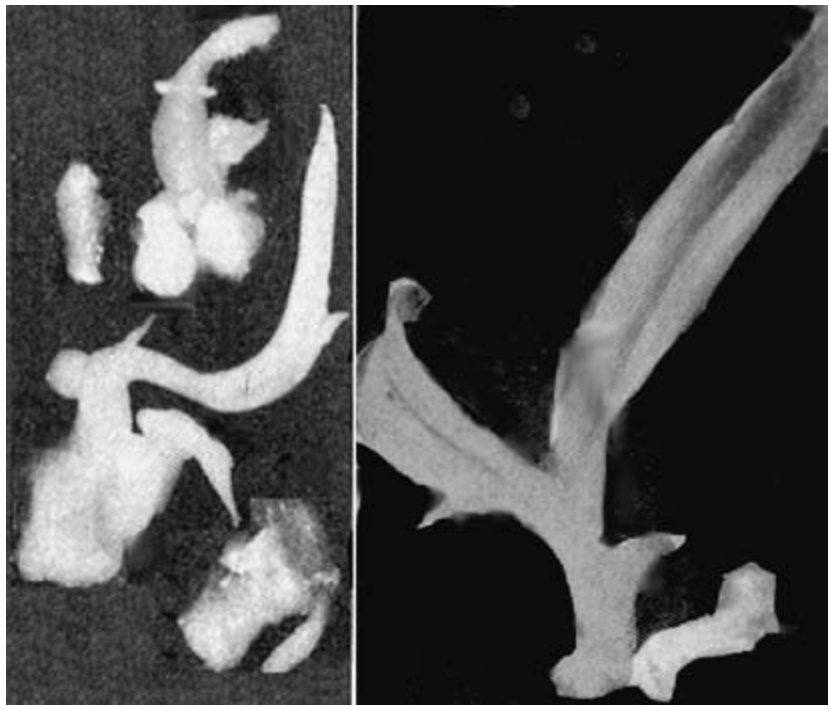


FIG. CYM-4. **Root-tip culture of *Cymbidium mastersii*. A. The explant starts to elongate and swell. B. PLB and bud formation. C. Bud formation. D. Early stages of plantlet formation. E. Plantlet. (Vij, 1993.)**

Plant Material. Root tips, 5–10 mm long, were taken from 16–30-week-old asymbiotic seedlings.

Surface Sterilization. There is no need to surface-sterilize explants taken from asymbiotic seedlings. However, the explants should be washed with sterile distilled water to remove residual agar and/or medium if any.

Culture Vessels. Test tubes (25 × 150 mm) and Erlenmeyer flasks (125-ml capacity) containing medium equivalent to one-fifth of their volume can be used for initial cultures (Fig. Cym-4A–C). Larger vessels are preferable for developing (Fig. Cym-4D) and larger (Fig Cym-4E) plantlets.

Culture Conditions. In the original research the cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx (the source is not described). Standard laboratory conditions are also suitable.

Culture Media. Modified MS medium (Murashige and Skoog, 1962) containing 1 mg l^{-1} each of kinetin and IAA (Table Cym-35) should be used for initial culture of explants and early plantlet development (Fig. Cym-4A–E). Another modification

TABLE CYM-35. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as used for the culture of *Cymbidium mastersii* root-tip explants (Vij, 1993, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indoleacetic acid (IAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Kinetin	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH as required, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

of MS containing only 1 mg NAA l^{-1} is also suitable. Although not suggested in the original report, standard or modified Knudson C (see Tables Aranda-7, Aranda-8, and Aranda-10) and Vacin and Went (see Tables Aranda-2, Aranda-6, and Arnth-3) media can be used for the culture of larger plantlets.

Procedure. Explants (Fig. Cym-4A) are taken from seedlings, washed with sterile distilled water, placed on the first medium (Table Cym-35), and left on it until callus, PLBs, and/or plantlets develop. Callus, PLBs, and plantlets can be subcultured onto the same medium (Table Cym-35). Larger plantlets can be moved to fresh medium, of either the same (Table Cym-35) or different (see Tables Aranda-2, Aranda-6, Aranda-7, Aranda-8, Aranda-10, and Arnth-3) composition.

Developmental Sequence. Initial explants (Fig. Cym-4A) swell and form new tissue or PLBs (Fig. Cym-4B, C). This is followed by plantlet development (Fig. Cym-4D, E).

General Comments. Since root explants are more difficult to culture than shoot tips, this method represents a significant advance in orchid micropropagation. It cannot be used to select desirable forms because the explants are taken from seedlings, but it can serve as a starting point for culture protocols that utilize root-tip explants from mature plants. It is not known if this procedure is suitable for root tips of mature plants. Should an attempt be made to culture root tips from mature plants, they must be free of mycorrhiza.

Plantlet Production from Root Tips of *Cymbidium pendulum*

Root tips of *Cymbidium pendulum* were cultured by Professor S. P. Vij (Vij, 1993).

Plant Material. Root tips, 5–10 mm long, were excised from 16–30-week-old asymbiotically germinated seedlings growing axenically in vitro.

Surface Sterilization. Explants taken from asymbiotic seedlings do not require surface sterilization, but they should be washed with sterile distilled water to remove agar and/or medium residue if any.

Culture Vessels. Erlenmeyer flasks (125-ml capacity) or test tubes (25×150 mm) containing medium equivalent to approximately 20% of their volume can be used for initial cultures. Larger vessels are more suitable for bigger plantlets.

Culture Conditions. The cultures were maintained at $25 \pm 2°C$ under 12-h photoperiods of 3500 lx (the source is not described) during the original research. Standard laboratory conditions are also appropriate.

Culture Media. Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) containing 1 mg IBA l^{-1} and 1 g peptone l^{-1} (Table Cym-36) should be used to culture the explants and for early plantlet development. Other modifications of

TABLE CYM-36. **Mitra, Prassad, and Roychowdhury medium (Mitra et al., 1976) as modified for the culture of root tips of *Cymbidium pendulum* (Vij, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, NaH_2PO_4	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavin (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Indolebutyric acid (IBA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
15	Peptone	1.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[f]	To 1000 ml			
	Solidifier				
18	Agar[f]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original recipe calls for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.6, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976; Vij, 1993).

MPR can also be used (Vij, 1993). Despite not being suggested in the original report, Knudson C (see Tables Aranda-7, Aranda-8, and Aranda-10) and Vacin and Went (see Tables Aranda-2, Aranda-6, and Arnth-3) media may prove suitable for the culture of larger plantlets.

Procedure. Explants taken from seedlings should be washed with sterile distilled water and cultured on the first medium (Table Cym-36) until callus, PLBs, and/or plantlets are formed. These can be subcultured onto the same medium (Table Cym-36). Larger plantlets may be cultured either on the same (Table Cym-35) or a different (see Tables Aranda-2, Aranda-6, Aranda-7, Aranda-8, Aranda-10, and Arnth-3) medium.

Developmental Sequence. The explants swell and form PLBs that produce plantlets.

General Comments. Root tips are more difficult to culture than shoot or leaf explants. Therefore this method is a major achievement. However, it cannot be used to select specific forms because the explants are taken from seedlings, but it can serve as a starting point for culture methods for root tips from mature plants. If root tips from mature plants are excised for culture, they must be free of mycorrhiza.

Plantlet Production of *Cymbidium pendulum* from Nodal Explants

An epiphytic species found at the foot of the eastern Himalaya, Khasi, and Jainitia Hills in India, *Cymbidium pendulum* is valued as both an ornamental and therapeutic plant. Because of that and due to overcollection the species is becoming rarer. Therefore a micropropagation method was developed cooperatively by the Orchid Laboratory at Panjab University and the Laboratory of Plant Chromosomes and Genes Stock at Hiroshima University (Vij et al., 1994*a*).

Plant Material. Etiolated and young shoots were taken from greenhouse-grown plants and from seedlings growing axenically in vitro.

Surface Sterilization. Explants from axenic seedlings growing in vitro do not require surface sterilization, but they must be rinsed to remove culture medium residue. Sources of explants from greenhouse-grown plants were dipped in 70% ethanol (74 ml of 95% ethanol diluted to 100 ml with distilled water) for 30 s, sterilized with 0.1% streptomycin (0.1 g 100 ml^{-1}) for 25 min and 0.1% $HgCl_2$ (0.1 g 100 ml^{-1}) for 5 min and washed thoroughly with sterile distilled water after that. $HgCl_2$ is highly toxic and must be handled with great care.

Culture Vessels. Test tubes, 22 × 150 mm, Erlenmeyer flasks, 125-ml capacity, or other containers filled with culture medium to approximately 20% of their capacity can be used for the initial explants. Larger containers should be used when the explants, callus masses, PLB proliferations, shoot bud groups, and/or plantlets become bigger.

Culture Conditions. The research cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx (the source of illumination was not described). Standard culture room conditions are also suitable.

Culture Media. Modified Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) was used in the original research for both the initial explants and further growth (Table Cym-37). However, larger plantlets may be cultured either on the same (Table Cym-37) or a different (see Tables Aranda-2, Aranda-6 to Aranda-8, Aranda-10, and Arnth-3) medium.

Procedure. Explants should be removed from the donor plants, sterilized if necessary, cut to one node size, and placed in culture (Table Cym-37). When propagules or plantlets form they should be transplanted to new medium (see Tables Aranda-2, Aranda-6 to Aranda-8, Aranda-10, Arnth-3, or Cym-37). After plants become large enough they should be potted in potting mix in pots.

Developmental Sequence. PLBs (Fig. Cym-5A) and shoot buds (Fig. Cym-5B, E) form and develop plantlets (Fig. Cym-5C, D, F), which become larger (Fig. Cym-5G, H).

General Comments. This procedure can be used to propagate desirable forms of *C. pendulum* and/or to simply multiply plants.

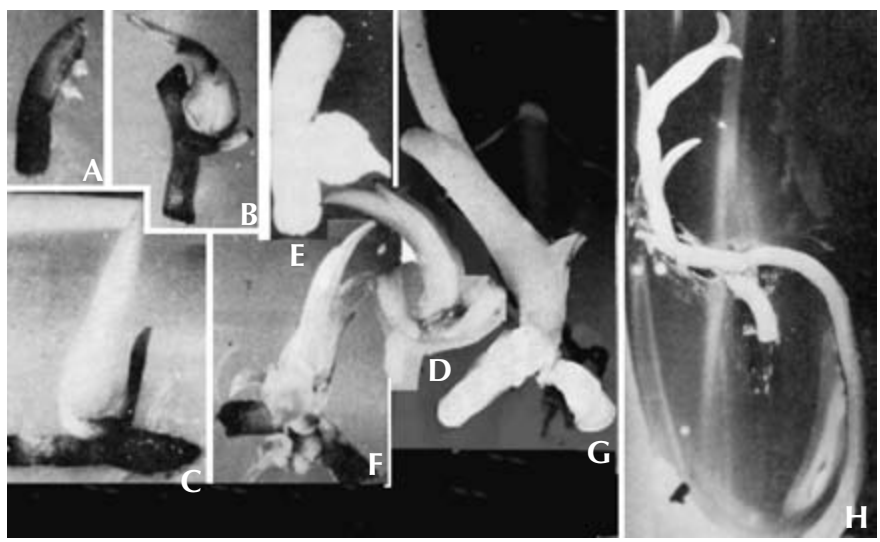


FIG. CYM-5. **Node culture of *Cymbidium pendulum*. A. PLB formation at an internode region. B. Vegetative bud (shoot bud) at an internodal area. C, D. Early stages of plantlet formation. E. Bud with shoot tip. F. Plantlet during early stages of development with a cluster of buds at its base. G. Plantlet with two roots. H. Eight-week-old plant. (Vij, 1993.)**

TABLE CYM-37. **Mitra, Prassad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of shoot tips of *Cymbidium pendulum* (Vij et al., 1994*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Indoleacetic acid (IAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Agar[f]	7.0 g	No stock	No stock	Weigh
	Darkening agent				
18	Activated charcoal	2.0 g	No stock	No Stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original recipe calls for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses. If the auxin fails to dissolve add a few drops of 0.1N KOH.

[f]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved add the charcoal (item 18) with vigorous and constant stirring, pour the solution into culture vessels, and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976; Vij, 1993; Vij et al., 1994*a*).

Propagation of *Cymbidium giganteum* through the Culture of Shoot Apices and Encapsulation of Protocorm-like Bodies in Sodium Alginate

Micropropagation of orchids would be simpler if PLBs could be grown in soil and/or sand rather than sterile culture media. A method involving encapsulation of PLBs in sodium alginate makes this possible (Corrie and Tandon, 1993).

Plant Material. Apices, 1–2 mm in length, are excised from 1-cm-long shoot tips and cultured.

Surface Sterilization. In the original research the shoot tips were washed with running tap water for 1 h (this may be somewhat long; a shorter wash with water and a mild detergent followed by several rinses will probably be sufficient). After that they were surface-sterilized by submerging them with agitation in 0.57% sodium hypochlorite (9.5 or 11 ml of a household bleach such as Clorox which contains 6 or 5.25% sodium hypochlorite, respectively, diluted to 100 ml with distilled water; plus a few drops of mild household detergent as a wetting agent) for 5 min and rinsed several times with sterile distilled water.

Culture Vessels. Test tubes, 25 × 150 mm, or 125-ml Erlenmeyer flasks filled with medium to 20–30% of their capacity are suitable for initial cultures. Larger containers may be needed for further proliferation.

Culture Conditions. Cultures were maintained at 25 ± 2°C under 14-h photoperiods of 2000 lx (light sources were not described) and 80% relative humidity (RH). Standard culture room conditions are also suitable. Encapsulated PLBs were grown for 2 weeks in pots covered with polyethylene bags and sprayed with water twice daily. After 2 weeks the polyethylene bags were removed and the pots were transferred to a greenhouse at 20–25°C and 60–70% RH.

Culture Media. Explants should be cultured on modified MS medium (Murashige and Skoog, 1962; Table Cym-38). A different modification of MS (Table Cym-39) is required for encapsulated PLBs. Sterile sand (0.3 mm grain size) or a sterilized soil mixture [sand : garden soil : cow dung in a ratio of 1 : 1 : 1 (v/v/v) plus a few small brick pieces] should be used.

Encapsulation Solutions. Two modified MS solutions, both containing fungicides and bactericides, are used for encapsulation. One (Table Cym-40) has 4% sodium alginate (4 g sodium salt of alginic acid made to 1 l with liquid MS solution; sodium alginate as low-viscosity "alginic acid sodium salt, sodium alginate" can be obtained from Sigma, P. O. Box. 14508, St. Louis, MO, 63178, USA, www.sigmaaldrich.com). The other contains 100 mmol of calcium nitrate (Table Cym-41).

Procedure. Shoot tips must be washed and surface-sterilized following removal from the plant. Their apices should be excised under sterile conditions after that and placed in culture on the first medium (Table Cym-38). Once PLBs form they can be

TABLE CYM-38. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Cymbidium giganteum* shoot tips (Corrie and Tandon, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.2–5.5, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE CYM-39. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of encapsulated protocorm-like bodies of *Cymbidium giganteum* (Corrie and Tandon, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, $KH_2PO_4 \cdot H_2O$	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.2–5.5, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE CYM-40. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the encapsulation of protocorm-like bodies of *Cymbidium giganteum* (Corrie and Tandon, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 2{\cdot}H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2{\cdot}6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Encapsulation reagent				
15	Sodium alginate	40.0 g	No stock	No stock	Weigh
	Bactericides				
16	Soframycin	0.5	50 mg 100 ml^{-1} 70% ethanol[e]	1	
17	Rose Bengal	0.1	10 mg 100 ml^{-1} 70% ethanol[e]	1	
	Fungicide				
18	Dithane	4.0	400 mg 100 ml^{-1} 70% ethanol[e]	1	
	Solvent[f]				
19	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4{\cdot}4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4{\cdot}7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses. Soframycin is a Hoechst product (www.hoechst.com, www.aventis.com). Rose Bengal is available from Sigma Chemicals (www.sigmaaldrich.com). Dithane is produced by Dow Agrosciences (www.dowagro.com).

[f]If the auxin or cytokinin does not dissolve add a few drops of 0.1N KOH or HCl, respectively.

[g]Add all items to 900 ml of distilled water (item 19), adjust pH to 5.8, and adjust volume to 1000 ml with distilled water (item 19). Pour the solution into a 2-l flask, autoclave, cool, and use as needed.

TABLE CYM-41. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the encapsulation of protocorm-like bodies of *Cymbidium giganteum* (Corrie and Tandon, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 2{\cdot}H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2{\cdot}6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Encapsulation reagent				
15	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	23.65 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4{\cdot}4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4{\cdot}7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve add 0.1N KOH or HCl, respectively.

[g]Add all items to 900 ml of distilled water (item 16), adjust pH to 5.8, and adjust volume to 1000 ml with distilled water (item 16). Pour the solution into a 2-l flask, autoclave, cool, and use as needed.

subcultured a few times should it be necessary to increase their number. Overproliferation should be avoided because it can cause undesirable mutations.

When PLBs reach 3–4 mm in size they can be encapsulated. This is done by mixing them with the alginate solution (Table Cym-40) and dropping the mixture (with

the help of a sterile small spoon or spatula) into the calcium nitrate solution (Table Cym-41). Gelling and bead (firm, round, 6–9 mm in diameter) formation require approximately 30 min. The beads can be separated with a sieve after that and placed on MS medium in vitro (Table Cym-39), sand or soil. The conditions used for explant culture are also suitable for culturing beads on MS. Those in pots should be grown as outlined above.

Developmental Sequence. Explants in culture produce PLBs. On being subcultured, the PLBs proliferate. Encapsulated PLBs produce plantlets in pots or in vitro.

General Comments. This procedure simplifies plantlet production because encapsulated PLBs can be grown in pots.

Plantlet Production from *Cymbidium goeringii* Flower Buds

Cymbidium goeringii, one of the temperate oriental cymbidiums, is mostly grown as an ornamental, but the flowers are also used for an alcoholic drink, as components of a soup recipe, and in a tea. The species is in demand, but propagation through division is slow. Like other oriental *Cymbidium* species, *C. goeringii* has not been easy to propagate through seed germination and tissue culture. However, a method was developed for plantlet production from young flower buds (Shimasaki and Uemoto, 1991).

Plant Material. Apical flower buds, 2 mm long, taken from 5-cm long inflorescences are used as explants.

Surface Sterilization. The inflorescences must first be washed with running water. Following the wash and after excision, the apical sections should be immersed for 10 min in 1% sodium hypochlorite (17 or 19 ml of Clorox or another household bleach which contains 6 or 5.25% sodium hypochlorite diluted to 100 ml with distilled water) containing 0.01% Tween 20 (0.1 ml or a few drops per 1000 ml; available from Acros Organics at www.fishersci.com; a few drops of mild household detergent can be used instead of Tween 20). After the soaking, the now surface-sterilized sections should be washed three times with sterile distilled water.

Culture Vessels. Flat bottom vials (Fig. Cym-6C) and bottles (Fig. Cym-6D) were used in the original research. Other vessels are also suitable.

Culture Conditions. In the original research cultures were maintained at 25 ± 1°C under continuous illumination of 75 μmol m^{-2} s^{-1} provided by Toshiba Bio-Light fluorescent lamps. Standard culture room conditions are probably also suitable.

Culture Media. One modification of the MS medium (Murashige and Skoog, 1962) is suitable for initial culture of the explants (Table Cym-42). A second modification should be used for rhizome development and subsequent plantlet growth (Table Cym-43).

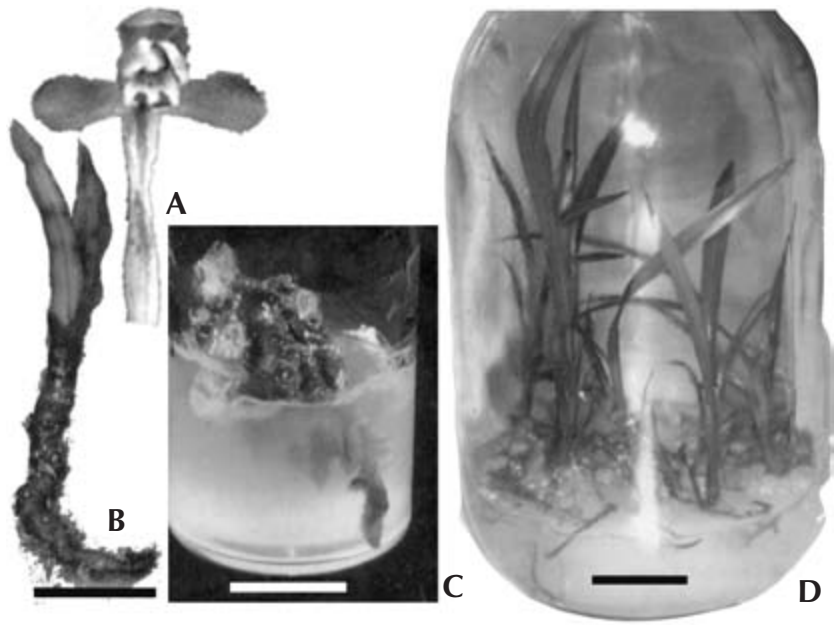


FIG. CYM-6. **Culture of *Cymbidium goeringii* flower buds. A. Flower. B. Rhizome and shoot development after 107 days (bar = 2 mm). C. Branching rhizome (bar = 10 mm). D. Multiple shoots on rhizome branches following 6 months of subculture (bar = 10 mm). (Shimasaki and Uemoto, 1991.)**

Procedure. Apical segments containing flower buds at their apices should be excised from the washed inflorescences and surface-sterilized and then washed with sterile distilled water. After that it is necessary to remove excess flower stalk tissues before placing the explants horizontally on the agar surface of the first medium (Table Cym-42). Rhizomes that form on this medium should be transferred to the second one (Table Cym-43) for plantlet formation and growth.

Developmental Sequence. Buds become swollen by the first week. Some of the swollen buds produce chlorophyll within 2 weeks of culture. Explants on appropriate media can start to produce elongated rhizomes after approximately 8 weeks of culture (Fig. Cym-6B). The rhizomes may produce multiple shoots and branches (Fig. Cym-6C, D). Shoots form within 15 weeks on the first medium (Table Cym-42). Roots appear and plantlets form following transfer to the second medium (Table Cym-43). When these plantlets become large enough, they can be moved to pots.

General Comments. The major advantage of this procedure is that removal of the explant does not endanger the donor plant. A second advantage is explant abundance. Third, the procedure is relatively simple and easy to master. And, fourth, for those who wish to use this procedure, the original paper is clear, concise, well written, and contains all relevant information.

TABLE CYM-42. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the culture of *Cymbidium goeringii* flower bud explants (Shimasaki and Uemoto, 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indoleacetic acid (IAA)	1.0–30	100–300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1N KOH.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.2–5.6, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE CYM-43. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for plantlet production from *Cymbidium goeringii* rhizomes derived from flower bud explants (Shimasaki and Uemoto, 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.2–5.6, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

Shoot Proliferation in *Cymbidium aloifolium*

A frequent complaint by those suffering through the frequent orchid name changes is that these gymnastics are the work of taxonomists who sit in musty herbaria and look at dry and dead plants, which are often hard to distinguish from each other. A good example of this is *Cymbidium aloifolium* which has been confused with *Cymbidum finlaysonianum*, *Cymbidium pendulum*, *Cymbidium pubescens*, and especially *Cymbidium bicolor* (Du Puy and Cribb, 1988). Living plants in the field of these two species are easy to tell apart (Comber, 1990). And contradictory statements by one and the same taxonomist about the distribution of this species have added to the confusion: "*C. aloifolium* extends to W. Malaysia and Java . . . , but has not been recorded from Sumatra and Borneo" (Du Puy and Cribb, 1988) versus "Distribution: India, Sri Lanka, Burma and S. China to Java and Sumatra" (Bechtel et al., 1986, 1992). The confounding of information about distribution is a result of inattention to detail (the two contradictory statements share an author – see citations above) and/or insufficient knowledge because the species was collected at 1100 m in Lubuk Sikaping in West Sumatra by H. A. B. Bünnenmeijer (Comber, 2002), who was a collector for the Bogor Herbarium between 1916 and 1921, and was reported to occur in Sumatra (Latif, 1960; Holttum, 1964) and Borneo (Latif, 1960 who added the proviso that *C. aloifolium* is "also known by the name *Cymbidium pubescens*"). Regardless of the confusion about its nomenclature and distribution, *C. aloifolium* is of interest to growers and is therefore found in commerce. Because of this, a micropropagation method has been developed for it (Nayak et al., 1997*b*).

Plant Material. Shoot explants, 6–15 mm long, with 1–2 nodes were taken from "plants raised in vitro." They were placed in culture after all leaves "along with their sheathing bases were removed."

Surface Sterilization. There is no need to surface-sterilize explants taken from plants growing in vitro, but agar medium residues must be washed away with sterile distilled water.

Culture Vessels. Erlenmeyer flasks, 150-ml capacity, containing 35 ml of medium were used in the original research. Other culture vessels can also be suitable.

Culture Conditions. The research cultures were maintained at $25 \pm 1°C$ and 55–60% relative humidity (RH) under 16-h photoperiods of 35 $\mu E\ m^{-1}\ s^{-1}$ provided by cool white fluorescent tubes (Philips, India). Donor plants were maintained under the same conditions.

Culture Media. Explants should be placed first on a modification of MS medium (Murashige and Skoog, 1962) containing TDZ and no other hormones (Table Cym-44). After buds form (about 10–12 days following the start of culture), the explants should be transferred to a BA-containing medium (Table Cym-45). The elongated shoots (15–15 days after the transfer) develop roots on a medium (Table Cym-46) containing IBA.

TABLE CYM-44. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of shoot explants of *Cymbidium aloifolium* (Nayak et al., 1997*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Thidiazuron (TDZ)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Dissolve the thidiazuron (TDZ) in a small volume of 0.5N HCl and make up to volume (100 ml) with 70 or 95% ethanol. The amount of TDZ used in the original research was 2.2 µmol (485 µg) l^{-1}. A slightly larger amount, 500 µg (0.5 mg, 2.26 µmol), is recommended here for convenience (0.5 mg is easier to weigh than 0.485 mg). The difference (0.015 mg, 15 µg, or 0.06 µmol) is insignificant and can be ignored. Sticklers for accuracy and those with infinite patience and/or plenty of time on their hands may still use 0.485 mg l^{-1}.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH as required, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE CYM-45. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of new shoots from shoot explants of *Cymbidium aloifolium* (Nayak et al., 1997*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, $KH_2PO_4 \cdot H_2O$	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	2.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively. In the original research the auxin used was 10.8 µmol (2010.9 µg or 2.011 mg) l^{-1}. The amount recommended here is 2 mg (10.74 µmol) l^{-1}. This differs from the original by an insignificant 0.011 mg or 0.06 µmol which can be ignored. Benzyladenine (BA) was used at 2.2 µmol (495.66 µg or 0.496 mg) l^{-1} in the original research. The amount recommended here, 0.500 mg (2.22 µmol), differs from the original by 4 µg (0.004 mg or 0.02 µg). This is insignificant and can be ignored by all except sticklers for accuracy.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH as required, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE CYM-46. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the rooting of *Cymbidium aloifolium* shoots obtained from shoot tips in vitro (Nayak et al., 1997*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 2{\cdot}H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	62.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2{\cdot}6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indolebutyric acid (IBA)	2.2	220 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4{\cdot}4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4{\cdot}7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1N KOH. In the original research 10.8 µmol (2.196 mg) were added. The amount recommended here, 2.2 mg (10.85 µmol), differs from the original by an insignificant 0.004 mg or 0.05 µmol. Those who insist on absolute and total accuracy can use the original amounts which fall within experimental error and are beyond the reproducibility limits of most laboratory balances.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH as required, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

Procedure. Explants must be freed of leaves after they are excised from the seedlings. The explants should then be placed on the first medium (Table Cym-44) and allowed to remain on it until new shoots form. These shoots should be transplanted onto the second medium (Table Cym-45) and allowed to elongate. The elongated shoots must be moved to the third medium (Table Cym-46) for rooting.

Developmental Sequence. Vegetative buds form on the explants 10–20 days after they are placed on the first medium (Table Cym-44). Their development and elongation continues until the eighth week. Following their transfer to the second medium (Table Cym-45), the shoots elongate within 12–15 days. Roots are formed only after the elongated shoots are moved to the third medium (Table Cym-46).

General Comments. This is an interesting method which cannot be used for the selection of desirable cultivars because the explants are taken from seedlings. Still, it should be tried with explants taken from the shoot tips of mature plants.

Rapid Micropropagation of *Cymbidium aloifolium* through the Culture of Thin Cross Sections

Excision of shoot tips for micropropagation may endanger the donor plant. Leaf tips and bases cannot always be cultured or made to produce PLBs and plantlets. The culture of flower stalk buds is possible in only a few orchids. Abundant explants are frequently not available. Therefore tissue culture scientists are constantly searching for new explants that can be used for micropropagation. Thin cross sections (TCSs) of stems are among the explants being explored more recently (Nayak et al., 2002).

Plant Material. Shoots, each with 1–2 nodes, were taken from asymbiotic seedlings cultured on a modification of the MS medium (Murashige and Skoog, 1962; Table Cym-47) at 25 ± 1°C, 60% relative humidity (RH), and under continuous illumination of 35 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Philips, India). Standard culture room conditions may also be suitable but continuous illumination is advisable. When PLBs produced by these shoots were 60 days old they were used as sources of explants. Serial transverse TCSs of these PLBs made by hand with sterile razor blades were cultured. Sterilized scalpels can also be used to section the PLBs.

Surface Sterilization. There is no need to surface-sterilize explants from plants taken from in vitro cultures, but they must be washed to remove agar and medium residues. Razor blades or scalpels used to section the PLBs must be sterilized initially by autoclaving. During use it is necessary to frequently flame and/or dip them in alcohol.

Culture Vessels. Erlenmeyer flasks, 150-ml capacity, were used in the original research. Other containers can also be used. Culture vessels should be filled with medium to 20–30% of their capacity.

TABLE CYM-47. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the culture of shoots of *Cymbidium aloifolium* (Nayak et al., 2002)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzyladenine (BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1N HCl.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.8, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. According to the original paper the medium was "semisolid." This suggests a lower amount of agar than the usual 10 g l^{-1}. The paper suggests 8 g agar l^{-1} for another medium. That is why this amount is suggested here. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

Culture Conditions. In the original research the cultures were maintained at 25 ± 1°C, 60% RH, and continuous illumination of 35 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Philips, India). The TCSs were cultured in liquid medium on a rotatory shaker at 80 rpm. TCSs can also be cultured on semisolid medium (8 g agar l^{-1}), but proliferation will be less than in liquid.

Culture Media. The explants are cultured on a modification of the MS medium (Murashige and Skoog, 1962) containing the cytokinin zeatin riboside (Table Cym-48). Shoots produced by the TCSs are cultured on a modification of MS containing the auxin IBA (Table Cym-49).

Procedure. Shoots should be taken from seedlings, freed of their leaves, and cultured on semisolid modified MS (Table Cym-47) for 50–60 days until they produce PLBs. These PLBs should be resectioned into TCSs, 0.5 mm thick, which are cultured in liquid modified MS (Table Cym-48) until they produce shoots. To bring about root formation, the shoots must be transferred to solid modified MS (Table Cym-49).

Rooted plantlets with three to four well-developed leaves should be removed from the culture, washed well to remove all agar, and potted in potting mix consisting of peat moss, wood charcoal, and brick (in a ratio of 1 : 1 : 1) in 7.5-cm diameter pots. Originally, the potted plants were placed in a growth chamber at 25 ± 1°C and 80% RH under a light intensity of 35 μmol m^{-2} s^{-1} for 8–10 weeks before being moved to the field.

Developmental Sequence. The explants first become swollen and then turn green. Small protuberances appear on the swollen green explants 15 days after the start of culture. These protuberances develop into PLBs approximately 10 weeks after the cultures are initiated. The PLBs produce shoots and plantlets after about 12 weeks of culture. On being transferred to the rooting medium, plantlets with two to three expanded leaves produce roots in about 30 days. The rooted plantlets acclimatize after being grown in a growth chamber for 8–10 weeks.

General Comments. The use of thin sections is a novel and interesting approach. However, this procedure is more interesting as a research tool than a micropropagation method because the initial explants are taken from seedlings. Adapting the TCS method to adult plants will be a worthwhile advance.

TABLE CYM-48. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the culture of thin cross section explants of *Cymbidium aloifolium* (Nayak et al., 2002)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Zeatin riboside (ZR)	5.0	500 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	[Solidifier]				
[16	Agar[g]	8.0 g	No stock	No stock	Weigh]

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.8, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15) **[**bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved**],** pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. More explants proliferate in liquid than on solid medium (95 vs 89%) and produce a higher number of protocorm-like bodies (33.5 vs 28.2 PLBs per explant). That is why this medium should be liquid. However, if a shaker is not available the explants can be cultured on a semisolid medium. To allow for such an eventuality the agar (item 16) in the table and instructions regarding its incorporation in this footnote are in bold square brackets.

TABLE CYM-49. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the rooting of shoot produced by thin cross section explants of *Cymbidium aloifolium* (Nayak et al., 2002)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	62.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indolebutyric acid (IBA)	2.0	500 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.8, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

Protocorm-like Body Formation from Epidermal and Subepidermal Cells of *Cymbidium*

Cymbidium was the third orchid to be propagated through tissue culture in vitro and the second to have its shoot tip cultured, not the first as claimed erroneously since 1960 (Morel, 1960; see Chapter 1 and for a review see Arditti and Krikorian, 1996). However, it seems to be the first orchid whose epidermal and subepidermal cells and tissues produced PLBs in vitro (Begum et al., 1993; Begum et al., 1994*a*).

Plant Material. PLBs of *Cymbidium* Thanksgiving 'Nativity' derived from shoot-tip cultures were cultured and subcultured at monthly intervals in liquid MS medium (Murashige and Skoog, 1962; Table Cym-50) on a rotary shaker at 1 rpm and 25 ± 1°C under continuous illumination of 20 $\mu m^{-2}\ s^{-1}$ provided by white fluorescent tubes (FL 20 S. W., Toshiba, Tokyo). PLBs, 3–4 mm in diameter, were sectioned horizontally at a right angle to the main axis into two or three sections. Outer tissue (OT) explants were peeled from the PLB sections by removing a sheet composed of 10–12 epidermal and subepidermal cell layers. OT sections lacking buds or outgrows were cultured.

Surface Sterilization. Explants taken from PLBs grown axenically in vitro do not require surface sterilization. However, excision and placing of explants in culture must be carried out under sterile conditions with sterilized tools. While being used, these tools must be dipped frequently in alcohol and/or flamed to maintain sterility.

Culture Vessels. The original paper states only that "culture flasks" or "flasks" were used. Test tubes and/or Erlenmeyer flasks are suitable for liquid medium on a shaker. Several different containers can be used for cultures on a solid medium.

Culture Conditions. OT as well as tissues and structures derived from them were cultured at 25 ± 1°C under 16-h photoperiods of approximately 30 $\mu m^{-2}\ s^{-1}$ provided by white fluorescent tubes (FL 20 S. W., Toshiba, Tokyo). Standard laboratory conditions are also suitable.

Culture Media. OT should be cultured initially on a modification of MS (Table Cym-51). PLBs that form on this medium develop into plantlets when cultured on a second modification of MS (Table Cym-52).

Procedure. PLBs derived from shoot-tip cultures should be increased in number on liquid MS (Table Cym-50) by subculturing them monthly. Overproliferation of PLBs must be avoided since it can cause undesirable mutations. PLBs from liquid cultures should be taken out under sterile conditions with sterilized tools and sectioned. This can only be done under a dissecting stereomicroscope. After the sections are peeled, those lacking buds or any other growths should be placed in culture on liquid MS (Table Cym-51). PLBs that form on this medium should be subcultured onto a different MS modification (Table Cym-52) for plantlet production.

TABLE CYM-50. **Liquid-modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for proliferation of protocorm-like bodies derived from shoot tips (Begum et al., 1994*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	α-Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Kinetin	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively. The original paper states that liquid medium was used but does not indicate whether any hormones were used and if so how much. The amounts suggested here are estimates.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH as required, add sugar (item 15), raise volume to 1000 ml with distilled water (item 16), pour the solution into a 2-l flask, and autoclave. Add the amino acid (item 8), hormones (items 10 and 11) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE CYM-51. **Liquid-modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of epidermal cell peels (outer tissue) of *Cymbidium* (Begum et al., 1994*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	α-Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH as required, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE CYM-52. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of and plantlet production from protocorm-like bodies produced by epidermal (outer) layers of *Cymbidium* (Begum et al., 1994*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	α-Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH as required, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

Developmental Sequence. PLBs in liquid medium on a shaker proliferate and multiply. When OT sections are placed in culture there are many cell divisions on the surface and deeper in the tissue. The explants turn light brown after seven days in culture. Gray transparent protuberances appear following an additional 7 days. These protuberances become globular and turn green (i.e., become PLBs) after a total of 21 days in culture. Shoots moved to the second solid medium develop into plantlets within 8 weeks.

General Comments. As an advance in orchid tissue culture and the identification of yet another explant source, this procedure is significant and interesting. However, it is of limited value as a micropropagation method because the PLBs which are used as explant sources can be grown into plantlets without the added steps of subculturing, slicing, peeling, culturing the peels, and then producing plants from the newly produced bodies. The value of this procedure will increase enormously if it can be adapted to epidermal peels from mature plants.

Plant Production through Callus Culture of *Cymbidium ensifolium* var. *misericors*

This orchid is another case that requires a consideration of taxonomy and nomenclature. *Cymbidium ensifolium* is a variable species which has been cultivated in China for a long time (Chen and Tang, 1982). Chinese taxonomists who had more opportunities than other systematists to observe different variants of the species recognized many subspecies, forms, and naturally occurring varieties (for one example see Yen, 1964). Taxonomists who studied the species elsewhere (for reviews see Seth and Cribb, 1984; Du Puy and Cribb, 1988) also observed varieties. One form was accorded varietal status as *C. ensifolium* var. *misericors* by some and elevated to species level, *Cymbidium misericors*, by others. Two British taxonomists who presumably depended heavily on herbarium specimens at the Royal Botanic Gardens, Kew, but had more limited access than Chinese botanists to a large number of living plants, relegated *C. misericors* to synonymy under *C. ensifolium* without a satisfactory explanation (Seth and Cribb, 1984; Du Puy and Cribb, 1988). A recent review from Korea uses *C. misericors* as the name of the species stating that it "is synonymous with *C. ensifolium*" (Paek and Murthy, 2002) also without giving any reason for their pronouncement. It is not clear where all these classifications and reclassifications leave *C. ensifolium* var. *misericors* and it does not really matter. Its flowers are and will continue to be beautiful and delightfully fragrant regardless of its taxonomic status. Be all this as it may, a method for clonal propagation was developed for this orchid in Taiwan (Chang and Chang, 1998).

Plant Material. Segments, 1 cm in length, of roots as well as pseudobulbs and rhizomes with lateral buds taken from 7–8-cm-long seedlings produced callus masses when cultured. Similar leaf explants did not produce callus.

Surface Sterilization. Explants taken from seedlings in axenic cultures do not require surface sterilization, but they must be rinsed thoroughly with sterile distilled

water to wash away medium and agar residues. All tools and work areas must be sterile.

Culture Vessels. Test tubes, Erlenmeyer flasks, and other containers can be used. They should contain medium equivalent to one-fifth of their volume.

Culture Conditions. The original paper states that "cultures were maintained for 6 months at 25 ± 2°C in darkness or exposed to artificial light (daylight fluorescent tubes FL-30D/29, 40 W, China Electic Co., Taipei) at 28–26 μmol m^{-2} s^{-1} with light dark cycle of 14/10 h[ours] at 26 ± 2°C." However the paper does not make clear which explants were exposed to light or kept in the dark. Therefore in practical applications of this procedure it will be necessary to keep some initial explants in the light and others in the dark. Callus derived from rhizomes should be maintained in the dark until sections are subcultured for plantlet production. The latter should be cultured at 26 ± 2°C under 14-h photoperiods of 28–26 μmol m^{-2} s^{-1} provided by daylight fluorescent tubes as above.

Culture Media. Initial explants intended for callus production should be cultured on modified MS medium (Murashige and Skoog, 1962) that contains an auxin and a cytokinin (Table Cym-53). A second modification of MS (Table Cym-54) is suitable for the maintenance of callus masses. However the original paper is not entirely clear in this respect. The text states that "a medium supplemented with 3.3 mg/l 2,4-D and 0.1 mg/l TDZ was selected as the standard maintenance medium for . . . calli." But the caption of a table which shows results obtained with different hormone concentrations and includes levels selected for the standard maintenance medium (Table Cym-54) refers only to "rhizome-derived calli." In the absence of another medium, the standard maintenance medium (Table Cym-54) should be used to maintain calli regardless of their origin. After four subcultures it is necessary to subculture callus sections onto a basal medium free of hormones (Table Cym-55). Globular bodies produced on this medium (Table Cym-55) produce rhizomes when transferred to a very dilute substrate (Table Cym-56). These rhizomes produce plants when moved to yet another modification of MS (Table Cym-57).

Procedure. Several steps are part of this somewhat complex method:

1. Explants are removed from seedlings and are cultured on the first medium (Table Cym-53) until callus is produced.
2. Callus masses produced from the explants are subcultured 2–3 times on the first medium (Table Cym-53) at 6-month intervals.
3. Sections of the callus are subcultured and maintained on the second medium (Table Cym-54).
4. After the callus has been subcultured four times on the maintenance medium, a small portion of about 500 mg should be moved to the third solution (Table Cym-55).
5. Globular bodies that arise on the third solution produce rhizomes when placed on the fourth medium (Table Cym-56).
6. The rhizomes should be moved to a final medium (Table Cym-57) for plantlet production.

TABLE CYM-53. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the culture of *Cymbidium ensifolium* var. *misericors* explants (Chang and Chang, 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	18.7	1.87 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	13.9	1.39 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One soultion
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.415	4.15 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	2,4-Dichlorophenoxyaceticacid (2,4-D)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
12	Thidiazuron (TDZ)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additive				
17	Peptone	1.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The concentrations of macroelements and microelements in this table are half those in the original formulation of the Murashige–Skoog medium

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–8, 10, and 17 to 900 ml of distilled water (item 18), adjust pH to 5.2, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 18). Add Gelrite as described in footnote *h* below. When the Gelrite is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 9), hormones (items 11 and 12), and vitamins (items 13–15) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Solidifier (agar or Gelrite) is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

TABLE CYM-54. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the maintenance of *Cymbidium ensifolium* var. *misericors* callus (Chang and Chang, 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	18.7	1.87 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	13.9	1.39 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.415	4.15 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	2,4-Dichlorophenoxyaceticacid (2,4-D)	3.3	33 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
12	Thidiazuron (TDZ)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additive				
17	Peptone[g]	1.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[h]	To 1000 ml			
	Solidifier				
19	Gelrite[h,i]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The concentrations of macroelements and microelements in this table are half those in the original formulation of the Murashige–Skoog medium. The original paper does not make clear whether this medium contains NaH_2PO_4. It is included in this table because its presence or absence probably makes no difference.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]It is not clear from the original paper if this medium contains peptone. Should this procedure be used for practical purposes it is advisable to initially try media with and without peptone.

[h]Add items 1–8, 10, and 17 to 900 ml of distilled water (item 18), adjust pH to 5.2, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 18). Add Gelrite in accordance with the instructions in footnote *i* below. When the Gelrite is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 9), hormones (items 11 and 12), and vitamins (items 13–15) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Solidifier (agar or Gelrite) is not added to liquid media.

[i]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

TABLE CYM-55. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the induction of globular granule masses from callus of *Cymbidium ensifolium* var. *misericors* (Chang and Chang, 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	18.7	1.87 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	13.9	1.39 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.415	4.15 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[f]	1	
	Sugar				
13	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additive				
14	Peptone[g]	1.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Gelrite[g,h,i]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The concentrations of macroelements and microelements in this table are half those in the original formulation of the Murashige–Skoog medium.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]The callus will produce the "globular granules" (a picture in the original paper suggests that the granules are actually protocorm-like bodies) on a medium which is free of growth regulators or contains 1 mg TDZ l^{-1} or 5 mg benzyladenine (BA) l^{-1}. A medium without growth regulators is preferable.

[f]Keep frozen between uses.

[g]It is not clear from the original paper if this medium contains peptone. Should this procedure be used for practical purposes it is advisable to initially try media with and without peptone.

[h]Add items 1–7, 9, and 16 to 900 ml of distilled water (item 15), adjust pH to 5.2, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 15). Dissolve the Gelrite in accordance with the instructions in footnote *i* below. When the Gelrite is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone if used, and vitamins (items 10–12) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Solidifier (agar or Gelrite) is not added to liquid media.

[i]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

TABLE CYM-56. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for rhizome induction from globular granules of *Cymbidium ensifolium* var. *misericors* (Chang and Chang, 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	165.0	16.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	44.0	4.4 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	37.0	3.7 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	190.0	19.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	17.0	17.0 g l^{-1}		
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	3.73	0.373 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	2.78	0.278 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additives				
9	Peptone	1.0 g	No stock	No stock	Weigh
10	Coconut water[e]	150.0 ml	No stock	No stock	Measure
	Solvent				
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Gelrite[f,g]	2.2 g	No stock	No stock	Weigh
	Darkening agent				
13	Activated charcoal[h]	1.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. What the original paper refers to as globular granules are probably protocorm-like bodies.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]This is probably the liquid endosperm of coconuts which should be referred to as coconut water. It is best to use water from green (i.e., immature) nuts. However water from mature nuts can also be used. Both immature (with the green outer covering removed so that they are white) and mature (brown) nuts can be found in food stores (especially those specializing in Asian foods) where coconuts do not grow. Canned or frozen coconut water or milk (grated and homogenized endosperm which is sometimes called meat) should not be used because they usually contain sugar and preservatives.

[f]Add items 1–7, 9, and 10 to 750 ml of distilled water (item 11), adjust pH to 5.2, add sugar (item 8), and raise volume to 1000 ml with distilled water (item 11). Dissolve the Gelrite in accordance with the instructions in footnote *g* below. When the Gelrite is completely dissolved add the chacoal slowly with vigorous stirring, pour the medium into culture vessels, and autoclave.

[g]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

[h]When the agar is completely dissolved add the darkening agent (item 13) slowly with vigorous stirring. When the charcoal is completely dispersed pour the solution into culture vessels and autoclave.

TABLE CYM-57. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for plantlet production of *Cymbidium ensifolium* var. *misericors* explants (Chang and Chang, 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	18.7	1.87 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	13.9	1.39 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.415	4.15 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Cytokinin				
8	Benzyladenine (BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water	To 1000 ml			
	Solidifier				
11	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The concentrations of macroelements and microelements in this table are half those in the original formulation of the Murashige–Skoog medium.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]It is not clear from the original paper whether sucrose is present in this medium. A test may be required to determine whether sucrose is needed or not.

[f]Add items 1–7 to 900 ml of distilled water (item 10), adjust pH to 5.2, add sugar if it is used (item 9), and adjust volume to 1000 ml with distilled water (item 10). Dissolve the Gelrite in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave.

[g]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

7 After 4 months on the last medium, the plantlets reach 5 cm in height and can be moved to pots in a greenhouse.
8 The potted plants grow to marketing size in 3 months.

Developmental Sequence. Soft, yellow calli form on the cut areas of root explants on the first medium (Table Cym-53). The lateral buds on rhizome and pseudobulb

explants produce swollen and granular proliferations which form soft, yellow callus after two or three subcultures on the initial solution (Table Cym-53). This soft, yellow callus proliferates and remains totipotent on the maintenance medium (Table Cym-54). Globular granules are produced by the callus on a medium that does not contain growth regulators (Table Cym-55). These globules develop into rhizomes on the fourth medium (Table Cym-56). When moved to the fifth medium (Table Cym-57), the rhizomes produce plants which can be moved to pots. The potted plants grow, become larger, and reach commercial size in a greenhouse.

General Comments. This is a very complex procedure which cannot be used to select and propagate outstanding cultivars because the explants are taken from seedlings. It can only be used to increase the number of plants. Therefore it may be superfluous if enough seedlings can be obtained through seed germination. There are also possible problems with this procedure. One is the high (10 mg l^{-1}) and therefore possibly mutagenic level of 2,4-D in the initial medium (Table Cym-53). A second problem is the presence of 2,4-D (3.3 mg l^{-1}) in the maintenance medium (Table Cym-54). This concentration may also be mutagenic, if not in itself then because it follows a high 2,4-D level in the initial medium. The multiple subcultures and proliferations, especially in media that contain so much 2,4-D, are a third problem because they may also cause mutations. Still, the procedure is interesting because it may serve as a starting point for the formulation of a method that could be used to propagate mature plants of selected cultivars.

Micropropagation of *Cymbidium ensifolium* var. *misericors* through Callus-derived Rhizomes

The previously outlined method for micropropagation of *Cymbidium ensifolium* var. *misericors* (Chang and Chang, 1998) was modified, starting with the callus-derived rhizome step (Chang and Chang, 2000a).

Plant Material. Apical segments, 1-cm long, of rhizomes produced by the previous procedure (see Tables Cym-53 to Cym-57) should be cultured in the first medium (Table Cym-58). Vegetative (shoot) buds taken from explants in the first medium (Table Cym-58) are used for the second medium (Table Cym-59).

Surface Sterilization. Explants taken from axenic cultures do not require surface sterilization, but they should be rinsed with sterile distilled water to remove medium residues.

Culture Vessels. In the original research, 400-ml culture flasks containing 25 apical sections in 40 ml of medium were used for liquid cultures (Table Cym-58). Smaller flasks with smaller volumes of medium and fewer explants can also be used. Test tubes or other containers can be used for the second medium (Table Cym-59).

Culture Conditions. The liquid cultures (Table Cym-58) must be placed on a 60-rpm rotary shaker at 25 ± 1°C under 16-h photoperiods of 10 μmol m^{-2} s^{-1}. In

TABLE CYM-58. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the culture of *Cymbidium ensifolium* var. *misericors* rhizome explants (Chang and Chang, 2000*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	18.7	1.87 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	13.9	1.39 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.415	4.15 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	Naphthaleneacetic acid (NAA)	0.3	30 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinins				
12	Thidiazuron (TDZ)	0.04	4 mg 100 ml^{-1} 95% ethanol[e,f]	1	
13	2-Isopentenyl adenine (2iP)	0.04	4 mg 100 ml^{-1} 95% ethanol[e,f]	1	
14	Benzyladenine	7.4 mg	740 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
15	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
16	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
17	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
18	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additive				
19	Peptone	1.0 g	No stock	No stock	Weigh
	Solvent				
20	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise. The concentrations of macroelements and microelements in this table are half those in the original formulation of the Murashige–Skoog medium.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses. The actual amounts used in the original research were 0.17 µmol of TDZ and 2iP or 37.45 and 34.54 µg, respectively. Both are rounded up here. The differences (2.55 and 5.46 µg respectively) are insignificant. The original paper states that one of the cytokinins added to this medium was "6-aminopurine adenine." Adenine is 6-aminopurine. So, writing "6-aminopurine adenine" is like saying adenine adenine. Chances are that the authors meant N^6-benzyladenine which is usually referred to as benzyladenine or BA as suggested in this table. The actual amount used by the authors is 7.44 mg. It was rounded here to 7.4 mg.

[f]If the auxin or cytokinins do not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]Add items 1–8, 10, and 19 to 900 ml of distilled water (item 20), adjust pH to 5.2, add sugar (item 18), raise volume to 1000 ml with distilled water (item 20), pour the solution into a 2-l flask, and autoclave. Add the amino acid (item 9), hormones (items 11–14), and vitamins (items 15–17) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Solidifier (agar or Gelrite) is not added to liquid media.

the original research the illumination was provided by 40-W tubes (China Electric Co., fluorescent tubes FL-30D). Similar tubes (40-W fluorescent tubes of some type) produced by other manufacturers can also be used. It is necessary to maintain solid cultures (Table Cym-59) under the same conditions, but without placing them on a shaker. After being potted, plantlets produced in the second medium (Table Cym-59) should be maintained under mist and 50% shade for the first 2 months and then moved to standard greenhouse conditions.

Culture Media. Explants taken from rhizomes produced by the previous procedure (see Tables Cym-53 to Cym-57) must be cultured in a liquid medium (Table Cym-58). A solid medium (Table Cym-59) is used for explants taken from rhizomes and maintained in the liquid medium (Table Cym-58). Plantlets produced on the second medium should be potted in sphagnum moss in 2.5-cm diameter pots.

Procedure. Explants taken from rhizomes produced by the previous procedure (see Tables Cym-53 to Cym-57) should be cultured in liquid medium (Table Cym-58) and allowed to develop for 3–6 weeks. After that it is necessary to take vegetative (shoot) buds from the rhizomes in the liquid solution (Fig. Cym-7B–D and Table Cym-58), place them on the second medium (Table Cym-59), and allow them to form plantlets. On reaching a height of 60–65 mm these plantlets should be taken out of the culture, washed to remove all remnants of gel that may adhere to them, and potted in the sphagnum moss (Fig. Cym-7F). The pots should be maintained under mist and 50% shade for 2 months and after that moved to standard greenhouse conditions.

Developmental Sequence. The explants form buds in the liquid medium. When excised and cultured on the second medium these buds develop plantlets.

General Comments. This procedure is a refinement of the method described in the section above, Plant Production through Callus Culture of *Cymbidium ensifolium* var. *misericors* (see Tables Cym-53 to Cym-57).

Cymbidium pendulum

TABLE CYM-59. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for plantlet production from *Cymbidium ensifolium* var. *misericors* rhizome explants (Chang and Chang, 2000*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	18.7	1.87 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	13.9	1.39 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.415	4.15 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	Naphthaleneacetic acid (NAA)	0.3	30 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinins				
12	Thidiazuron (TDZ)	0.04	4 mg 100 ml^{-1} 95% ethanol[e,f]	1	
13	2-Isopentenyl adenine (2iP)	0.04	4 mg 100 ml^{-1} 95% ethanol[e,f]	1	
14	Benzyladenine	7.4 mg	740 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
15	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
16	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
17	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
18	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additives				
19	Peptone	1.0 g	No stock	No stock	Weigh
20	Ripe banana pulp[g]	50.0 g	No stock	No stock	Weigh
	Solvent				
21	Water, distilled[h]	To 1000 ml			
	Solidifier				
22	Gelrite[i]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The concentrations of mineral salts in this table are half those in the original MS formulation.

[b]Solutions containing ammonium and/or nitrate can become contaminated. Stock solutions should not be prepared. If made they must be stored frozen.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses. The actual amounts used in the original research were 0.17 µmol of TDZ and 2iP or 37.45 and 34.54 µg, respectively. Both are rounded up here. The differences (2.55 and 5.46 µg respectively) are insignificant. The original paper states that one of the cytokinins added to this medium was "6-aminopurine adenine." Adenine is 6-aminopurine. Chances are that the authors meant N^6-benzyladenine which is usually referred to as benzyladenine or BA as suggested in this table. The actual amount used by the authors is 7.44 mg and was rounded down here to 7.4 mg.

[f]If the auxin or cytokinins do not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]The original paper states that "green ripen banana pulp" was used. As a rule, ripe (i.e., the most commonly used) bananas for orchid media are not green when ripe. They are yellow. Also, the pulp is homogenized. The recommendation in this table is to use ripe banana and homogenize it. Opinions differ regarding the use of banana in culture media. The predominant opinion is that only homogenized pulp of ripe banana should be used. Other views are that: (1) unripe (green) bananas are preferable, (2) the peel rather than the pulp should be used, (3) unpeeled banana homogenate should be incorporated in media, and (4) slices, ripe or unripe, peeled or unpeeled, rather than homogenate are most effective. Opinions also differ regarding which is the most effective banana variety (Gros Michel, Raja, Pisang Susu, and others). Since there is no experimental evidence to support any view, the best approach is to follow the recommendations in the original reports and use what is suggested there.

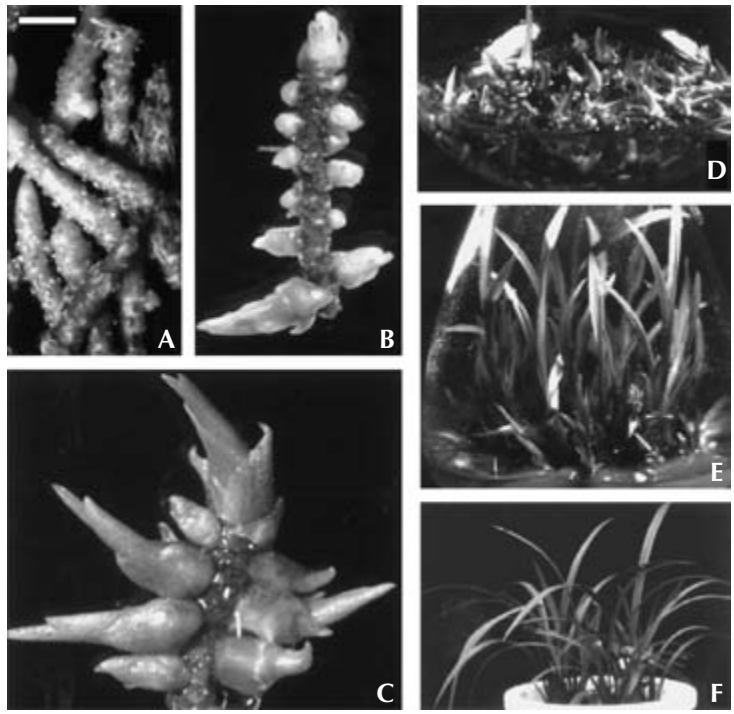


FIG. CYM-7. **Plantlet production from callus-derived rhizomes of *Cymbidium ensifolium* var. *misericors*. A. Callus-derived rhizomes (bar = 3 mm). B–D. Vegetative (shoot) buds on rhizomes from liquid medium after 21 days (B) and 45 days (C, D) of culture. E. Plantlets on solid medium. F. Plantlets in pots. (Chang and Chang, 2000*a*.)**

Plantlet Production from Thidiazuron-induced Buds on Rhizomes of *Cymbidium sinense*

Cymbidium sinense, a terrestrial species, is one of the so-called oriental cymbidiums which are not easy to propagate in vitro (Paek et al., 1993; Paek and Kozai, 1998). It produces fragrant red flowers and is sold as a potted plant. Its seeds can be germinated in vitro, but they produce rhizomes rather than protocorms (Paek et al., 1990; Paek and Murthy, 2002). Propagation through division is slow (Chang and Chang, 2000*b*). Shoot-tip cultures also produce rhizomes (Paek and Murthy, 2002). Therefore a micropropagation method utilizing TDZ-induced buds on seed-derived rhizomes was developed in Taiwan (Chang and Chang, 2002*b*).

[h]Add items 1–8, 10, 19, and 20 to 800 ml of distilled water (item 21), homogenate the pulp thoroughly, adjust pH to 5.2, add sugar (item 18), and raise volume to 1000 ml with distilled water (item 21). Dissolve the Gelrite as indicated in footnote *i* below, pour the solution into a 2-l flask, and autoclave. Add the amino acid (item 9), hormones (items 11–14), and vitamins (items 15–17) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels.

[i]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

TABLE CYM-60. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for proliferation of explants from *Cymbidium sinense* seed-derived rhizomes (Chang and Chang, 2000*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.415	4.15 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	Naphthaleneacetic acid (NAA)	3.0	300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additive				
16	Peptone	1.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh
	Darkening agent				
19	Activated charcoal[i]	1.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The concentrations of macroelements and microelements in this table are half those in the original formulation of the Murashige–Skoog medium.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1N KOH.

[g]Add items 1–8, 10, and 16 to 900 ml of distilled water (item 17), adjust pH to 5.2, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 17). Dissolve the Gelrite in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved add the charcoal (item 19) slowly with vigorous stirring, pour the solution into a 2-l flask and autoclave. Add the amino acid (item 9), hormone (item 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Solidifier (agar or Gelrite) is not added to liquid media.

Plant Material. Rhizome tip explants, 1–1.5 cm in length, were taken from seedling cultures "during a 4–8 months period."

Surface Sterilization. Explants taken from axenic cultures in vitro do not require surface sterilization. However, all tools and work areas must be sterile.

Culture Vessels. Test tubes, 20×145 mm, were used in the original research. Other containers are also suitable. Plantlets in sphagnum and a potting mix were grown in pots.

Culture Conditions. All cultures were maintained at $26 \pm 1°C$ under 14-h photoperiods. Explants in the proliferation (Table Cym-60) and shoot-tip (Table Cym-61) media were maintained under a light intensity of 5–10 μmol m^{-2} s^{-1}. Bud explants in the plant development medium (Table Cym-62) were placed under 28–36 μmol m^{-2} s^{-1}. Similar (even if not exactly the same) illumination levels are also suitable. Plantlets in moss were grown under mist for 6 months. After being potted in a potting mix plants were cultivated in a greenhouse.

Culture Media. Explants from seedling-derived rhizomes were cultured in modified half-strength MS medium (Murashige and Skoog, 1962) supplemented with 3 mg NAA l^{-1} and darkened with charcoal (Table Cym-60). Apical segments from proliferated rhizomes were cultured in TDZ-containing medium (Table Cym-61). Plantlets were produced in a third medium containing 0.5 mg NAA l^{-1} and also darkened (Table Cym-62). Well-developed plantlets taken from the third medium were potted in sphagnum. Larger plants were moved to soil.

Procedure. Tip explants from seedling-derived rhizomes are cultured in the first medium (Table Cym-60). They should be subcultured every 6 months. The original paper does not suggest how many times to subculture them. As a rule too many subcultures and extensive proliferation must be avoided because they may cause undesirable mutations. Tip explants from the proliferated rhizomes should be cultured in the TDZ-containing medium (Table Cym-61) for 2 months to bring about the formation of vegetative (shoot) buds. The buds must be moved to the last medium (Table Cym-62) for plantlet formation. After 4 months in culture, the plantlets reach

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

[f]When the agar is completely dissolved add the darkening agent (item 19) slowly with vigorous stirring. When the charcoal is completely dispersed pour the solution into culture vessels and autoclave. Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite which are very different in nature should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution, and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

TABLE CYM-61. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for vegetative (shoot) bud induction on *Cymbidium sinense* rhizome explants (Chang and Chang, 2000*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.415	4.15 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
11	Thidiazuron (TDZ)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additive				
16	Peptone	1.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The concentrations of macroelements and microelements in this table are half those in the original formulation of the Murashige–Skoog medium.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If thidiazuron does not dissolve, add a few drops of 0.1N KOH.

[g]Add items 1–8, 10, and 16 to 900 ml of distilled water (item 17), adjust pH to 5.2, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 17). Dissolve the Gelrite in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 9), hormone (item 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Solidifier (agar or Gelrite) is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

TABLE CYM-62. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for plantlet production from buds on *Cymbidium sinense* rhizome explants (Chang and Chang, 2000b)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	18.7	1.87 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	13.9	1.39 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.415	4.15 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	Naphthaleneacetic acid (NNA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
12	Thidiazuron (TDZ)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additive				
17	Peptone	1.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh
	Darkening agent				
20	Activated charcoal[i]	1.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The concentrations of macroelements and microelements in this table are half those in the original formulation of the Murashige–Skoog medium.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–8, 10, and 17 to 900 ml of distilled water (item 18), adjust pH to 5.2, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 18). Dissolve the Gelrite in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved add the charcoal (item 20) slowly with vigorous stirring, pour the solution into a 2-l flask and autoclave. Add the amino acid (item 9), hormones (items 11 and 12), and vitamins (items 13–15) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Solidifier (agar or Gelrite) is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

[i]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite which are very different in nature should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution, and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

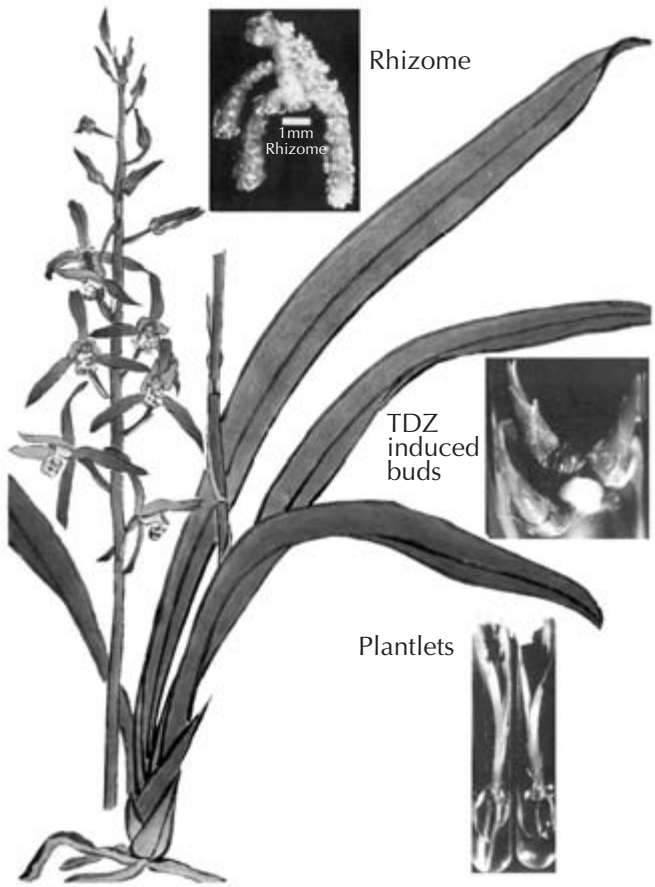


FIG. CYM-8. ***Cymbidium sinense*** **flowering plant, rhizome, buds, and plantlets (photographs from Chang and Chang, 2002*b*; drawing from Yen, 1964).**

a height of 10 cm. At that point they should be taken out of culture, potted in sphagnum, and placed under mist for 6 months. Following that they can be moved to soil and grown in a greenhouse.

Developmental Sequence. The germinating seeds form rhizomes (Fig. Cym-8). Tip explants from these rhizomes can be taken after 4–8 months of growth. These explants proliferate and form new rhizomes with repeated subculturing. Tips from these rhizomes form buds when cultured in a TDZ-containing medium. In the third medium, these buds form plantlets that reach a height of 10 cm in 4 months. When potted first in sphagnum and then in soil and grown in a greenhouse, some of these plants flower in 2 years. After 3 years the plants develop 5–6 shoots, grow to 43 cm in height, and form about 20 roots; 73% of these plants flower normally.

General Comments. This method can be useful for the propagation of *C. sinense*. It shortens the juvenile phase to 2–3 years. This is an important advance in the

cultivation of these slow growing orchids. On the negative side, the original paper is not well written and fails to explain the rationale behind some steps in the procedure.

Production of *Cymbidium aloifolium* Plantlets from Explants of Seed-derived Rhizomes

Several methods exist for shoot proliferation and culture of thin cross sections of *Cymbidium aloifolium*. The group that developed these procedures also formulated a method for the culture of seed-derived rhizomes (Nayak et al., 1998).

Plant Material. Seeds from mature, undehisced fruits were germinated on MS medium (Murashige and Skoog, 1962) containing 2.0 mg NAA l^{-1} and 0.2 mg BA l^{-1} at 25 ± 1°C, 55–60% relative humidity, and continuous illumination of 50 μmol m^{-2} s^{-1} provided by cool white (Philips India, Ltd.) fluorescent tubes. The seed produced protocorms (erroneously referred to as protocorm-like bodies in the original paper) which were transferred to MS containing 2.0 mg NAA l^{-1}. Rhizomes developed on this medium reached a size of 1.0 cm within 2–3 months and were used as explants.

Surface Sterilization. There is no need to sterilize explants taken from plants that are grown in vitro under axenic conditions.

Culture Vessels. Erlenmeyer flasks, 150-ml capacity, containing 40 ml medium, were used in the original research. Other vessels containing medium to approximately 20% of their capacity can also be used.

Culture Conditions. Cultures were maintained under the conditions used for seed germination above. Standard culture room conditions are also suitable.

Culture Media. The rhizomes were placed on a modification of MS for growth, proliferation, and plantlet production (Table Cym-63).

Procedure. Rhizomes are taken from the seedling cultures and placed on the culture medium. Plantlets should be potted and kept in the culture room for 4 weeks and under shade for 3 weeks after that, before being moved outdoors.

Developmental Sequence. The rhizomes developed shoots and roots within seven weeks. Complete plantlets formed in 2 months. These plantlets were hardened in pots in the culture room and outdoors.

General Comments. Seeds of epiphytic species are generally easy to germinate on Knudson C medium. The seedlings grow well on this medium and there does not seem to be a need for an elaborate micropropagation procedure like the one described here. In fact this procedure is an elaborate seed propagation method not micropropagation in a strict sense. It is presented here only because it may be of some use for species that are difficult to germinate or for instances in which only a few seedlings can be obtained from a capsule.

TABLE CYM-63. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for plantlet production from seedling-derived rhizomes (Nayak et al., 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	N^6-benzyladenine (BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.8, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

Induction of Early Flowering in *Cymbidium niveo-marginatum* in Vitro

Orchids can take a long time to flower. An extreme example is *Aranda* Lucy Laycock which flowered in Singapore when the plants were 13 years, 3 months old (Wee, 1971). Other examples are *Vandachnis* Scarlet Runner (11 years, 10 months, 11 days), *Vanda* John Warne (10 years, 2 months, 3 days), *Renantanda* Joan Mah (9 years exactly), and *Arachnopsis* Eric Holttum (7 years, 5 months, 2 days). Plants of the hybrid *Cymbidium* Faridah Hashim flowered at the age of 5 years, 20 days (for a review see Goh et al., 1982). Plants of *Cymbidium niveo-marginatum* flower when they are 4–7 years old. Therefore a protocol that induces early flowering of this species in vitro was developed in Korea (Kostenyuk et al., 1999).

Plant Material. The plants used in these experiments were unusual. Therefore direct quotes from the original paper will be used to describe them: "Rhizomes (3–4 months old) of *C. niveo-marginatum* Mak and 3- to 5-months-old plants (3–5 months after the initiation of regeneration from rhizomes of *C. niveo-marginatum*) were used in the present experiments. The rhizomes had been cultured in vitro for about 22 years." Since no comparisons were made with rhizomes newly produced from seeds and ones that were not propagated in NAA-containing medium it is impossible to determine what effects the long culture period and the propagation in vitro may have had on the results of the experiments. It is also not possible to predict whether this protocol will be effective with newly produced rhizomes.

Surface Sterilization. Explants taken from in vitro culture do not require surface sterilization.

Culture Vessels. Glass bottles, 1-l capacity, containing 250 ml of medium, were used in the original research. Other large containers can also be used.

Culture Conditions. Cultures were maintained at 25–26°C and 16-h photoperiods of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps.

Culture Medium. Rhizomes were propagated on MS medium containing 40 g sucrose l^{-1} and 5 mg NAA l^{-1} (Table Cym-64). Plants flowered in vitro when cultured on modified MS containing 40 mg sucrose l^{-1}, one-twentieth the original concentration of nitrogen (but the original paper does not indicate how the reduction was obtained), five times the level of phosphorus, and 10 mg BA l^{-1} (Table Cym-65).

Procedure. Rhizomes were propagated on the NAA-containing medium (Table Cym-64). Shoots produced on any of the media were moved to the flower-inducing medium (Table Cym-65) after their roots were pruned.

Developmental Sequence. Rhizomes were propagated in the NAA-containing medium. Although the original paper does not state this very clearly, it indicates that shoots were produced on this medium (Table Cym-64) and on the flower-inducing solution (Table Cym-65). When shoots were transferred to the flower-inducing medium

TABLE CYM-64. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the proliferation of *Cymbidium niveo-marginatum* rhizomes**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzyladenine (BA)	10.0	1.0 g 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	40.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Gelrite[g,h]	2.5 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1N HCl.

[g]Add items 1–7, 9, and 10 to 900 ml of distilled water (item 15), adjust pH to 5.2–5.4, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Add the Gelrite in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8) and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Solidifier is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

TABLE CYM-65. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the proliferation of *Cymbidium niveo-marginatum* rhizomes**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	82.44	8.24 g l^{-1}	10	$^1/_{20}$ of MS
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	95.0	9.5 g l^{-1}	10	$^1/_{20}$ of MS
5	Monopotassium phosphate, KH_2PO_4	850.0	85.0 g l^{-1}	10	5 times MS
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	5.0	500 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	40.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Gelrite[g,h]	2.5 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The fractions ($^1/_{20}$) and multiple (5×) of MS are assumptions based on statements in the original paper which stated "Final nitrogen concentration . . . [and] Final phosphorus concentration . . . [in] . . . the inductive medium compared to that of basal MS medium," without describing actual salt levels. No effort was made to adjust the recipe in this table for potassium levels.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1N KOH.

[g]Add items 1–7, 9, and 10 to 900 ml of distilled water (item 15), adjust pH to 5.2–5.4, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Add the Gelrite in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8) and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Solidifier is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

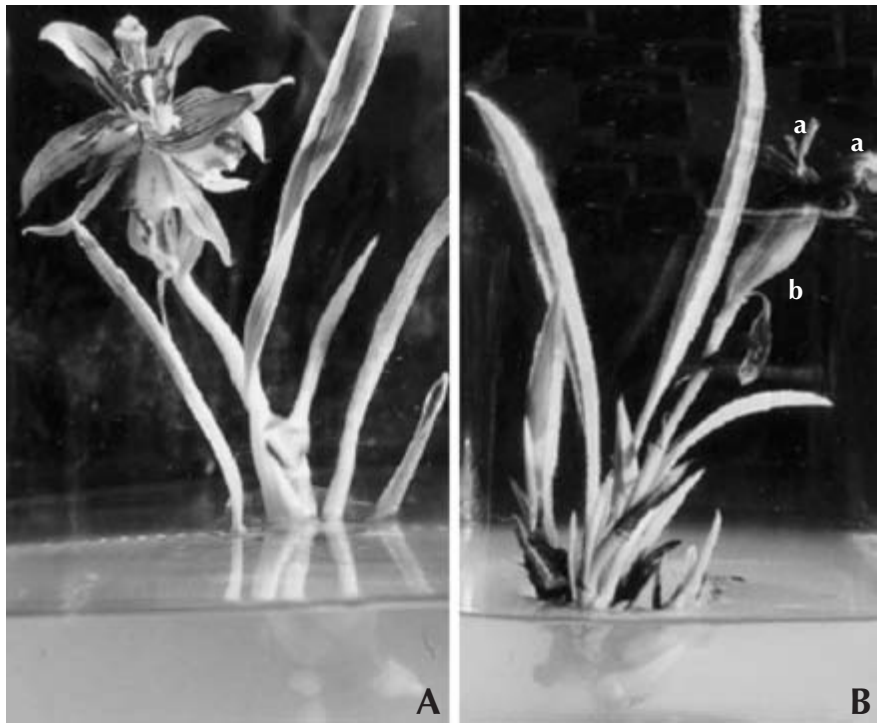


FIG. CYM-9. ***Cymbidium niveo-marginatum*** **flowering (A) and fruiting (B) in vitro. A, Remnants of perinath; B, fruit. (Kostenyuk et al., 1999.)**

after their roots were pruned, they grew and 90% of them flowered 60 days post treatment (Fig. Cym-9A). A few self-pollinated flowers set fruit (Fig. Cym-9B), but the original paper does not indicate whether the fruits matured and produced viable seeds.

General Comments. This is an interesting and pioneering procedure. Therefore, more is the pity that the original paper is not more detailed and better organized.

Flower Induction in *Cymbidium ensifolium* in Vitro

The first well-documented reports regarding flower induction in oriental *Cymbidium* in vitro are by Xiong Wang who was then at the Shanghai Institute of Plant Physiology (Chia et al., 1999). I visited her in 1987, saw her cultures and small *Cymbidium* plants flowering in vitro, and was amazed [my son Jonathan, 3 years old at the time (19 when this was written and 22 at the time of publication), and being carried by me was not impressed and may have resented having to share my attention with what to him were probably strange glass bottles with green things inside]. Shortly after our visit she moved to the Diaoyutai State Guest House in Beijing

and continued to lecture and report on her work. She lectured on flower induction in vitro in *Cymbidium ensifolium* at the Nagoya International Orchid Show which was held from March 8 until March 11, 1990 (Wang, 1990).

Plant Material. Tips were taken from young shoots, 2–3 cm long, and from their axillary buds.

Surface Sterilization. The shoots were scrubbed with a very soft toothbrush under running water. Their roots and leaves were removed following the wash and they were sprayed with 70% ethanol (74 ml of 95% ethanol diluted to 100 ml with distilled water). Axillary buds and shoot tips with innermost leaves were removed after that and immersed first in 70% ethanol for 30 s, then in 5% sodium hypochlorite for 30 min (95 ml Clorox or another household bleach which contains 5.25% sodium hypochlorite diluted to 100 ml with distilled water) and washed six times with sterile distilled water. All leaves were removed from the shoot tips and their meristems, and the axillary buds were excised and cultured.

Culture Vessels. In the original research the explants were first cultured on solid medium in flasks. For proliferation and differentiation, PLBs were cultured in T-shaped tubes (the horizontal part was 130 mm long and 35 mm in diameter, whereas the vertical portion measured 30×18 mm) which contained 10 ml liquid medium. These culture tubes were placed on a rotary shaker. If such tubes and a shaker are not available, Erlenmeyer flasks and a different type of a shaker can also be used. Shoots were cultured in flat-bottomed tubes (Fig. Cym-10) for flowering. If such tubes are not available, standard culture containers can be used.

FIG. CYM-10. ***Cymbidium ensifolium*** **flowers in vitro, in a pot, and in drawings. A. Plant flowering in vitro on March 6, 1984. B. A plant flowering in vitro in 1989. C. Flowering in vitro. D. Drawings of flowers (a) and a bud (b). E. Potted plant in bloom. F. Xiong Wang.**

TABLE CYM-66. **Half-strength Murahige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Cymbidium ensifolium* shoot-tip and axillary bud explants (Wang, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	5.0	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Complex additives				
15	Casein hydrolysate	1.0 g	No stock	No stock	Weigh
16	Coconut water[g]	100.0 ml	No stock	No stock	Measure
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar[g]	6.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same one liter of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1N KOH.

[g]The correct term for the liquid endosperm of coconuts is coconut water (CW), not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Filtered CW must be stored in a freezer until it is used. Canned or commercially frozen CW must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

Culture Conditions. Liquid cultures were placed on a rotary shaker at 1 rpm. If a different type of shaker is used it should rotate or oscillate at the same speed. Appropriate illumination is 2000–4000 lx. Temperature should be maintained at 25°C.

Culture Media. Shoot-tip and axillary bud explants should be cultured in half-strength MS medium (Murashige and Skoog, 1962) supplemented with 5 mg NAA l^{-1} and 10% coconut water (Table Cym-66). Sections of the PLBs produced on the first medium proliferate and produce shoots or plantlets on a liquid modification of MS which also contains 10% coconut water, but no NAA (Table Cym-67). When cultured on solid half-strength MS (Table Cym-68) the plants flower (Fig. Cym-10).

Procedure. Tips are excised from shoot tips and axillary buds following sterilization and placed in culture on the first medium (Table Cym-66). PLBs produced on this medium should be sectioned into several pieces, which proliferate and produce shoots and plantlets when subcultured in liquid medium (Table Cym-67). The plantlets should be moved to solid half-strength MS (Table Cym-68) to induce flowering.

Developmental Sequence. The excised tips produce bright green PLBs after 3–4 months on the first medium. Sections of these develop rosettes of new PLBs within a month. When sectioned and subcultured, sections of the new PLBs proliferate again. This process can be repeated almost indefinitely. However, excessive proliferation is not advisable. Plantlets are produced on the tips of the PLBs. More than 90% of these plantlets flower after 2 months on the solid half-strength medium.

General Comments. Orchids have flowered in vitro before this method was developed, but only occasionally and spontaneously. When they flowered around 1983, Xiong Wang's plants were the first to be induced to flower in vitro intentionally. Some of them were placed in Queen Elizabeth's room when she visited China. Dr. Wang was justly proud of that when she showed me her flasks in 1987. She had then, and still has now, a right to be proud. An effort by someone else (Goh, 1996) to claim this achievement has no basis in science or history.

The name *Cymbidium* is derived from the Greek kymbe (κύμβη) which means boat-shaped cup. This is an allusion to the boat-shaped labellum (Schultes and Pease, 1963).

[b]Add items 1–7, 9, 15, and 16 to 800 ml of distilled water (item 17), adjust pH to 5, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE CYM-67. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for proliferation of plantlets and shoot production from *Cymbidium ensifolium* protocorm-like bodies obtained from shoot tip and axillary bud explants (Wang, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 2{\cdot}H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2{\cdot}6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Complex additives				
14	Casein hydrolysate	1.0 g	No stock	No stock	Weigh
15	Coconut water[f]	100.0 ml	No stock	No stock	Measure
	Solvent				
16	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4{\cdot}4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4{\cdot}7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water (CW), not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Filtered CW must be stored in a freezer until it is used. Canned or commercially frozen CW must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[g]Add items 1–7, 9, 15, and 16 to 800 ml of distilled water (item 16), adjust pH to 5, add sugar (item 13), adjust volume to 1000 ml with distilled water (item 16). Pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), and vitamins (items 10–12) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE CYM-68. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as used for the culture of *Cymbidium ensifolium* plantlets which flower in vitro (Wang, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 2{\cdot}H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2{\cdot}6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	11.2	1.12 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[e]	To 1000 ml			
	Solidifier				
15	Agar[e]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4{\cdot}4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4{\cdot}7H_2O$ at the same concentration, a difference probably without much effect.

[e]Add items 1–7 and 9 to 900 ml of distilled water (item 14), adjust pH to 5.0, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into 2-l flask and autoclave. Add the amino acid (item 8) and vitamins (items 10–12) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

In 1988 Du Puy and Cribb reduced *Cymbidium niveo-marginatum* Mak to synonymy under *Cymbidium ensifolium* (L.) Sw.

Growth Promotion of *Cymbidium* Plantlets by High Light Intensity, Elevated Carbon Dioxide Levels, and Increased Air Exchange

Plantlets of *Cymbidium kanran* and *Cymbidium goeringii* grown for 40 days on sugar-free MS medium (Murashige and Skoog, 1962) at 25°C under a relative humidity of 70 ± 5%, 16-h photoperiods of 100 μmol m^{-2} s^{-1} (provided by fluorescent tubes and metal halide lamps), carbon dioxide (CO_2) concentration of 1000 ± 50 μmol mol^{-1}, and 4.5 air exchanges per hour (conditions described as photoautotrophic) produced 1.5 times the dry mass (i.e., grew better) than ones grown under on MS with 30 g sucrose l^{-1} under illumination of 50 μmol m^{-2} s^{-1} (heterotrophic conditions). This simply means that plantlets grown under the photoautotrophic conditions grew 1.5 times better (Hahn and Paek, 2001). And, indeed, they seem to have more leaves and roots and to be taller (Fig. Cym-11). Given the cost of increasing the light intensity, CO_2 levels, and air exchange, this procedure may not be suitable for commercial applications.

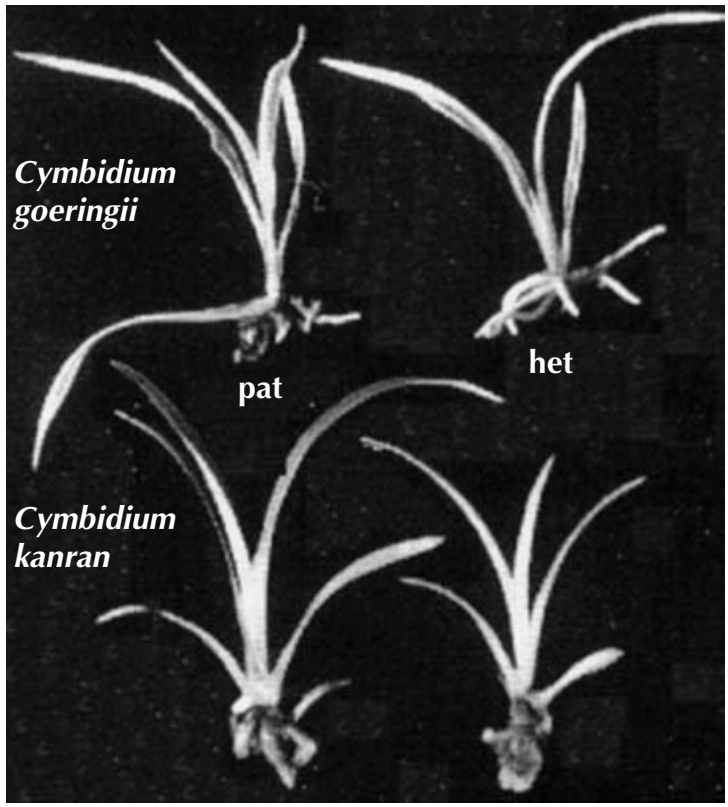


FIG. CYM-11. **Effects of photoautotrophic (pat, left) and heterotrophic (het, right) culture conditions on the growth of *Cymbidium* plantlets (Hahn and Paek, 2001).**

Effects of Benzyladenine on the Induction of Upright Shoots in *Cymbidium ensifolium*

Rhizome formation can be induced easily in shoot-tip cultures of *Cymbidium ensifolium* Yuh Hwa maintained at 26 ± 1°C under 16-h photoperiods of 50 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) provided by cool white fluorescent lamps on a horizontal 50 rpm shaker in a liquid medium consisting of one-third strength inorganic salts and full-strength vitamins of the MS medium (Murashige and Skoog, 1962) containing 20 g sucrose l^{-1} and activated charcoal (wrapped in Whatman No. 2 filter paper), and adjusted to pH 5.7 before autoclaving. The induction of upright shoots is more difficult (Lu et al., 2001). Experiments with BA showed that exposures to concentrations:

> . . . greater than 2.5 μM BA for 12 weeks or longer inhibited both elongation and upright, aerial shoots and root growth. Through transfer experiments, it was determined that the commitment to induction of upright shoots occurred after 10–14 days of exposure to 2.5 μM BA. When was BA supplied at 20 μM during the first 14 days of culture it induced upright shoot formation, but shoot elongation did not continue under these conditions. Using radiolabeled BA, adenine was found to be a major metabolite . . . in the rhizome tissue at day 14. . . . (Lu et al., 2001)

The importance and great value of these experiments is their quantitative, physiological, and metabolic rather than empirical approach to tissue culture and micropropagation.

Regeneration of *Cymbidium* Orchids from Leaf and Root Explants

> Explants were taken from plants [species or hybrid not listed], cultivated in vitro for more than a year [under] 16 [hour photoperiods], and 22°C–25°C. They consisted of small, 2 mm long basal fragments of young leaves or similar pieces of aerial roots. The explants were placed on agar solidified basal medium [Kozak, 1991 cited by Pindel and Miczynski, 1996*b*], with 30 g sucrose, 2 mg adenine sulphate, 5 mg BAP, and 1 mg NAA per [liter]. (Pindel and Miczyński, 1996*b*)

Despite citing a reference in the text, this abstract did not have a list of references. A search on the World Wide Web for "Kozak" resulted in 105,000 hits. Searching within the results for "1991" reduced the number to a daunting 11,400. Even searches using more limiting words did not identify a possible source of the medium used in this study. A search for the first author showed that she is a Professor of Botany at the Agricultural University in Krakow, Poland but their website does not provide e-mail addresses. There was no reply to a message sent to a general address on the website. This is why only quotes from this abstract are presented here. Those who may wish to use this procedure can try it with the Knudson C, MS, or Vacin and Went media.

> After 2 months of cultivation shoots appeared on 86 percent of leaf explants, and on 20 percent of root explants callus formation was visible. In subsequent transfers the media were supplied additionally with 10 mg/l ascorbic acid +60 mg/l cysteine to counteract darkening of the solutions. In such conditions after ca. 4 months of

> culture, the leaf explants developed on average 3.2 shoots per explant, while root explants formed at the same time still undifferentiated protocorm-like structures, which, however, regenerated shoots after next transfer onto fresh media.
>
> The selected medium supported only the formation of shoots. Induction of [roots] was obtained after transfer of plantlets to the same medium supplied with 1.0–1.2 mg/l of ancymidol, or 0.1–10 mg/l of abscissic [sic] acid. Media with ancymidol induced root formation usually after 2 months of culture, and at concentration of 1.25 mg/l of that compound all the tested plantlets developed roots. On the other hand, abscisic [sic] acid was more effective at lower concentrations at which 90 percent of explants formed roots. They also developed quicker, and were much stronger, than those cultivated at higher concentrations of ABA. (Pindel and Miczyński, 1996*a*)

Unfortunately the abstract does not list all concentration(s). Those who would like to use this method can try adding ABA at concentrations of 0.1, 0.5, 1, 2.5, 5, and 10 mg l^{-1}. More details are available in a full paper (Pindel and Miczyński, 1996*b*) and an e-mail from Dr. Pindel (ropindel@cyf-kr.edu.pl), received when the manuscript of this book was nearly complete. The abstract of the paper states that *Cymbidium* can be regenerated from "basal parts of leaves or aerial root sections of a *Cymbidium* hybrid cultivated in vitro for more than a year." The Kozak medium is actually a modification of the MS solution consisting of basal MS medium (macronutrients, micronutrients, and organic constituents) supplemented with 88 mmol sucrose, 11 µmol adenine sulfate, 22.5 µmol BA, and 5.5 µmol NAA (Pindel and Miczyński, 1996*b*, pers. comm.). The pH of the medium "was brought to . . . 5.5 before autoclaving. . . . cultures were kept at 16 h[our] daylength under fluorescent lamps of 55–60 µmol m^{-2} s^{-1} intensity and temperature of 22–25°C. In further steps the material was transferred every 6 weeks to the modified K[ozak]-medium, to which 55 µ[mol] of ascorbic acid [i.e., vitamin C] and 5 m[mol] cysteine were added to prevent darkening of the agar. . . . Rooting was obtained after transfer of regenerated shoots to the same medium supplied with 4–5 µ[mol] ancymidol or 0.4–4 µ[mol] ABA [i.e., abscisic acid]" (Pindel and Miczyński, 1996*b*). The optimal level of ancymidol was 5 mmol. ABA was most effective at 0.4 µmol l^{-1}. Molecular weights are: ABA, 264.32; adenine sulfate, 368.24; ancymidol, 256.30; ascorbic acid, 176.12; BA, 225.26; cysteine, 121.15; NAA, 186.21; and sucrose, 342.30.

Ancymidol is a plant growth regulator used to produce container-grown plants which are more compact. Its effects are the result of inhibition of gibberellin biosynthesis.

In Vitro Propagation of *Cymbidium bicolor* in Bangladesh

> Various . . . explants namely shoot tip, leaf tip and axillary bud of *Cymbidium bicolor* were tried for large scale multiplication. Both agar solidified and liquid media of Murashige and Skoog, Vacin and Went and Knudson C were used for this purpose. Of the three media tried higher percentages of protocorm development were obtained from . . . shoot tip and axillary bud explants of *Cymbidium bicolor* . . . in Vacin and Went medium containing 400 mg/l casein hydrolysate +20% coconut water +1.0 mg/l IAA +1.0 mg/l BAP. Growth and subsequent multiplication of protocorms were found better in the liquid medium compared to solid medium. (Sarker and Roy, 1993)

The proper term in this case is PLB, not protocorm. By definition protocorms are produced only by germinating seeds and never by explants.

Effect of Initial Sugar Concentration on in Vitro Growth of *Cymbidium* Protocorm-like Bodies

> The glucose concentration in [liquid half-strength MS medium] decreased with time in all . . . treatments. The fresh and dry weights of PLB were greater under higher glucose concentration conditions. It should be noted that the mean relative growth rate (RGR) of PLB for the entire culture period was similar when the initial amount of sugar per fresh weight of PLB was the same, regardless of the sugar concentrations of the media. (Kubota and Kozai, 1991)

Photoautotrophic Micropropagation of Protocorm-like Bodies of *Cymbidium*

> *Cymbidium* (cv. Lisa rose [sic]) PLB (protocorm-like bodies) were cultured in liquid 1/2 MS medium with/without 20 mg g^{-1} sucrose under continuous lighting conditions. The vessels were shaken at 100 rpm under PPF (photosynthetic photon flux) of 20 and 2000 μmol m^{-2} s^{-1} and CO_2 concentrations outside the vessel (C_{out}) of 450 and 2000 μmol mol^{-1} conditions. Photoautrophic growth was obtained at high PPF and high C_{out}. Chlorophyll content of the PLB in the medium without sucrose at high PPF and high C_{out} was almost 3 times that with sucrose at low PPF and low C_{out}. The number of newly developed PLB with sucrose at low PPF and low C_{out} was 1.6 times that without sucrose at high PPF and high C_{out}; the dry weight per unit PLB with sucrose at low PPF and C_{out} was almost 3 times that without sucrose at high PPF and high C_{out}. Photoautrophic growth of the PLB might be further promoted at higher CO_2 concentration (> 1%). (Kubota et al., 1992)

These comparisons (Kubota et al., 1992) would have been more useful and easier to understand if given in a table with chlorophyll content, newly developed PLBs, and dry weight listed for all combinations of high and low photosynthetic photon flux and high and low carbon dioxide concentrations outside the vessel, all with and without sucrose. If the results could be understood more easily, they might have been more useful.

Substrate-specific Hydrolysis of Aromatic and Aromatic–Aliphatic Esters in *Cymbidium* Tissue Culture

> In tissue cultures of orchids (*Cymbidium* 'Saint Pierre' and *Dendrobium phalaenopsis*) acetates of phenols and aromatic–aliphatic alcohols were hydrolyzed whereas methyl esters of aromatic and aromatic and aliphatic acids did not undergo this reaction. Acetates of racemic aromatic–aliphatic alcohols were hydrolyzed with distinct enantiospecificity. (Mironowicz et al., 1993)

These findings represent good and interesting research that should be of interest to phytochemists and plant physiologists, but is of no relevance to micropropagation.

Comparisons of *Cymbidium* Protocorm-like Body and Plantlet Formation on Solid and Liquid Media.

PLBs were cultured for multiplication on solid substrate or in liquid substrate consisting of Knudson C medium (Knudson, 1946) plus the minor elements of the MS solution (Murashige and Skoog, 1962): 20 g sucrose l^{-1}, 0.1 mg NAA l^{-1}, and 0.01 mg BA l^{-1}. Solid media were solidified with 7 g agar l^{-1}. Cultures were maintained at 25°C under 18-h photoperiods of 14 W m^{-2} light intensity (the source of the illumination was not described). Liquid cultures were rotated at 2 rpm. A 2-l Osmotek plastic bioreactor was used for some cultures (Pamfil, 2002). The results (Table Cym-69) are not surprising.

The data show the following:

1 Proliferation is more extensive in a liquid medium (this has been well known for a long time).
2 The differences in fresh PLB weight between liquid agitated, non-agitated, and bioreactor cultures are not statistically significant. In practical terms the weight of PLBs is not as important as normal differentiation of plantlets.
3 Proliferation is most extensive in a bioreactor. However, there is no statistically significant difference between proliferation in a bioreactor (5.1 ± 0.5)and that in liquid cultures on a shaker (4.6 ± 0.3). This suggests that in practical terms the costs involved in the purchase, maintenance, and operation of a bioreactor must be compared to the outlays for a shaker.
4 Necrosis is highest in a bioreactor. This must also be considered in considering the use of a bioreactor versus a shaker.
5 In terms of fresh weight, the best growth of seedlings is on a solid medium. However, as mentioned above, in a practical laboratory proper differentiation and plantlet formation are much more important than weight of PLBs.

TABLE CYM-69. **Effects of the state of the medium on the growth of protocorm-like bodies and plantlets**

	Medium			
Factor	Agar-solidified	Liquid, stationary	Liquid, agitated	Bioreactor
Protocorm-like bodies				
Final fresh weight, mg	161.9 ± 12	177.6 ± 16	182.4 ± 15	192.1 ± 21
Multiplication factor, mg	3.6 ± 0.2	4.1 ± 0.3	4.6 ± 0.3	5.1 ± 0.5
Necrosis, percent	2.3	3.7	3.5	5.3
Plantlets				
Initial fresh weight, mg	44.4 ± 3.4	39.8 ± 3.6	38.7 ± 4.2	35.2 ± 2.8
Final fresh weight, mg	159.3 ± 11	148.1 ± 13	151.7 ± 8	140.4 ± 7
Hyperhydricity, percent[a]	3.8	6.2	8.5	11.8

[a]This term that is spelled "hyperhydricity" in the table and "hiperhydricity" in the text is not defined. There is also no description of the method by which the data were obtained (Pamfil, 2002).

Starch and α-Amylase Levels During Protocorm-like Body Formation from Foliar Explants of *Cymbidium* Burgundian Chateau

> Leaf bases measuring 7–10 mm were [taken] from the second and third leaves of shoots . . . [cultured on] . . . Vacin and Went (VW) medium supplemented with BA, 0.2 mg/l and agar 0.8%. . . . [These explants were cultured on a medium consisting of] . . . salts and sugar of VW medium, 10% (v/v) tender coconut water, 2 mg/l naphthaleneacetic acid (NAA) and 1 mg/l benzyl adenine (BA). The pH of the medium [was adjusted] to 5.2 before adding agar. After autoclaving at 120°C for 18 min, aliquots (15 ml) of the medium were dispensed into 9 cm petri dishes. The cultures were maintained in the dark at 26 ± 1°C and 60 ± 5% RH in a Lab-Line model 8482 plant growth chamber. A large pool of sugars [which] formed after two days of culture disappeared at the initiation of tissue growth (10 days). Maximum accumulation of starch occurred when the growth centres were already formed and PLBs were emerging (22 days). Rapid depletion of the starch reserve, consequent rise and then fall in free sugars and high α-amylotic activity characterized the rapid phase (20–30 days) of development of the PLBs. Evidence for early appearance of a highly persistent isozyme of α-amylase, a_1 and a transient, probably a marker isozyme a_2, confined to the period of high intensity metabolism (15–30 days) during the initiation and rapid development of the PLBs were obtained. Mature PLBs contained negligible levels of starch and lacked the second isozyme. (Gopalan, Krishnan and Seeni, 1992)

This report is valuable because it identified periods during which PLBs require sugar and this information can be used to formulate culture media.

Growth of Tissue-cultured *Cymbidium goeringii* and *Cymbidium kanran* in Korean Native Orchid Habitat Soils

Tissue-cultured *Cymbidium goeringii* grew well in pine bark and soils collected from Korean native orchid habitats. Plantlets of *Cymbidium kanran* grew better in non-sterilized soil (Lee et al., 1997). These findings reflect the formation of appropriate mycorrhizal associations. They should be considered in moving plantlets from flasks to horticultural conditions.

Growth of *Cymbidium* Plantlets in Vitro under Blue and Red Light-emitting Diodes

"Red light [emitted by red LEDs] promoted leaf growth [of *Cymbidium* plantlets in vitro], but decreased chlorophyll content. This was reversed by blue light [emitted by blue LEDs]. The growth of *Cymbidium* plantlets in terms of increase in total shoot and root weights was comparable under red plus blue LEDs [45 $\mu mol\ m^{-2}\ s^{-1}$] and the fluorescent system . . ." (Tanaka et al., 1998) consisted of Homo-Lux lamps (National Electric Co., Ltd.). Photoperiods were 16 h and the temperature was 25 ± 1°C. These findings are not surprising because the light intensity and spectrum provided by the two sources of illumination were very similar. What these experiments show has been known for a long time, that is, plants grown under

appropriate illumination in terms of intensity, spectrum, and photoperiods grow well. Given the cost of light-emitting diodes (LEDs), there does not seem to be any reason to start using them rather than fluorescent lights to illuminate *Cymbidium* and other tissue cultures of orchids.

Proliferation of *Cymbidium* Protocorm-like Bodies

PLBs obtained from a commercial orchid establishment were proliferated on MS medium (Murashige and Skoog, 1962) containing 30 g sucrose l^{-1}, 4 mg NAA l^{-1}, and 0.05 mg kinetin l^{-1}, solidified with 8 g agar l^{-1}, with the pH adjusted to 5.8–6.0 prior to autoclaving. Cultures were maintained at 26 ± 1°C. There is no information in the original paper regarding photoperiods and light intensity. Plantlets were produced by the PLBs. The plants used are described as "*Emken* × *Cymbidium*" (Prasad and Verma, 2001). A search in several data bases (including the excellent WildCatt, www.wildcattdata.com) could not find an orchid named *Emken*. This is not a complete procedure since the PLBs were produced from shoots elsewhere. It is also not necessary because most published methods include a proliferation step. And, since excessive proliferation may cause undesirable mutations, using this method is not advisable. It is listed here solely for the purpose of presenting or at least mentioning as many published reports of orchid propagation as possible.

Eradication of Odontoglossum Ringspot Virus from in Vitro Cultures of *Cymbidium*

A widely accepted and often repeated (in the orchid world), but completely incorrect, myth is that Professor Georges Morel (1916–1973) proposed and implemented the idea that plants can be freed of virus by culturing their shoot tips. This "proven" and widely accepted "truth" was originated by Morel himself and driven into the minds of orchid growers and scientists by him, starting in 1960 and continuing until his death in 1973. Once this dogma became accepted, others spread it and resisted or even beat down challenges to it (see Arditti and Krikorian, 1996 for a review). In fact the idea is at least 60 years old. In 1943 Arthur W. Dimock (1908–1972) published a method for establishing *Verticillium*-free clones of chrysanthemum by rooting shoot-tip cuttings. After that he refined the method and extended it to other diseases. Similar methods were used to free carnations, dahlias, and other plants from fungal and virus diseases (see Arditti and Krikorian, 1996 for a review). Therefore it is not surprising that when viral diseases caused problems for potatoes and dahlias in France, Pierre Limaset and Pierre Cornuet suggested to Morel that cultivating shoot meristems may free these plants of viruses. Morel did it, was successful, extended the method to orchids (Arditti and Krikorian, 1996), and created the erroneous impression that he developed concepts and techniques that were originated by others.

After Morel published and promoted his findings, growers assumed that shoot-tip cultures could be used for mass, rapid, clonal propagation – freeing orchids from viruses. That was a misconception. Before "mericloning" orchid viruses were less of a problem than after its advent. Careless culturing of shoot tips actually propagated

viruses as well as orchids. Now the viruses are more common and widespread (Arditti and Krikorian, 1996). Because of this, virus eradication methods are being coupled with micropropagation. One such method for *Cymbidium* was developed in Belgium (Toussaint et al., 1993).

Plant Material. PLBs produced by standard methods (Morel, 1963; Vanseveren and Fréson, 1969; also described in the first edition of this book, Arditti and Ernst, 1993) from shoot-tip cultures of *Cymbidium* plants infected with odontoglossum ringspot virus (ORSV) were proliferated and used in the original research.

Surface Sterilization. PLBs taken from in vitro cultures do not require surface sterilization. However they should be rinsed with sterile distilled water to remove medium residue.

Culture Vessels. Bellco test tubes, 2.5 × 15 cm, containing 10 ml of medium were used during the ORSV eradication research. Other containers are also suitable.

Culture Conditions. The conditions in a standard culture room are appropriate.

Culture Medium. Liquid ribavirin-containing Knudson C medium (Knudson, 1946) should be used to free plants from virus (Table Cym-70). To produce plantlets PLBs should be cultured in Knudson C medium containing 5 g sucrose l^{-1} (Table Cym-71).

Antiviral Agent. Virazole® (commercial name of ribavirin, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, MW 244.2) should be used at a concentration of 35 mg (143.5 μmol) l^{-1}. This concentration was selected because it is a good balance between antiviral effects and toxicity. The maximum solubility in water of Virazole is 142 mg ml^{-1} (which is slightly more than needed for 4 l of medium). It cannot be autoclaved and filter sterilization is too complex for a practical laboratory. Therefore, 35 mg ribavirin (available from Sigma-Aldrich, www.sigmaaldrich.com, as item R 9644 at a cost in mid 2002 of US$68.25 for 50 mg) should be dissolved in 1 ml of water and brought to 4 ml with 95% ethanol. This solution should be added to the autoclaved culture medium (Table Cym-70) while it is still warm and liquid. The medium should be swirled well or stirred with a sterile stirrer to ensure even dispersal of the ribavirin.

TABLE CYM-70. **Number of virus-free plants as a function of the number of subcultures and time in ribavirin-containing medium**

Number of subcultures	Number of plants per culture	Time in ribavirin, days	Number of healthy plants
4	14	18	13
5	28	47	25
6	25	66	28
Total	**100**		**90**

TABLE CYM-71. **Ribavirin-containing Knudson C (KC) medium (Knudson, 1946) for the eradication of virus from protocorm-like bodies of *Cymbidium* (Toussaint et al., 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement[d]				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Antiviral agent				
7	Ribavirin (Virazole®)[d]	35.0	35 mg in 1 ml water adjusted to 4 ml with 95% ethanol[d]	4	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Kundson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$, l^{-1} but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored.

[d]Ribavirin (Virazole®) cannot be autoclaved and filter sterilization is too complex for a practical laboratory. Therefore 35 mg (143.5 µmol) ribavirin (Virazole®) should be dissolved in 1 ml of distilled water and brought to 4 ml with 95% ethanol. This mixture should be prepared immediately before use.

[e]Add items 1–6 to 900 ml of distilled water (item 9), adjust pH to 5–5.2, add sugar (item 8), raise volume to 1000 ml with distilled water (item 9), pour the solution into a 2-l flask, and autoclave. After the autoclaved solution has cooled add the ribavirin (item 7), mix well by swirling, and distribute into sterile culture vessels. There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946). It is not clear whether the addition of such nutrients would be of benefit. If the incorporation of additional microelements is necessary, those used in the Murashige–Skoog (MS) medium are suitable.

Procedure. PLBs must be taken for culture on ribavirin-containing medium (Table Cym-70) when they are small and before leaf primordia are evident. The original paper reports that 4–7 subcultures (over a period of 18–71 days) were needed to obtain a high proportion of virus-free plants (Table Cym-72). The time in ribavirin-containing solutions should be balanced against the possibility that long exposure to the antiviral agent can lead to teratogenic effects. In the original research the presence of virus after culturing PLBs in ribavirin-containing media was determined using ORSV antibodies in a procedure called enzyme-linked immunosorbent assay (ELISA), which may be too complex for practical laboratories. Those interested in learning the procedure should consult *The ELISA Guidebook* by J. R. Crowther, published in 2001 by Humana Press. It can be ordered from www.sigmaaldrich.com. ORSV test kits and assays are available from Agdia Inc., www.agdia.com. Tests are also available from http://plantpath.osu.edu/cweppdc/plantdis.html#tests.

When protocorms become (or are assumed to be) virus-free they should be moved to solid Knudson C (Table Cym-71) and grown until plantlets are ready to be

TABLE CYM-72. **Knudson C (KC) medium (Knudson, 1946) for plantlet production from protocorm-like bodies of *Cymbidium* that have been freed from odontoglossum ringspot virus (Toussaint et al., 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement[d]				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
7	Sucrose	5.0 g	No stock	No stock	Weigh
	Solvent				
8	Water, distilled[d]	To 1000 ml			
	Solidifier				
9	Agar[d]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Kundson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$, l^{-1} but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored.

[d]Add items 1–6 to 900 ml of distilled water (item 8), adjust pH to 5.2, add sugar (item 7), and raise volume to 1000 ml with distilled water (item 8). Bring the solution to a gentle boil and add the agar (item 9) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946). It is not clear whether the addition of such nutrients would be of benefit. If the incorporation of additional microelements is necessary, those used in the Murashige–Skoog (MS) medium are suitable.

potted. Well-developed plantlets should be potted in small pots in a greenhouse at 22–25°C, repotted several times, and grown to maturity.

Developmental Sequence. PLBs proliferate in the liquid ribavirin-containing medium while the antiviral agent eradicates the virus. Plantlets form on the solid medium.

General Comments. This is a useful procedure that can free infected clones of virus. The only problem with it is its complexity, especially the ELISA.

Production of Virus-free *Cymbidium* through the Use of Vidarabine in Vitro

Orchid viruses are spread mostly through contaminated tools, pots, and manual contact. There are at least 25 orchid viruses (for an excellent review see Wong, 2002).

Two of the most prevalent orchid viruses, and among the first to be described, are *Cymbidium* mosaic virus (CyMV) and odontoglossum ringspot virus (ORSV). They can cause death of plants, inhibit growth, and distort flowers. Temperate *Cymbidium* species like *Cymbidium gyokuchin* and *Cymbidium ensifolium* are more susceptible than tropical taxa. That is why a method for the production of virus-free plants was developed in Korea (Paek et al., 1997).

Plant Material. Shoot tips of *Cymbidium* Golden Gate were used in the original research. However other orchids can probably be freed of virus using this method provided they can withstand the high concentration of the antiviral agent.

Surface Sterilization. The surface sterilization procedure, which is part of Clonal Propagation of *Cymbidium* through Shoot-tip Meristem Culture (Sagawa et al., 1966), can be used.

Culture Vessels. Test tubes containing 10 ml of medium were used in the original research (size of tubes not indicated, but 25×150 mm is suitable). Other containers can also be employed.

Culture Conditions. The conditions used during the original research were 25°C under 16-h photoperiods of 35–50 μmol m^{-2} s^{-1} provided by fluorescent lamps (the type used is not described). Conditions in a standard culture room are also suitable.

Culture Media. "Murashige–Skoog basal medium containing 3% sucrose and solidified with 0.2% Gelrite" and vidarabine (Table Cym-73) was used in the original research. The term "basal medium" implies that only the mineral components of the MS medium (Murashige and Skoog, 1962) were used. This is in line with the previous procedure (Toussaint et al., 1993). It uses the Knudson (KC) medium (Knudson, 1946) which does not contain vitamins and hormones. Vidarabine-free medium should be used to grow plantlets after the initial 6–8 weeks. The medium used in the previous procedure is suitable (Table Cym-71).

Antiviral Agent. Vidarabine (VIRA-A is its trade name and it is also known as adenine arabinoside, 9*H*-purin-6-amine, 9-β-D-arabinofuranosyl, MW 285.25) at 100 mg l^{-1} is used to eliminate CyMV and ORSV. It can be obtained from Sigma-Aldrich (www.sigmaaldrich.com) as item number 01832 under the names adenine 9-β-D-arabinofuranoside or ARA-A.

This compound is not heat-stable and cannot be autoclaved. Filter sterilization is too complex for a practical laboratory. Therefore 100 mg vidarabine should be suspended in 5 ml of 70% ethanol (74 ml of 95% ethanol adjusted to 100 ml with distilled water) in an autoclaved 10–20-ml-capacity vial regardless of whether it will dissolve completely or not, and shaken vigorously several times, allowed to stand for 5 min, and agitated again. This should be repeated 4–6 times over a 30-min period to sterilize the vidarabine even if it will not dissolve the powder completely. All of the suspension should be added to a still-warm 1 l of culture medium after it was autoclaved. The vial must be rinsed several times with warm culture medium until all particulate matter has been washed away and added to the culture medium. After

TABLE CYM-73. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for virus eradication from *Cymbidium* (Paek et al., 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, $KH_2PO_4 \cdot H_2O$	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	62.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Antiviral agent				
8	Vidarabine[e]	100.0	No stock	No stock	See footnote *e*
	Sugar				
9	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Gelrite[f,g]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]This compound is not heat stable and cannot be autoclaved. Filter sterilization is too complex for a practical laboratory. Therefore 100 mg vidarabine should be suspended in 5 ml of 70% ethanol (74 ml of 95% ethanol adjusted to 100 ml with distilled water) in an autoclaved 10–20-ml capacity vial regardless of whether it will dissolve completely or not and shaken vigorously several times, allowed to stand for 5 min, and agitated again. This process must be repeated 4–6 times over a 30-min period. It will sterilize the powder whether it dissolves or not. The suspension should be added to 1 l of still warm culture medium after it was autoclaved and the vial must be rinsed several times with warm culture medium until all particulate matter has been washed away and added to the culture medium. After that the medium should be swirled or stirred vigorously with sterile stirrers before being dispensed into preautoclaved culture vessels.

[f]Add items 1–7 to 900 ml of distilled water (item 10), adjust pH to 5.2–5.4, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Dissolve the Gelrite in accordance with the instructions in footnote *g*. When the Gelrite is completely dissolved, pour the solution into a 2-l flask and autoclave. Add the antiviral agent as indicated in footnote *e* above under sterile conditions, mix well, and distribute the medium to preautoclaved culture vessels. Solidifier is not added to liquid media.

[g]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved, pour the medium into culture vessels and autoclave.

that the medium should be swirled or stirred vigorously with sterile stirrers before being dispensed into preautoclaved culture vessels.

The concentration of vidarabine (100 mg l^{-1}) used in this procedure is high enough to kill 50% of the shoot tips placed on the medium and to "increase the occurrence of abnormal PLB," but it does free of virus most of the surviving ones. A higher concentration (150 mg vidarabine l^{-1}) causes abnormal growth such as swollen PLBs.

Levels of CyMV and ORSV in plants cultured on vidarabine-containing MS were assayed using ELISA (Bio-tek Instruments, EL-307). ORSV kits and assays are available from Agdia Inc., www.agdia.com. Tests are also available from http://plantpath.osu.edu/cweppdc/plantdis.html#tests. The ELISA may be too complex for practical laboratories.

Procedure. Shoot tips, one per culture vessel, should be placed on the culture medium and allowed to grow for 6–8 weeks or until they reach a height of 7–10 cm. Then the plantlets should be moved to fresh, vidarabine-free medium in a larger vessel and grown until they are large enough to be moved to pots. These plantlets must be maintained in an enclosure with enough space between each plantlet so they do not touch; they should be handled with sterile tools and observed for signs of virus infection. Any that show symptoms must be destroyed by burning or autoclaving. Before any plants can be sold as virus-free it is necessary to assay them in some fashion.

Developmental Sequence. The shoot tips develop into PLBs, which form plantlets. Hopefully most of the plantlets will be virus-free.

General Comments. This procedure is simple enough to be suitable for practical laboratories. The only complex step is ELISA. Kits and testing are available from www.agdia.com and http://plantpath.osu.edu/cweppdc/plantdis.html#tests.

Cymbidium Protoplast Culture

Protoplasts of *Cymbidium* can be isolated and cultured using the method suggested for *Dendrobium* (Yasugi, 1990).

In Vitro Morphogenesis of *Cymbidium*

Pseudobulb, rhizome, and root sections of *Cymbidium ensifolium* var. *misericors* formed totipotent calli on modified MS medium containing 2,4-D and TDZ. On being subcultured onto MS supplemented with BA and TDZ, the calli formed embryoids. These embryoids developed rhizomes which eventually produced plantlets (Chang et al., 2001).

In Vitro Storage of Protocorm-like Bodies

This paper (Kim et al., 1999) is in Korean. Only part of the English summary is presented here:

> Excellent storage of PLB was obtained from moist condition using [a medium] with 3 g l^{-1} hypones and 4 g l^{-1} peptone . . . in the dark. The optimal [storage] temperature was 5 C in [dry] storage and 10 C in moist condition storage. The preservation periods . . . were 6 weeks in gradual dry, 3 weeks in rapid dry storage and 18 weeks in moist storage condition without drying pretreatment.

Effect of Auxin and Ethylene on Rhizome Formation from Shoot Cultures of *Cymbidium kanran*

Only the summary of this report (Shimasaki, 1993) is in English:

> Application of Ethephon promoted rhizome formation from shoot cultures of *Cymbidium kanran*. Naphthaleneacetic acid (NAA) in the culture medium enhanced rhizome formation from shoot cultures, whereas simultaneous addition of aminoethoxyvinylglycine (AVG) suppressed rhizome formation. Ethylene evolution from shoot cultures was enhanced by NAA, but reduced by AVG.

Ethephon (2-chloroethylphosphonic acid or ethrel) acts by liberating ethylene. AVG is an ethylene inhibitor.

Effects of Benzyladenine, Gibberellin, and Paclobutrazol on Organogenesis in *Cymbidium* Species

This report (Shimasaki et al., 2002*a*) is in Japanese with an English summary:

> Addition of paclobutrazol (PAC) to modified MS medium induced protocorm-like body (PLB) formation from rhizome segment cultures of *Cymbidium kanran* Makino (terrestrial type). The combination of BA with PAC reduced the number of PLB. Application of BA induced shoot[s] (protocorm-like pseudobulb with short leaves) without root[s]. Single addition of 10 μM [sic] BA had a significant effects on induction and increased the number of shoots. PAC in culture medium inhibited BA-induced leaf formation from rhizome cultures.
>
> Application of GA_3 to PLB cultures of epiphytic type *Cymbidium* (*C.* Hiroshima Golden Cup 'Sunny Moon') resulted [in] rhizome formation. GA_3 treatment at 100 μM [sic] was effective for rhizome formation from PLB cultures. Single addition of 10 μM [sic] PAC was effective for proliferation of PLB from 'Sunny Moon'.

Paclobutrazol ($C_{15}H_{20}ClN_3O$), a plant growth regulator, is used to reduce terminal growth and to prune volume in trees not used for food production on sites such as utility rights-of-way, urban environments, and residential and non-crop areas. It is (2*RS*,3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1*H*-1,2,4-triazol-1-yl)pentan-3-ol.

Chitosan Effects on Organogenesis in Protocorm-like Bodies of *Cymbidium finlaysonianum*

This is yet another paper in Japanese (Shimasaki et al., 2003*c*) with an English summary:

> PLB of *Cymbidium finlaysonianum* Lidl. were explanted on modified Kano's medium (Hyponex medium) supplemented with two kinds of chitosans. Within 8 weeks of culture, proliferation of . . . PLBs and differentiation of shoots from PLBs were observed. Explants cultured on medium containing chitosans formed a mass of PLBs followed by differentiation of shoots. The number of PLBs and the number of shoots from proliferated PLBs were significantly increased by application of chitosans.

Chitosan is a biocompatible, antibacterial preparation which is an environmentally friendly polyelectrolyte. It has a number of applications including: additive for cosmetics, antimicrobial textile treatment, aqueous thickener, biodegradable films, biomedical devices, chromatography, encapsulating agent, flocculant, microcapsule implants for controlled release drug delivery, novel fibers for textiles, photographic papers, protein precipitation, and water treatment. It forms gels with multivalent anions and produces clear solutions which dry to form strong and clear films. It is available from www.sigmaaldrich.com.

Jasmonic Acid, Methyl Jasmonate, and Cucurbic Acid Effects on Organogenesis in Rhizome Segments of *Cymbidium kanran*

This report (Shimasaki et al., 2002*b*) is in Japanese with an English summary:

> Differentiation of rhizome into shoots occurs in Murashige–Skoog medium containing 0.01–0.03 μM [sic] jasmonic acid (JA), 0.01–0.03 μM [sic] methyl jasmonate (Me-JA) and 0.01–0.03 μM [sic] cucurbic acid (CUC), respectively. Differentiation was especially marked on at 0.03 μM [concentration] of JA and 0.03 μM [concentration of] Me-JA. Formation of protocorm-like bodies (PLB) from rhizome cultures [was] observed at all treatments except for control treatments (growth regulator free). Higher concentrations of JA, Me-JA and CUC were effective for PLB formation. The average number of PLBs was highest at 3 μM [concentration] of Me-JA. The rate of PLB formation was 100 percent at 1 and 3 μM Me-JA and 0.03 μM CUC treatments. Higher concentrations pf Me-JA and CUC inhibited branching of rhizome cultures. Branching of the rhizome was completely inhibited by 0.1–3 μM Me-JA and 1 and 3 μM CUC.

Cucurbic acid is a naturally occurring metabolite of 7-epi jasmonic acid. It is as a plant growth regulator. Jasmonic acid and its methyl esters are of ubiquitous distribution in plants. They have hormone properties and function in the regulation of plant growth and development, senescence of leaves, and in the defense against fungi.

Methyl jasmonate (Me-JA) is one of the main fragrance components (ca. 10^{-4} g per blossom) of jasmine along with jasmone. It comprises 2–3% of jasmine oil. A total of 15,000 blossoms are needed to produce 1.5 g of the oil. Me-JA is also a flavor ingredient of semiblack and black teas.

Methyl Jasmonate Effects on Organogenesis in Shoot Cultures of Epiphytic and Terrestrial *Cymbidium* Species

Since this paper (Shimasaki et al., 2003*b*) is in Japanese, only part of the abstract is presented here:

> The addition of Me-JA at concentrations below 1 μM effectively increased the number of PLBs from shoot cultures. A high concentration (10 μM) of Me-JA had little effects on the proliferation of PLB. Rhizome branches were formed from shoot cultures of *Cymbidium kanran* Makino (terrestrial species) and the largest number of rhizomes were developed at 1 μM Me-JA. Addition of Me-JA to culture medium inhibited shoot development from explants.

Effects of Cytokinin and Ethephon on Shoot Formation in Rhizome Cultures of *Cymbidium kanran*

The research that resulted in the method which is described in this section was carried out in an effort to determine the effects of and interactions between cytokinins and ethylene in organogenesis of rhizome cultures (Shimasaki, 1995).

Plant Material. The original report only states that apical segments, approximately 5 mm, were "prepared by . . . methods described previously." Shimasaki (1995) describes the preparation:

> Immature seeds (10 months after pollination) of *C. kanra*n were cultured on modified Kano medium . . . containing 3 g/l Hyponex, 2 g/l Difco bacto-peptone [and probably 20 g sucrose l^{-1}], pH 5.3 at 25 ± 1°C under total darkness for the first 10 months. One of the rhizomes was subcultured for two months to proliferate rhizome branches on the modified MS (Murashige and Skoog, 1962) medium containing 412.5 mg/l ammonium nitrate and 950 mg/l of potassium nitrate, 20 g/l sucrose and 20 g/l gelrite [this seems to be a very high level of Gelrite]. Approximately 10 mm of vegetative shoots formed on apical segments on rhizome branches of proliferated rhizomes cultured on the modified MS medium were used for explants.

Surface Sterilization. Explants taken from in vitro cultures do not require sterilization, but should be rinsed with sterile distilled water to remove agar residues.

Culture Vessels. Culture tubes, 25 × 120 mm, with plastic caps are suitable.

Culture Conditions. All research cultures were maintained for 8 weeks at 25 ± 1°C under 16-h photoperiods of 25 μE m^{-2} s^{-1}. The light source was not described. Standard culture room conditions are also suitable.

Culture Media. Several concentrations of BA and ethephon singly and in combination were added to liquid MS medium (Murashige and Skoog, 1962). Explant responses varied with the cytokinin–ethephon combination. Reasonable results can be obtained with the initial medium (Table Cym-74) suggested here. The original

TABLE CYM-74. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Cymbidium kanran* rhizome explants (Shimasaki, 1995)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Ethylene-generating substance				
10	Ethephon	1.45	145 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin				
11	Benzyladenine (BA)	0.023	2.3 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1N HCl.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH as required, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

report does not recommend a medium for the culture of shoots obtained on the initial substrate. A modified MS medium (Table Cym-75) should prove suitable.

Procedure. Seeds should be germinated on the Hyponex medium described above (under Plant Material) and maintained on the same medium (with transfers if

TABLE CYM-75. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Cymbidium kanran* shoots and possible root induction**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0000.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Napthaleneacetic acid (NAA)	1.0	100–300 mg 100 ml^{-1} 95% ethanol[e,f]	1	Add to induce root formatrion
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1N KOH. Auxin should be added if the shoots fail to form roots.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH as required, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels.

necessary) for 10 months. After that, several rhizomes should be cultured on modified MS (Table Cym-75). Explants from these rhizomes are cultured on the initial medium (Table Cym-74). Shoots that form when the rhizomes are cultured on the initial medium (Table Cym-74) should be moved to the modified MS (Table Cym-75). If the

shoots fail to produce roots, 1 mg NAA l^{-1} should be added to this medium (Table Cym-75) in the hope that this auxin may induce root formation.

Developmental Sequence. The seeds will germinate on the Hyponex medium and form rhizomes. When moved to the modified MS medium (Table Cym-75), rhizomes will produce tips which are excised and cultured on the initial medium (Table Cym-74). Shoots produced on this medium (Table Cym-74) should grow into plantlets on modified MS (Table Cym-75) with or without the addition of NAA. When plantlets are produced they should be moved to horticultural conditions.

General Comments. This procedure was developed as a means of investigating the effects of cytokinins and ethephon on branching and ethylene evolution by rhizome sections, but it can also be used for micropropagation.

Ethylene and Jasmonic Acid Effects on the Organogenesis of *Cymbidium*

Cytokinins and auxins are used to control organogenesis in *Cymbidium* explant culture. The procedure described here is a result of investigations aimed at determining the effects of ethylene and jasmonic acid (Shimasaki, 1996).

Plant Material. As in the previous procedure, the plant material was prepared like this:

> Immature seeds (10 months after pollination) of *C. kanra*n were cultured on modified Kano medium . . . containing 3 g/l Hyponex, 2 g/l Difco bacto-peptone [and probably 20 g sucrose l^{-1}], pH 5.3 at 25 ± 1°C under total darkness for the first 10 months. One of the rhizomes was subcultured for two months to proliferate rhizome branches on the modified MS (Murashige and Skoog, 1962) medium containing 412.5 mg/l ammonium nitrate and 950 mg/l of potassium nitrate, 20 g/l sucrose and 20 g/l gelrite [this seems to be a very high level of Gelrite]. Approximately 10 mm of vegetative shoots formed on apical segments on rhizome branches of proliferated rhizomes cultured on the modified MS medium were used for explants. (Shimasaki, 1996)

Surface Sterilization. Explants taken from in vitro cultures do not require sterilization. However, they should be rinsed with sterile distilled water to remove medium and agar residues.

Culture Vessels. UM culture bottles (Iuchi, Osaka, Japan), of 500-ml capacity, each containing 50 ml of solid medium, were used in the original research. Other containers are also suitable.

Culture Conditions. All research cultures were maintained for 8 weeks at 25 ± 1°C under 16-h photoperiods of 54 μE m^{-2} s^{-1} provided by "white fluorescent light."

Culture Media. Explants should be cultured initially in modified MS medium (Murashige and Skoog, 1962) free of hormones (Table Cym-76), but containing

TABLE CYM-76. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of rhizome tips of *Cymbidium kanran* (Shimasaki, 1995)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	412.5	41.25 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Growth regulator				
10	Jasmonic acid (JA)	0.210	21 mg 100 ml^{-1} 95% ethanol[e]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[f]	To 1000 ml			
	Solidifier				
16	Gelriter[f,g]	20.0 g	No stock	No stock	Weigh. Seems too high

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH as required, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Dissolve the Gelrite in accordance with the instructions in footnote *g*. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), growth regulatgor (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

[g]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave. The amount suggested in the original report seems too high by one order of magnitude; 2 g l^{-1} seems more appropriate. The error may be due to a misplaced decimal point.

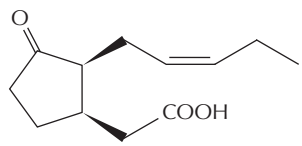
1 μmol jasmonic acid l^{-1}. Shoots obtained on this medium can be cultured on modified MS (Table Cym-75).

Procedure. Seeds of *Cymbidium kanran* should be germinated on the Hyponex medium described above (under Plant Material) and maintained on the same medium (with transfers if necessary) for 10 months. After that, several rhizomes should be cultured on modified MS (Table Cym-75). Explants from these rhizomes are cultured on the initial medium (Table Cym-76). Shoots that form when the rhizomes are cultured on the initial medium (Table Cym-76) should be moved to modified MS (Table Cym-75). If the shoots fail to produce roots, 1 mg NAA l^{-1} should be added to this medium (Table Cym-75) in the hope that this auxin may induce root formation. Shoot cultures of standard *Cymbidium* hybrids (*Cymbidium* Hiroshima Golden Cup 'Sunny Moon') should be cultured on a different initial medium (Table Cym-77). Shoots or plantlets produced by these cultures should be cultured on modified MS (Table Cym-75).

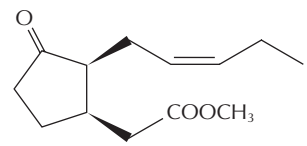
Developmental Sequence. Seeds of *C. kanran* will germinate on the Hyponex medium and form rhizomes. When moved to modified MS medium (Table Cym-75) rhizomes will produce tips which are excised and cultured on the initial medium (Table Cym-76). Shoots and/or PLBs produced on this medium (Table Cym-76) should grow into plantlets on modified MS (Table Cym-75) with or without the addition of NAA. Shoots of *Cymbidium* Hiroshima Golden Cup 'Sunny Moon' form PLBs on the initial medium (Table Cym-77). These PLBs can be expected to form plantlets on the second medium (Table Cym-75).

General Comments. This procedure was developed as a means of investigating the effects of jasmonic acid and ethylene on organogenesis, but it can also be used for micropropagation.

Initially isolated as inhibitors, jasmonic acid (JA) and related substances are now known to be associated with several physiological processes and to function in the defense reactions of plants. Interestingly, JA is structurally similar to the mammalian hormones, prostaglandins. JA was first isolated as a growth inhibitor in 1971 from the fungus *Botryodoploidii theobromae*. Cucurbic acid was first isolated in 1977 from immature pumpkin seeds. It was also considered to be a growth inhibitor. Methyl jasmonate (Me-JA) and JA were found to have growth retarding and senescence promoting characteristics in the early 1980s. The active substances in plants are (+)-7-isojasmonate and (+)-7-jasmonate. In 1989, 12-hydroxyjasmonic acid (12-*O*-β-glucoside of tuberonic acid) was found to be a tuber-inducing factor in potatoes.



(+)-7-isojasmonic acid



Methyl (+)-7-isojasmonate

TABLE CYM-77. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of rhizome tips of *Cymbidium ensifolium* and *Cymbidium kanran* (Ogura and Okubo, 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	412.5	41.25 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[f]	To 1000 ml			
	Solidifier				
15	Agar[f]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Add items 1–7 and 9 to 900 ml of distilled water (item 14), adjust pH as required, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8) and vitamins (items 10–12) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

Shoot Formation from Rhizome Apices of *Cymbidium ensifolium* and *Cymbidium kanran*

Temperate *Cymbidium* species are difficult to germinate and propagate in vitro. An in vitro method for shoot formation from apical segments was developed at the Laboratory of Horticultural Science, Division of Agricultural Botany, Department of

Plant Resources, Faculty of Agriculture, Kyushu University, Fujuoka 812-8581, Japan (Ogura and Okubo, 2003).

Plant Material. Apical segments, 5 and 10 mm long, of rhizomes were taken from plants of *Cymbidium ensifolium* and *Cymbidium kanran* growing in vitro on MS medium (Murashige and Skoog, 1962).

Surface Sterilization. Explants taken from plants in vitro do not require surface sterilization. However, they should be rinsed with sterile distilled water to remove medium residues.

Culture Vessels. Erlenmeyer flasks, 100-ml capacity, containing 30 ml medium were used in the original research. Other containers are also suitable.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under continuous illumination of 44.73 μmol m^{-2} s^{-1} or standard laboratory conditions.

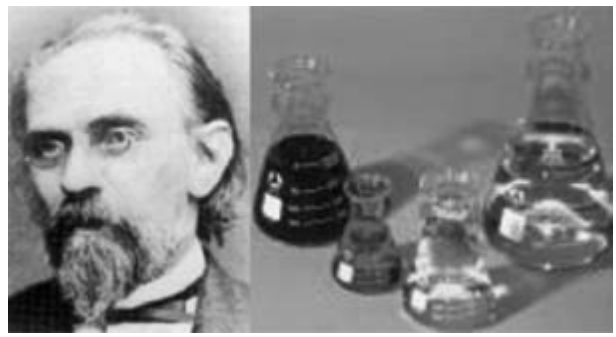
Culture Media. The original paper is not entirely clear regarding the use and combinations of media and species. However it seems that shoot formation in both species occurred on MS (Table Cym-77) with reduced levels of ammonium nitrate (25% of standard MS) and potassium nitrate (50% of basal). It would seem that, as in the preceding two procedures, the newly produced shoot could be cultured on MS (Table Cym-75).

Procedure. Rhizome segments from in vitro plants should be cultured on standard MS (the medium in Table Cym-75 is suitable) for 3–4 months to produce explant sources. Shoot tips are taken from these sources and cultured on the reduced-nitrogen medium (Table Cym-77) for 1–2 months and then transferred to MS (Table Cym-75) for further growth.

Developmental Sequence. The rhizomes grow on MS and produce tips which can be excised. These excised tips grow shoots on the reduced-nitrogen medium (Table Cym-77). These shoots grow further on MS (Table Cym-75).

General Comments. This is an interesting procedure that can be used to propagate two temperate, terrestrial *Cymbidium* species. However, the original report states

Richard August Carl Emil Erlenmeyer (June 28, 1825 to January 22, 1909) invented the Erlenmeyer flask (right). Sources: photograph, www.ch.tu.de; flasks, www.indigo.com.



only that the plants used as explant sources "have been maintained on our laboratory by subcultures for long years." It is not clear if these plants were produced by seeds or through explant culture.

Cytokinin-induced Flowering of *Cymbidium ensifolium* var. *misericors* in Vitro

There are several reports in the literature of orchid seedlings that flowered spontaneously in vitro (for a review see Chia et al., 1999). *Cymbidium ensifolium* is one of the first orchids to flower in vitro, ca. 1984, after being propagated through tissue culture by Xiong Wang at the Shanghai Plant Physiology Institute. After that, other orchids were brought into flower in vitro following micropropagation (Chia et al., 1999). The most recent report regarding the flowering of callus-derived orchids is that of *Cymbidium ensifolium* var. *misericors* at the National Museum of Natural Science in Taichung, Taiwan and the Institute of Botany, Academia Sinica, Taipei, Taiwan (Chang and Chang, 2003).

Plant Material. Callus-derived rhizomes, 3–5 mm long, were used as explants. See elsewhere in the *Cymbidium* section for a procedure (Chang and Chang, 1998) that produces rhizomes from *Cymbidium ensifolium* var. *misericors* callus.

Surface Sterilization. Explants taken from in vitro cultures do not require surface sterilization. However, they should be rinsed with sterile distilled water to remove medium residues.

Culture Vessels. Photographs in the original report suggest that culture tubes (approximate diameter 3–4 cm) were used. Other containers are also suitable.

Culture Conditions. The research cultures were maintained for 100 days at 25 ± 1°C under 16-h photoperiods of 10 μmol m^{-2} s^{-1} provided by daylight fluorescent lights (FL-30D/29 40W, China Electric Co., Taipei, Taiwan). Standard tissue culture conditions may be suitable, however attention must be paid to the light/dark cycle (16/8 h) because photoperiod length may be critical.

Culture Medium. Half-strength MS medium supplemented with peptone, sodium phosphate (NaH_2PO_4), NAA, TDZ or N^6-(2-isopentenyl) adenine (2iP), and solidified with Gelrite should be used (Table Cym-78).

Procedure. Explants are placed on the medium and cultured for 100 days.

Developmental Sequence. Rhizomes (Fig. Cym-12A) in culture produced gravitropic rhizomes covered with trichomes (Fig. Cym-12B), vegetative buds that contained small and true leaves (Fig. Cym-12C), and inflorescences that had bracts at their nodes and florets at the tips (Fig. Cym-12D). In the presence of cytokinins, the rhizomes produced undersized (Fig. Cym-13A), erect (Fig. Cym-13B), gravitropic (Fig. Cym-13F), and clumped (Fig. Cym-13E) inflorescences, which had normal flowers with, sepals, petals, labella and gynostemia (Fig. Cym-13C, D, G, H).

TABLE CYM-78. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	17.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	Naphthaleneacetic acid (NAA)	0.42	42 mg 100 ml^{-1} 95% ethanol[e,f]	1	Use NAA and TDZ or 2-iP, not NAA plus 2-iP
	Cytokinin				
12	N^6-(2-isopentenyl adenine (2-iP)	4.5	450 mg 100 ml^{-1} 95% ethanol[e,f]	1	Use 2-iP or TDZ, not both
	or thidiazuron (TDZ)	1.5	150 mg 100 ml^{-1} 95% ethanol[e,f]	1	Use TDZ or 2-iP, not both
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
16	Peptone	1.0	No stock	No stock	Weigh
	Sugar				
17	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Gelrite[g]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively. Use NAA and 2-iP or TDZ, not NAA plus 2-iP and TDZ.

[g]Add items 1–8 and 9 to 900 ml of distilled water (item 18), adjust pH as required, add sugar (item 17), and raise volume to 1000 ml with distilled water (item 18). Dissolve the Gelrite (item 19) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved, pour the solution into a 2-l flask and autoclave. Add the amino acid (item 9), hormones (items 11 and 12; use TD or 2-iP, not both), and vitamins (items 13–15) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Solidifier is not added to liquid media.

[h]Gellan gum (Phytagel or Gelrite are available from www.caissonlabs.com or www.sigmaaldrich.com) is a solidifier which produces a firm and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for

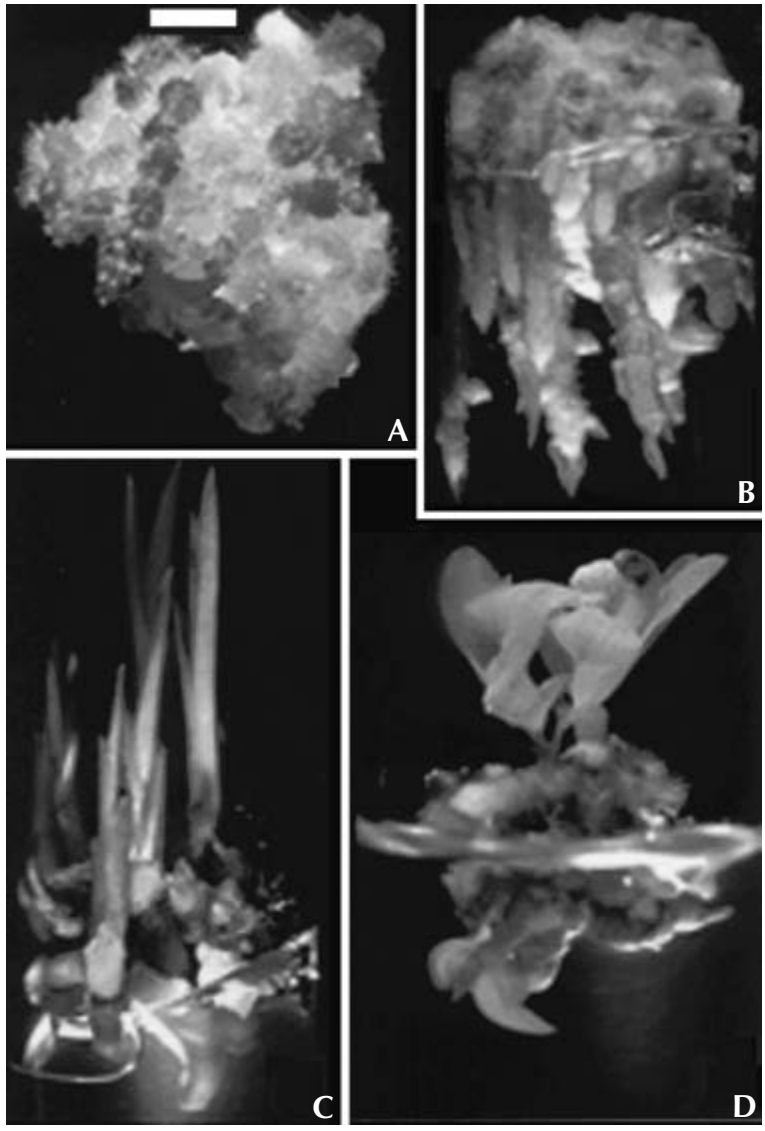


FIG. CYM-12. **Induction of flowering in vitro in *Cymbidium ensifolium* var. *misericors*. A. Rhizome explant (bar = 3 mm). B. Proliferated rhizome after 100 days of culture on hormone-free medium (bar = 6 mm). C. Shoots which formed on medium containing 3.3 µmol TDZ l^{-1} and 1.5 µmol NAA l^{-1} after 100 days of culture (bar = 10 mm). D. Inflorescence which formed on medium containing 10 µmol BA l^{-1} and 1.5 µmol NAA l^{-1}after 100 days in culture (bar = 8 mm). The bar in A refers to all the illustrations, but the relative length is different in each.**

gelation. Dilute media may require higher concentrations of gellan gum or additional calcium or magnesium salts. Gellan gum must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} may have to be used for media which must be very hard or contain low cation levels. After the gellan gum is dissolved the medium is poured into culture vessels and autoclaved.

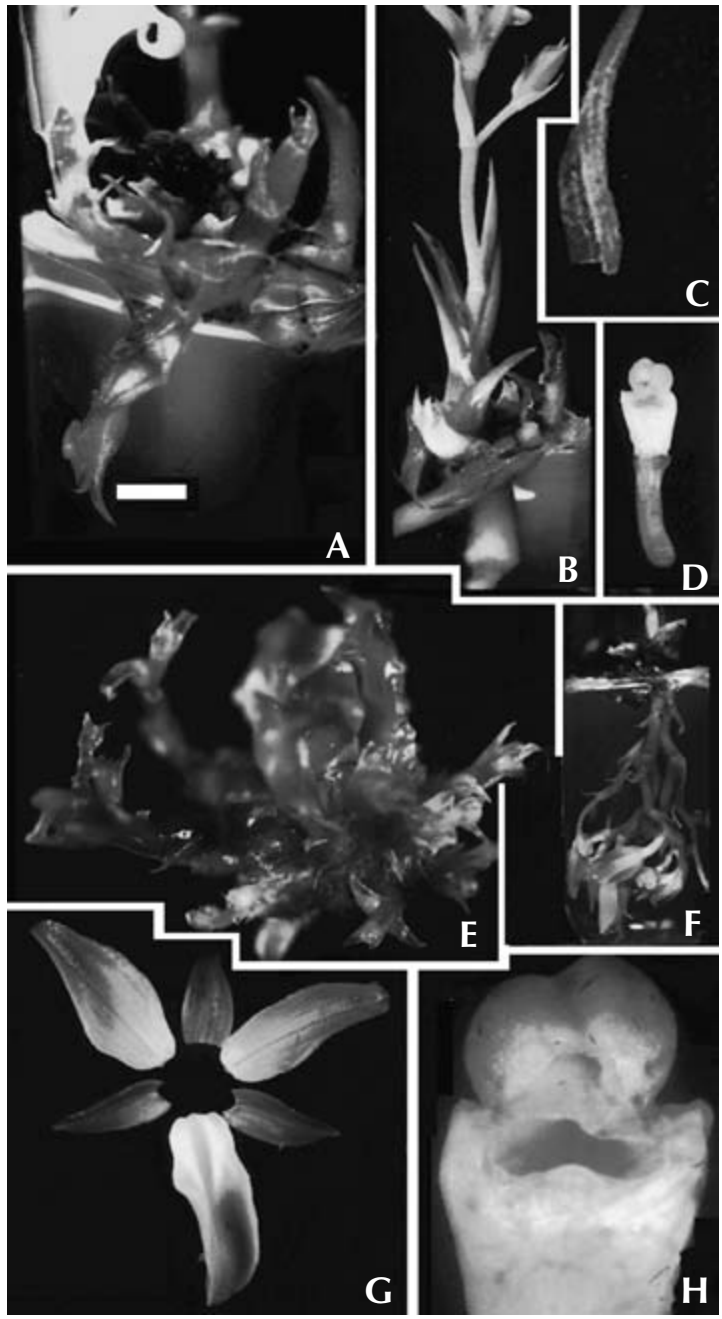


FIG. CYM-13. **Flowering of *Cymbidium ensifolium* var. *misericors* in vitro. A. Undersized inflorescence derived from apex and lateral buds of a rhizome (bar = 3.2 mm). B. Erect raceme inflorescence (bar = 8 mm). C. Flower segment (bar = 3 mm). D. Column and ovary (bar = 3 mm). E. Inflorescence clumps (bar = 4 mm). F. Inflorescence (bar = 4 mm). G. Sepals and petals (bar = 3 mm). H. Column with anther and stigma (bar = 0.7 mm). The bar in A refers to all the illustrations, but the relative length is different in each.**

General Comments. This is an interesting and unique method for flower induction in vitro. The authors should be commended for providing complete details, which should make it possible for others to repeat the procedure and perhaps even apply it to other cymbidiums or orchids. This approach stands in marked and commendable contrast to the bizarre content of a previous paper on the induction of *Dendrobium* flowers in vitro (Goh, 1996) and instructions by Professor Goh that no one should be allowed to read two honors theses by his students (Ng, 1997, being one of them).

Eradication of Odontoglossum Ring Spot (ORSV) and *Cymbidium* Mosaic (CyMV) Viruses through Chemotherapy in Vitro

Infections of *Cymbidium* plants with CyMV and ORSV have been of major economic importance for a long time. An eradication method using Virazole was developed nearly two decades ago (Albouy et al., 1988).

Plant Material. Apices were excised from "a Cymbidium [sic] miniature hybrid."

Surface Sterilization. The original report presents no information regarding surface sterilization. Methods for other *Cymbidium* explants (Wimber, 1963, 1965; Sagawa et al., 1966) are suitable.

Culture Vessels. Culture tubes, 20 × 120 mm, were used in the original research. They should be filled to 20–30% of their volume with medium. Other containers are also suitable.

Culture Conditions. Cultures should be maintained at 25°C during light periods and 20°C in the dark under 16-h photoperiods of 3500–6000 lx. Sources of illumination were not described. Standard culture room conditions are also suitable.

Culture Media. "The basic medium is that of the producer (Ets Vacherot Lecoufle) solidified with Gr (8 g/l): M[edium] I with 2 g active charcoal and M[edium] II without, supplemented with 25 or 35 ppm Virazole . . . or (0) for the control. . . ." This information is useless because the content of medium or media used by Vacherot and Lecoufle was never disclosed. They were given a recipe (or recipes) by their good friend Professor Georges Morel long before he published it (them). Appropriate media are probably those used in other procedures (see Tables Cym-1 to Cym-4). Thus:

1. M-I will be one of these media (see Tables Cym-1 to Cym-4), supplemented with 2 g active charcoal (AC) l^{-1}.
2. M-II will be one of these media (see Tables Cym-1 to Cym-4), supplemented with 25 or 35 ppm Virazole.
3. M-III will be one of these media (see Tables Cym-1 to Cym-4), without AC or Virazole.
4. M-IV will be one of these media (see Tables Cym-1 to Cym-4), plus 0.1 mg IAA l^{-1}, 0.01 mg BA l^{-1}, 20 g sucrose l^{-1}, and 2 g AC l^{-1}. This medium should be used for the culture of PLBs.

5 M-V will be one of these media (see Tables Cym-1 to Cym-4), plus 1 mg IAA l^{-1}, 0.1 mg BA l^{-1}, and 20 g sucrose l^{-1}. This medium should be used for rooting plantlets.
6 M-VI will be one of these media (see Tables Cym-1 to Cym-4), plus 1 mg IAA l^{-1}, 0.1 mg BA l^{-1}, 1 mg gibberellin (GA_3) l^{-1}, 5 g sucrose l^{-1}, and 3 g AC l^{-1}. This medium should be used for the culture of plantlets.
7 M-VII will be one of these media (see Tables Cym-1 to Cym-4), plus 25 ppm Virazole. This medium should be used to culture protocorms for the purpose of freeing them from virus.

Procedure. PLBs produced by the explants (the original paper refers to them mistakenly as protocorms) should be subcultured five times on M-IV, but after 2–3 transplants they should be moved to a Virazole-free medium. The original report is not very clear regarding these steps: "In each assay new protocorms [sic] were transplanted 5 times to the same fresh medium, but after 2–3 subcultures they were transferred to a virazole free-medium for short time . . . Each subculture was 18 . . . or 30 days old. . . . For inducing the formation of shoots and roots we planted protocorms [sic] on modified medium [presumably M-V or M-VI]." The abstract of the report states that "After 5 subcultures of 18 days in presence of 25 ppm Virazole [presumably on M-VII] we obtained 95% virus free plantlets." Altogether the following seems to be a good procedure (or the best one that can be extracted from a relatively unclear paper):

1 Explants should be taken and cultured on an appropriate medium (see Tables Cym-1 to Cym-4) to produced PLBs.
2 PLBs should be cultured on medium M-IV 2–5 times.
3 PLBs from M-IV should be subcultured five times for 18 days each time on medium M-VII to free them from virus.
4 After culture on M-VII, PLBs should be moved to media M-V and/or M-VI to produce rooted plantlets.

Plantlets presumed to be virus-free should be assayed for virus. These assays are neither simple nor easy. Kits and testing are available from www.agdia.com and http://plantpath.osu.edu/cweppdc/plantdis.html#tests. Virus-free plantlets should be planted in potting mix in a greenhouse. Infected plantlets should be destroyed.

Developmental Sequence. Explants form PLBs on the initial medium (see Tables Cym-1 to Cym-4). These PLBs will presumably multiply on the second medium (M-IV). The PLBs will (hopefully) be freed of virus on medium M-VII. On transfer to media M-V and/or M-VI, the PLBs can be expected to produce rooted plantlets.

General Comments. This is an older procedure which may work. Newer procedures may be more effective. As in the newer procedures, the most difficult part will be the virus assays.

Cypripedium

Conrad Gesner (March 26, 1516 to December 13, 1565), who was honored with the title Plinius Germanicus (Wehner et al., 2002), was the first to describe a *Cypripedium* in the European botanical literature in his *Horti Germaniae* which was published in 1561. He also included a drawing of *Cypripedium calceolus* in his *Opera Botanica* which lay forgotten for 200 years and was published in 1751 (Wehner et al., 2002). And, a *C. calceolus* painting was among the many Gesner paintings which were discovered in 1929 at Erlangen University and published in his *Historia Plantarum* between 1972 and 1980 (Wehner et al., 2002).

The name *Cypripedium* was introduced by Carolus Linnaeus (1707–1778) in 1737 and for many years after that all "slipper orchids" or "slippers" were included in this genus. Species which are included in *Paphiopedilum* at present were removed from *Cypripedium* by Ernst Pfitzer (1846–1906) between 1886 and 1894. The taxonomic treatment of the slipper orchids was completed by Robert Alan Rolfe (1855–1921) in 1896.

The genus *Cypripedium* consists of approximately 45 species which are found in North America down to Mexico, temperate areas in northern Asia, Europe, and Japan (for a review of the genus see Cribb, 1997).

Cypripedium species are not as easy to grow as *Paphiopedilum*. Their seeds are more difficult to germinate in vitro. Their explants are often recalcitrant in tissue culture.

Plantlet Production from Root Tips of *Cypripedium calceolus*

A very attractive orchid, *Cypripedium calceolus* is an endangered species due to over-collection and loss of habitat. A micropropagation method was developed as part of cooperative research between Hirosaki University in Japan and the Royal Botanic Gardens, Kew in the UK (Tomita and Ramsay, 1998). Unfortunately the only description of the procedure is in an abstract.

Plant Material. Seedlings of *C. calceolus* were removed from aseptic culture and stored in (non-sterile) sealed vinyl bags in the dark at 5°C for approximately 3 months. Root tips, 5–10 mm long, taken from these seedlings are used as explants.

Surface Sterilization. Prior to taking explant seedlings from the vinyl bags they should be surface sterilized by immersing them in saturated calcium hypochlorite for 20 min and then washed three times with sterile distilled water. Surface sterilization can (and probably should) be avoided, either by storing the young plantlets in sterile containers or by taking explants from seedlings that are still in aseptic culture and maintained either under standard culture conditions or stored at 5°C. The sterilant is prepared by suspending 7 g of calcium hypochlorite [$CaCl_2O_2$ or $Ca(ClO)_2$] in 100 ml of water with vigorous stirring for 2–3 min, allowing the solution to stand until the precipitate settles, stirring again, and filtering or decanting the yellowish fluid which must be used within 12 h. This sterilant, known as Wilson's calcium hypochlorite

solution, was formulated in the laboratory of Professor Lewis Knudson (the Cornell University botanist who discovered the asymbiotic germination of orchid seeds) by one of his students (Wilson, 1915) who left botany to teach tennis (personal communication in the late 1980s by the late Giltner J. Knudson, Prof. Knudson's younger son).

Culture Vessels. Erlenmeyer flasks, test tubes or other containers are suitable. They should be filled with medium to about 20–30% of their volume.

Culture Conditions. The original paper is not very clear on this point. Cultures should be kept in the dark at 20°C at least at first. When callus is formed the cultures can probably be moved gradually to the light and/or standard culture room conditions.

Culture Media. Half-strength MS medium (Murashige and Skoog, 1962) containing 20 g sucrose l^{-1}, 0, 10 or 50 mg picloram l^{-1} (there is no recommendation for a specific amount), and 1 or 10 mg BA l^{-1} (an optimal level is not indicated), adjusted to pH 5.5 before autoclaving and solidified with 7 g agar l^{-1} or 3 g gellan gum (available as that from www.caissonlabs.com and sold as Phytagel by www.sigmaaldrich.com) l^{-1}, should be used as the initial substrate (Table Cyp-1). Shoots and plantlets should be moved to gellan gum (Phytagel)-solidified hormone-free half-strength MS (Table Cyp-2) after the initial 22 weeks.

Procedure. It is not clear if storing the seedlings in the dark at 5°C is a requirement for success in culture or is performed as a matter of convenience. Therefore it is advisable to start by taking seedlings from cultures and storing them as suggested before excising explants, but also by culturing root tips from seedlings which have not been removed from their original cultures. Root tips in culture should be kept in the dark at 20°C until callus is formed. After that the callus cultures should be moved gradually to light and standard laboratory conditions.

Developmental Sequence. Callus is formed by the root tips after 4 weeks of culture on the first medium (Table Cyp-1). Shoots develop after 16 weeks. Some shoots form roots after longer periods. Roots appear on most shoots after an additional 4 weeks of culture on the second medium (Table Cyp-2).

General Comments. These findings suggest that it may be possible to develop protocols for the micropropagation of mature plants of *C. calceolus*. However if root tips of mature plants are used it will be necessary to control or avoid the mycorrhizal fungi which are always present in orchid roots. The authors of this procedure suggest that picloram is preferable to 2,4-D as an auxin in the first culture medium because "it is effective at lower concentrations than 2,4-D and thus less likely to cause genetic changes." This suggestion may be valid. However, it is also possible that a lower level of picloram is needed because it is a more active analog of auxin. If so this compound could also cause mutations. In any case, mutagenicity is not necessarily and/or always correlated with effectiveness as an auxin and/or concentration.

TABLE CYP-1. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for culture of root tips of *Cypripedium calceolus* (Tomita and Ramsay, 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin analog				
10	Picloram	10.0 or 50	1000 or 5000 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	1.0 or 10	10 or 100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	7.0 g	No stock	No stock	Weigh
	or gellan gum (Phytagel)	3.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin analog or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. Gellan gum (Phytagel) must be added to water at room temperature slowly with rapid stirring to eliminate lumps before the medium is heated. If gellan gum (Phytagel) is added to warm or hot medium it will form clumps and fail to gel properly after autoclaving. Typical concentrations are 1.5–2.5 g l^{-1} which means that the amount used in this medium is slightly higher than usual. When the agar or gellan gum (Phytagel) is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10, 11) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels.

TABLE CYP-2. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for culture of root-tip-derived callus, shoots, and plantlets of *Cypripedium calceolus* (Tomita and Ramsay, 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[f]	To 1000 ml			
	Solidifier				
15	Agar[f]	7.0 g	No stock	No stock	Weigh
	or gellan gum (Phytagel)	3.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Add items 1–7 and 9 to 900 ml distilled water (item 14), adjust pH to 5.5, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. Gellan gum (Phytagel) must be added to room temperature water slowly with rapid stirring to eliminate lumps before the medium is heated. If gellan gum is added to warm or hot medium it will form clumps and fail to gel properly after autoclaving. Typical concentrations are 1.5–2.5 g l^{-1} which means that the amount used in this medium is slightly higher than usual. When the agar or gellan gum is completely dissolved pour the solution into culture vessels and autoclave.

Production of Plants from Seed-derived Callus of *Cypripedium macranthos* var. *taiwanianum*

Like many other orchid species *Cypripedium macranthos* has a checkered nomenclatural history that is complicated by a wide distribution (China, Japan, Korea, European and Asiatic Russia, including Kamchatka, the Kuril Islands, Sakhalin Island, and Siberia, as well as Taiwan) and some variability, both of which invited tinkering for nearly a quarter of a millennium by taxonomists of several nationalities. As a result there are two specific epithets (*Cypripedium macranthum* and *Cypripedium macranthos*) and several varieties. The species is a terrestrial herb which can be 15–40 cm tall. It produces attractive purple to pink flowers (Cribb, 1997). This species is currently endangered due to habitat loss and overcollection (Golovanov et al., 1988). Plant production from seed-derived callus of *C. macranthos* Swartz var. *taiwanianum* (Masamune) F. Maekawa *hort.* was studied by the husband and wife team of Masanori and Mina Tomita "to obtain basic information for the micropropagation of and future breeding of endangered *Cypripedium* species" (Tomita and Tomita, 1997).

Plant Material. Unripe capsules were harvested 7 weeks after pollination and the immature seeds were placed on a medium which favors callus induction. Callus masses should be moved to a second medium for plantlet production.

Surface Sterilization. No details beyond "standard method" are given in the original paper, but the steps recommended below are appropriate. The capsule should first be washed with tap water and a household detergent. Brushing with a soft (used) toothbrush is advisable. Following a rinse with tap water the capsule should be soaked in 70% ethanol (73–74 ml 95% ethyl alcohol diluted to 100 ml with distilled water) for 30–60 s (it does not matter if the alcohol kills the outer cell layers of the capsule because the seeds are inside, but it is important to make sure that there are no cracks in the fruit which will allow water or sterilants to reach the interior of the fruit). The next step must be several rinses with sterile distilled water. After that flame the capsule for 1–2 s in an alcohol flame. The flamed fruits should then be soaked for 10–20 min in a 10% dilution of household bleach (10 ml household bleach diluted to 100 ml with distilled water) and rinsed 2–3 times with sterile distilled water. An additional dip in 70% ethyl alcohol for 10–20 s followed by 2–3 rinses with sterile distilled water may be advisable, but are not necessary.

Scalpels and other tools that will be used to split the capsule and place the seeds on the culture medium must be sterilized by autoclaving or dipping them in alcohol and/or flaming. While working these tools should be dipped in alcohol and/or flamed often.

Culture Vessels. Test tubes, 25×150 mm, containing 20 ml of medium, are suitable for seed germination. Erlenmeyer flasks, 125, 250, or 500 ml, containing 25, 50, or 100 ml of medium respectively can be use to culture callus or produce plantlets. Other containers can also be used.

Culture Conditions. Seed cultures should be placed in the dark at 20°C. Conditions for the culture of callus are not indicated, but standard culture rooms should prove suitable after a short period at 20–25°C in the dark. Cultures should be placed under standard culture room conditions for plantlet production, growth, and development.

Culture Media. T medium (Tsutsui and Tomita, 1990) supplemented with yeast extract should be used for seed germination (Table Cyp-3). The same medium containing NAA and BA is used for plantlet induction (Table Cyp-4). Plantlets should be cultured first in hormone-free T medium (Table Cyp-3) and then transferred to sterile sand irrigated with the inorganic components of the T medium (Table Cyp-5).

TABLE CYP-3. **Tsutsui and Tomita (T) medium (Tsutsui and Tomita, 1990) for immature seed germinination and callus induction in *Cypripedium macranthos* var. *taiwanianum* (Tomita and Tomita, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	120.0	12.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	470.0	47.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	245.0	24.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	200.0	20.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	545.0	54.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50.0 mg l^{-1}		
	Complex additive				
8	Yeast extract	200.0	No stock	No stock	Weigh
	Sugar				
9	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier				
11	Gellan gum (Phytagel)[e]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Add items 1–8 to 900 ml of distilled water (item 10), adjust pH to 5.5, add sugar (item 9), and raise volume to 1000 ml with distilled water (item 10). Gellan gum (available as such from www.caissonlabs.com and as Phytagel from www.sigmaaldrich.com) is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Phytagel (gellan gum; item 11) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel) has dissolved pour the medium into culture vessels and autoclave.

TABLE CYP-4. **Tsutsui and Tomita (T) medium (Tsutsui and Tomita, 1990) for plantlet production from seed-derived callus of *Cypripedium macranthos* var. *taiwanianum* (Tomita and Tomita, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	120.0	12.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	470.0	47.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	245.0	24.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	200.0	20.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	545.0	54.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	0.5	50.0 mg l^{-1}		
	Auxin				
8	Naphthaleneacetic acid (NAA)	1.9	190 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin				
9	Benzylaminopurine (BAP)	0.26	26 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
10	Yeast extract	200.0	No stock	No stock	Weigh
	Sugar				
11	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[f]	To 1000 ml			
	Solidifier				
13	Gellan gum (Phytagel)[f]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]If the auxin or cytokinin fails to dissolve add a few drops of 0.1N NaOH or 0.1N KOH, or 0.1N HCl respectively. Keep frozen between uses.

[f]Add items 1–8 to 900 ml of distilled water (item 12), adjust pH to 5.5, add sugar (item 11), and raise volume to 1000 ml with distilled water (item 12). Gellan gum (available as such from www.caissonlabs.com and as Phytagel from www.sigmaaldrich.com) is an agar substitute (item 13) which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Phytagel (gellan gum) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into a 2-l Erlenmeyer flask and autoclave. Following the autoclaving use a sterile pipette to add the auxin to the medium while it is still liquid and warm, but no longer hot. Swirl or stir the medium with a sterile stirrer to mix the auxin thoroughly and pour the medium into presterilized culture vessels. Solidifier is not added to liquid media.

Procedure. The seeds must be sown on the first medium (Table Cyp-3) as soon as the unripe fruits have been harvested. After 16 weeks (ca. 4 months) of culture callus masses can be subcultured onto the same medium (Table Cyp-3) every 4 weeks (about a month) for 60 weeks (1 year and 2 months). To produce plants, callus masses should be cultured on the NAA- and BA-containing medium (Table Cyp-4). When

TABLE CYP-5. **Basal Tsutsui and Tomita (T) medium (Tsutsui and Tomita, 1990) for irrigating plantlets of *Cypripedium macranthos* var. *taiwanianum* cultured in sand (Tomita and Tomita, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	120.0	12.0 g l^{-1}	10	
2	Calcium nitrate $Ca(NO_3)_2 \cdot 4H_2O$	470.0	47.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	245.0	24.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	200.0	20.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	545.0	54.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50.0 mg l^{-1}		
	Solvent				
8	Water, distilled[e]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Add the macroelements (items 1–5), chelated iron (item 6), and microelements (item 7) to 750 ml of distilled water (item 8). Adjust pH to 5.5 and bring volume to 1000 ml (1 l) with distilled water (item 8).

plants are formed they should be cultured on the NAA- and BA-containing medium for 8 weeks and moved to a hormone-free substrate (Table Cyp-3). After 16 weeks on this medium the plantlets can be transferred to sand culture (Table Cyp-5). While the plants are on the sand medium they should be vernalized for 12 weeks at 5°C.

Developmental Sequence. Seed germination takes place on the first medium (Table Cyp-3). Some of the seeds form callus masses on this medium. These masses produce more callus on being subcultured on the same solution (Table Cyp-3). When cultured on the second medium (Table Cyp-4) the callus produces plantlets which develop after being moved to the first substrate (Table Cyp-3). The plantlets develop further on the sand medium and after they are vernalized under low temperature (5°C for 12 weeks).

General Comments. This is not a micropropagation procedure in the very strict sense because the callus masses are produced by seeds. Nevertheless it is presented here because it could serve as a starting point for an actual micropropagation protocol.

Micropropagation of *Cypripedium montanum* through Node Culture

North American *Cypripedium* species are no less beautiful than other members of the genus, but they have not received the attention they deserve as horticultural plants and in the laboratory. A micropropagation method using nodes from axenically cultured plants was developed at Hiroshima University in Japan (Hoshi et al., 1994).

Plant Material. Nodes were taken from more than 1-year-old seedlings grown in vitro obtained from Green Lab Co. (Nagano, Japan). No details are provided regarding the manner of cutting and size of explants.

Surface Sterilization. Explants taken from seedlings growing in vitro do not require surface sterilization.

Culture Vessels. Erlenmeyer flasks, 100-ml capacity, containing 30 ml of medium, were used in the original research. Other containers are also suitable.

Culture Conditions. The original cultures were maintained at 22°C under 16-h photoperiods of 400–800 lx provided by fluorescent lamps (type not indicated). Standard culture room conditions are also suitable.

Culture Media. No information was presented regarding the medium or media used to germinate seeds and/or maintain seedlings of *C. montanum*. Nodes were cultured on MS medium (Murashige and Skoog, 1962) containing 0.2 mg NAA l^{-1} and 2 mg BA l^{-1} (Table Cyp-6).

Procedure. Explants were cultured but no attempt was made to take the resulting plantlets out of the flasks and grow them.

Developmental Sequence. Explants produced well-branched rhizomes with 20 or more shoots (Fig. Cyp-1). "When shoot tips were cut and meristems were then removed, forthcoming interposes became shorter and thicker, producing several knob-like shoots which were subsequently divided and transplanted individually into different culture flasks."

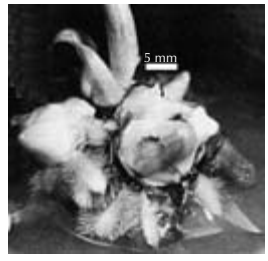


FIG. CYP-1. **Subculture, 30-day-old, of *Cypripedium montanum* node. Multiple shoots formed within 10 days (Hoshi et al., 1994).**

TABLE CYP-6. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Cypripedium montanum* nodes (Hoshi et al., 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.2	20 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH as required, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

General Comments. The authors of this procedure put this best: "The node culture may be a promising method for the micropropagation of *C. montanum*, however, it remains to be determined whether or not in vitro-grown seedlings [sic, the correct word here is plantlets] derived from this nodal technique can survive transfer to septic conditions."

Micropropagation of *Cypripedium yatabeanum* through Root-tip Culture

The Japanese botanist Tomitaro Makino (1862–1957) named *Cypripedium yatabeanum* in honor of Dr. Ryokichi Yatabe (1851–1899), who was the first professor of biology at Tokyo University. He is credited with discovering the species (Luer, 1975). It is found in the Aleutian Islands, Alaska, Japan, Kamchatka Peninsula, Kodiak Island, Kurile Islands, Sakhalin Island, and Siberia. In 1903 E. Pfitzer reduced *C. yatabeanum* to varietal status under *Cypripedium guttatum* and many taxonomists have treated it as such. Others retained the original name which will be used here because the report on the culture of its root tips uses it (Jo et al., 2001). The method was developed to facilitate conservation and meet commercial demands.

Plant Material. Root tips, 10 mm long, were excised from 30- to 40-mm-tall seedlings grown on a medium (Table Cyp-7) designated as JA (this medium is not named by/for Joseph Arditti; the designation "JA" is by Jo et al., 2001 for reasons of their own) for 17 months.

Surface Sterilization. There is no need to surface sterilize explants taken from seedlings which are growing in vitro. However they must be rinsed with sterile distilled water to remove medium residues.

Culture Vessels. Erlenmeyer flasks are most suitable for suspension cultures on shakers. Plastic or other containers can be used for solid cultures.

Culture Conditions. Cultures for callus inductions should be maintained at 23 ± 1°C in the dark. The same conditions should be used for suspension cultures which must be shaken at 90 rpm (there is no indication if the shaking must be rotatory or oscillatory). There is no information regarding culture conditions for what the paper calls "regeneration of the callus" and production of adventitious roots and shoots. A possible assumption is that these processes can take place under the same temperature, but may require illumination.

Culture Media. Seedlings should be grown on the JA (see note above about the "JA" name) medium (Table Cyp-7). "Adventitious roots and shoots could be regenerated from callus" on the same medium. Callus is induced on the B5 medium (Table Cyp-8) containing 1 mg l^{-1} each of 2,4-D and BA. Callus should be proliferated on liquid medium KM81 (Table Cyp-9) supplemented with 2 mg NAA l^{-1}, 0.2 mg BA l^{-1} and 0.5% polyvinylpyrolidone (PVP) l^{-1}. An unclear step is "regeneration of the callus of *C. yatabeanum* [which] could be induced by culture on B5 medium [Table

TABLE CYP-7. **JA medium (Jo et al., 2001) for culture of seedlings and plantlets of *Cypripedium yatabeanum***

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	60.0	6.0 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	100.0	10.0 g l^{-1}	10	
3	Monobasic potassium phosphate, KH_2PO_4	300.0	30.0 g l^{-1}	10	
4	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
5	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Cobaltous chloride, $CoCl_2$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50.0 mg l^{-1}		
	Amino acid				
6	L-Glutamine	100.0	No stock	No stock	Weigh
	Polyol				
7	*myo*-inositol	100.0	No stock	No stock	Weigh
	Complex additives				
8	Casamino acids	500.0	No stock	No stock	Weigh
9	Yeast extract	500.0	No stock	No stock	Weigh
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[e]	To 1000 ml			
	Solidifier				
12	Agar[e]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 4a) and the iron salt (item 4b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. Kundson's original recipe for this medium does not use chelated iron which is is preferable and therefore suggested here. The concentration is the same as the one used in the Murashige–Skoog medium.

[d]Add all microelements to the same 1 l of water.

[e]Add items 1–9 to 900 ml of distilled water (item 11), adjust pH to 5–5.2, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. The similarity between the initials of Joseph Arditti and the designation "JA" is purely coincidental. The medium was not formulated by Joseph Arditti and it is not named by or for him.

Cyp-10] supplemented with 1 mg l^{-1} IPA [indolepropionic acid, an auxin available from www.sigmaaldrich.com, www.fishersci.com, and www.caissonlabs.com] and 5% PVP [available from the same sources]." What this step could be is multiplication of the callus or induction of meristematic regions.

Procedure. The root tips are cultured first in the B5 medium with 1 mg l^{-1} each of 2,4-D and BA (Table Cyp-8) at 23 ± 1° in the dark. Callus which forms on this medium should be moved to the KM81 liquid medium (Table Cyp-9) on a shaker in the dark

TABLE CYP-8. **B5 medium (Gamborg et al., 1968) modified for callus induction from root-tip explants of *Cypripedium yatabeanum* (Jo et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	134.0	13.4 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 2{\cdot}H_2O$	150.0	15.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	2500.0	250.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4{\cdot}H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.0	300.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2{\cdot}6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}H_2O$	10.0	1.0 g l^{-1}		
(e)	Potassium iodide, KI	0.75	75.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	2.0	200.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
9	2,4-Dichlorophenoxyacetic acid (2,4-D)	1.0	10–100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
10	Benzyladenine (BA)	1.0	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	8.0 g	No stock	No stock	Agar or Phytagel, not both. Weigh one or the other
	or gellan gum (Phytagel)[h]	2.0–3.0	No stock	No stock	

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]Add items 1–8 to 900 ml of distilled water (item 15), adjust pH to 5.2–5.4, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring [see footnote *h* if gellan gum (Phytagel) is used]. The agar can also be added to the cold water which is then brought to a boil and stirred [see footnote *h* if gellan gum (Phytagel) is used]. When the agar or gellan gum is completely dissolved pour the solution into a 2-l flask and autoclave. Add items 9–13 to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels.

[h]Gellan gum (available as such from www.caissonlabs.com and as Phytagel from www.sigmaaldrich.com) is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Phytagel (gellan gum) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave. Or, if it is undesirable to autoclave hormones and vitamins pour the medium into a 2-l Erlenmeyer flask after the gellan gum has dissolved and autoclave. Following the autoclaving use sterile pipettes to add the auxin (item 9), cytokinin (item 10), and vitamins (items 11–13) to the medium while it is still liquid and warm, but no longer hot. Swirl or stir the medium with a sterile stirrer to mix the auxin thoroughly and pour the medium into presterilized culture vessels. Solidifier is not added to liquid media.

TABLE CYP-9. **Kao and Michayluck (KM81) medium (Kao and Michayluk, 1981) modified for proliferation of callus derived from root-tip explants of *Cypripedium yatabeanum* (Jo et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	600.0	60.0 g l^{-1}	10	
2	Ammonium sulfate, $(NH_4)_2SO_4$	67.0	6.7 g l^{-1}	10	
3	Calcium chloride, $CaCl_2 \cdot 2H_2O$	295.0	29.5 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	310.0	31.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	2200.0	220.0 g $^{-1}$	10	
7	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	75.0	7.5 g l^{-1}	10	
8	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
9	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.0	300.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	10.0	10.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.75	75.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.0	200.0 mg l^{-1}		
	Amino acids				
10	Alanine	4.0	400 mg 100 ml^{-1} 70% ethanol[e]	1	
11	Asparagine	8.0	800 mg 100 ml^{-1} 1.5-*M* HCl or NaOH in 70% ethanol[e]	1	Or no stock, weigh (may be hard to dissolve in ethanol). Try both concentrations or even 120 mg l^{-1} medium[f]
12	Glutamine	20.0 or 100	No stock		
13	Glycine	0.8	80 mg 100 ml^{-1} 95% ethanol[e]	1	
	Organic acids				
14	Citric acid	10.0	1 g 100 ml^{-1} 70% ethanol[e]	1	
15	Fumaric acid	10.0	1 g 100 ml^{-1} 70% ethanol[e]	1	
16	Malic acid	10.0	1 g 100 ml^{-1} 70% ethanol[e]	1	
17	Sodium pyruvate	5.0	500 mg 100 ml^{-1} 70% ethanol[e]	1	
	Polyol				
18	*myo*-inositol	500.0	No stock	No stock	Weigh
	Complex additives				
19	Casamino acids	500.0	No stock	No stock	Weigh (source: www.bd.com)
20	NZ-amine	500.0	No stock	No stock	www.sigma-aldrich.com
21	Yeast extract	200.0	No stock	No stock	www.sogma-aldrich.com
	Buffer				
22	Morpholinoethanesulfonic acid (MES), pH 5.8	50.0 ml[g]	See footnote *g*	Footnote *g*	www.sigma-aldrich.com
	Auxin				
23	Naphthaleneacetic acid (NAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,h]	1	
	Cytokinin				
24	Benzyladenine (BA)	0.2	20 mg 100 ml^{-1} 95% ethanol[e,h]	1	

TABLE CYP-9. (***Continued***)

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Vitamins				
25	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
26	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
27	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
28	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
29	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 8a) and the iron salt (item 8b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4{\cdot}4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4{\cdot}7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]The recipe as provided by one of the authors (Jo et al., 2001) is not clear because it lists glutamine in two places, once as 20 ml l^{-1} and a second time as 100 mg l^{-1}. As a rule components are listed only once per recipe.

[g]Dissolve 975 mg morpholinoethanesulfonic acid (MES, available from www.sigmaaldrich.com or www.fishersci.com) in 50 ml distilled water, adjust pH to 5.8 with 0.1 or 1 N NaOH (if pH is below 5.8) or HCl (if pH is above 5.8) and add this solution in its entirety to 1 l of medium.

[h]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[i]Add items 1–27 plus 5 g polyvinylpyrrolidone to 900 ml of distilled water (item 29), check pH and adjust to 5.8 if necessary, add sugar (item 28), and raise volume to 1000 ml with distilled water (item 29). Pour the solution into culture vessels and autoclave. As a rule amino acids (items 10–13), hormones (items 23 and 24), and vitamins (items 25–27) are added to media after autoclaving, but there is no indication that there is a need to do that in this case. Agar is not added to liquid media.

for proliferation. Once the callus is proliferated it is necessary to move it to the medium with IPA and PVP (Table Cyp-10). As indicated above, the original paper is not entirely clear regarding the function of this medium and the purpose of this step. However it seems that the callus mass may increase in size and shoots may be produced on it. After this step sections of the callus should be moved to the JA medium (Table Cyp-7) for plantlet production.

Developmental Sequence. Callus forms on the first version of the B5 medium (Table Cyp-8). It proliferates on medium KM81 (Table Cyp-9). It is not clear what happens on the second B5 medium (Table Cyp-10). Plantlets should form when callus is moved from the second B5 medium (Table Cyp-10) to the JA solution (Table Cyp-7).

General Comments. This is an interesting, potentially useful procedure that could have been even more useful if the paper had been clearer, with more details.

TABLE CYP-10. **B5 medium (Gamborg et al., 1968)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	134.0	13.4 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	150.0	15.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	2500.0	250.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.0	300.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	10.0	1.0 g l^{-1}		
(e)	Potassium iodide, KI	0.75	75.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.0	200.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
9	Indolepropionic acid (IPA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Phenolics absorber				
10	Polyvinylpyrrolidone (PVP)	5.0 g	No stock	No stock	
	Vitamins				
11	Niacin (nicotinic acid)	1.0	10 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.0–5.4, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Some hormones and vitamins are not heat-stable and it is generally advisable to add following autoclaving. There is no need to do that with this medium.

Plant Production from Protocorm-derived Callus of *Cypripedium formosanum*

"Slipper" orchids are still difficult if not impossible to propagate clonally through tissue culture. Methods to the extent that they are formulated use explants from

seedlings for the most part. This is also the case with a method which was developed recently at the Department of Horticulture, National Taiwan University, Taipei, Taiwan (Lee and Lee, 2003). Of the few methods for *Paphiopedilum* which use explants from mature plants, two (Bubeck, 1973; Stewart and Button, 1975) received considerable publicity (one of Ms. Bubeck's mentors was influential in the American Orchid Society whereas Joyce Stewart is an effective international orchid politician), despite being unreliable and not of much use. A third method (Huang, 1988) has not been used extensively despite being reproducible.

Plant Material. Plants of *C. formosanum* cultivated in a greenhouse located 2000 m above sea level were self-pollinated and the capsules were harvested 3 months after pollination. They were surface sterilized with 1% sodium hypochlorite (1.7 ml of Clorox which contains 6% sodium hypochlorite diluted to 100 ml with distilled water) for 20 min, rinsed three times with sterile distilled water, and cut open. The seeds were scooped out and germinated on modified (quarter-strength macroelements, full strength-microelements, and other additives) MS medium (Murashige and Skoog, 1962) solidified with Gelrite (Table Cyp-11). Seed cultures were maintained in the dark at 25 ± 2°C for 12 weeks. Small round protocorms developed during this period. The cultures were then cultured under illumination of 25–30 μmol m^{-2} s^{-1} (duration of photoperiods was not given) produced by daylight fluorescent tubes (FL-20D/18, 20W, China Electric Co., Taipei) for an additional 8 weeks. Following a total of 20 weeks in culture the protocorms were bisected transversely and the sections were used as explants.

Surface Sterilization. Explants taken from in vitro cultures do not require surface sterilization. However, the explants must be rinsed with sterile distilled water to remove culture medium residues.

Culture Vessels. Culture tubes, 25 × 100 mm, containing 10 ml of medium can be used for callus induction, PLB formation, and plantlet production. Other containers are also suitable.

Culture Conditions. All cultures should be maintained at 25 ± 2°C. Those intended to produce callus are kept in the dark. For PLB formation and plantlet production cultures should be maintained under the same intensity of illumination as the seeds and 12-h photoperiods, but the original report also includes the puzzling phrase "or in the dark."

Culture Media. Callus is produced on a modified MS medium (Table Cyp-12) containing 4.52 μmol 2,4-D and 4.54 μmol TDZ. PLBs form on another modification of MS medium (Table Cyp-13) containing 4.44 μmol BA. Plantlets develop on yet another medium (Table Cyp-14).

Procedure. The seeds are germinated and protocorms are cultured on the germination medium (Table Cyp-11) for 12 weeks in the dark. After that these cultures are illuminated for 8 weeks before the protocorms are cut in half transversely. The sections are cultured under illumination on the 2,4-D- and TDZ-containing medium

TABLE CYP-11. **Modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the germination of immature seeds of *Cypripedium formosanum* from green (unripe) 3-month-old fruits (Lee and Lee, 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	412.5	41.25 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	110.0	11.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	97.5	9.75 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	475.0	47.5 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	42.5	4.25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
13	Tryptone[f]	1000.0	No stock	No stock	Weigh
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]The original paper does not list the exact type and source of the tryptone. If available tryptone recommended for plant tissue culture should be used.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.5, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Add the Gelrite (item 16) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved dispense the medium into culture vessels and autoclave. Solidifier is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

TABLE CYP-12. **Modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for callus production from protocorms produced by immature seeds of *Cypripedium formosanum* (Lee and Lee, 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	412.5	41.25 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	110.0	11.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	97.5	9.75 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	475.0	47.5 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	42.5	4.25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Auxin				
13	2,4-Dichlorophenoxyacetic acid (2,4-D)[f]	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin				
14	Thidiazuron (TDZ)[f]	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin fails to dissolve add a few drops of 0.1 N KOH or 0.1 N HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Add the Gelrite (item 17) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved dispense the medium into culture vessels and autoclave. Solidifier is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

TABLE CYP-13. **Modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for protocorm-like body (PLB) production from callus produced by immature seeds of *Cypripedium formosanum* (Lee and Lee, 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	412.5	41.25 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	110.0	11.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	97.5	9.75 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	475.0	47.5 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	42.5	4.25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin				
13	Thidiazuron (TDZ)[f]	1.15	115 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the cytokinin fails to dissolve add a few drops of 0.1 N HCl.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.5, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Add the Gelrite (item 16) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved dispense the medium into culture vessels and autoclave. Solidifier is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

TABLE CYP-14. **Modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for plantlet production from protocorm-like bodies derived from callus generated from immature seeds of *Cypripedium formosanum* (Lee and Lee, 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	412.5	41.25 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	110.0	11.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	97.5	9.75 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	475.0	47.5 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	42.5	4.25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
13	Potato homogenate[f]	20.0	No stock	No stock	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh
	Darkening agent				
17	Activated charcoal[i]	1.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]The original paper does not provide instructions regarding the preparation of potato homogenate. One possibility is to place 20 g of peeled and cubed potatoes in a homogenizer, add 100 ml of distilled water, and homogenize the cubes completely.

[g]Add items 1–13 to 750 ml of distilled water (item 15), adjust pH to 5.5, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Add the Gelrite (item 16) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved dispense and add the charcoal with vigorous stirring to ensure complete dispersion. After that dispense the medium into culture vessels and autoclave. Solidifier is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

[i]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite which are very different in nature should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

(Table Cyp-12) until callus is formed. This callus can be subcultured onto the same medium (Table Cyp-12). Callus sections, 4 mm in diameter, are cultured on the BA medium (Table Cyp-13) to produce PLBs. The PLBs are placed on the last medium for plantlet production.

Developmental Sequence. The seeds germinate and form protocorms during the 12 weeks in the dark. These protocorms increase in size during the subsequent 8 weeks under illumination. Transverse sections taken from the protocorms produce callus (Fig. Cyp-2A). Sections of the callus give rise to PLBs (Fig. Cyp-2B, C). Up to 13 PLBs can be obtained from a callus section, 4 mm in diameter. The PLBs produce plantlets (Fig. Cyp-2D–F).

General Comments. This cannot be used for mass rapid propagation of desirable cultivars because the nature of seedlings is unknown. It can be used to multiply seedlings and as a starting point for experiments in the culture of explants from mature plants.

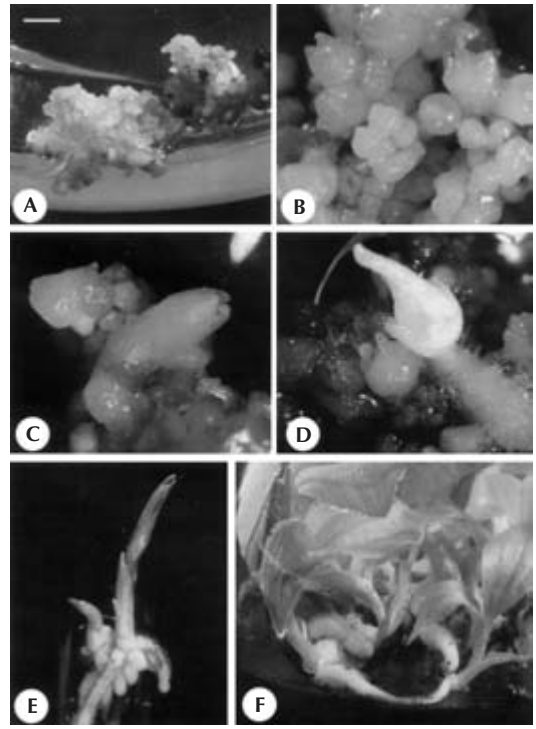


FIG. CYP-2. **Plantlet production from protocorm-derived callus of *Cypripedium formosanum*. A. Callus derived from a protocorm explant. B. PLBs derived from callus. C. Elongated PLB. D. A PLB which developed into a vegetative bud and formed a root. E. Young shoots with roots. F. Plantlets. (Lee and Lee, 2003.)**

Cyrtopodium

Attempts to develop micropropagation procedures using root-tip explants have been made in an effort to reduce the damage or threat to the donor plant. Only a few of these attempts have been successful. One of the more effective ones was developed using roots of *Cyrtopodium* cf. *punctatum*, a species native to the Argentine Chaco (Sanchez, 1988).

Plant Material. The original research was carried out with 2-mm-long root tips taken from 1-year-old seedlings growing axenically on Vacin and Went medium.

Surface Sterilization. Since the explants were taken from axenically grown plants, there was no need for surface sterilization.

Culture Vessels. Test tubes, 20×120 mm and containing 10 ml of medium, were used in the original research. Other culture vessels can also be used.

Culture Conditions. Cultures should be maintained under 16-h photoperiods of 1.8 W m^{-2} and $24 \pm 1°C$.

Culture Medium. Modified Vacin and Went medium (Vacin and Went, 1949) containing NAA and BA (Tables Cyrt-1 and Cyrt-2) should be used.

Procedure. Place explants on the appropriate medium. If necessary, subculture them every 30 days.

Developmental Sequence. After the root tips reach a length of 10 mm, PLBs form on the apical meristems of 20% of the explants. Plantlets develop on these PLBs 1 month after their formation.

General Comments. It is not clear whether this method can be used to culture root tips of mature plants of the same orchid, additional *Cyrtopodium* species, or other orchids. If explants from mature plants are to be used, they should be taken from roots that have not come into contact with the substrate to avoid the presence of mycorrhiza, which will contaminate the cultures. At least one investigator has suggested that orchid plants derived from roots may not fully resemble the parent plant.

TABLE CYRT-1. **Vacin and Went medium (Vacin and Went, 1949) modified for the culture of *Cyrtopodium* root tips, formation of protocorm-like bodies, and plantlet regeneration (Sanchez, 1988)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Peptone	1	No stock	No stock	Weigh
	Vitamin				
9	Thiamine (vitamin B_1)	500 µg	50 mg 100 ml^{-1} 95% ethanol[e]	1	Sugar
	Sugar				
10	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Agar[f]	8 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, stir, and/or heat until it dissolves. Add the other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution of chelated iron dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Keep refrigerated between uses.

[f]Add items 1 and 3–8 to the 500 ml distilled water (item 11) that contains the calcium phosphate (item 2), adjust pH as required, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring solution to a gentle boil, and add agar (item 12) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a gentle boil and stirred. When agar is completely dissolved, pour medium into an appropriate container and autoclave. Add the vitamin (item 9) to the warm, still liquid medium; mix well, and distribute into culture vessels. Omit agar if preparing liquid medium.

TABLE CYRT-2. **Effects of auxin, cytokinin, and coconut water on *Cyrtopodium* root tips in vitro (Sanchez, 1988)**

Medium	Effects
VW[a]	Root tips elongated and formed callus, PLBs,[b] and plantlets
VW + 0.5 ppm NAA + 0.5 ppm BA[b]	Root tips elongated and formed PLBs and plantlets
VW + 1 ppm NAA + 0.5 ppm BA	40% of explants formed roots and whitish basal calli that died
VW + 0.5 ppm NAA + 0.1 ppm BA	Root tips were more elongated on this medium than on other media; 20% formed calli that "showed tissue oxidation"
VW + 1 ppm + 0.1 ppm BA	Root tips elongated but did not form calli
VW + 0.5 ppm NAA + 1 BA	50% of root tips formed fasciated roots and later formed both normal and fasciated ones
VW + 10% CW[c]	30% of explants produced numerous PLBs without callus formation; 60% of this group (i.e., 18% of total) regenerated plantlets

[a]VW, Vacin and Went medium (see Table Cyrt-1). This medium is preferable to the others because it does not contain hormones and other additives that could increase the number of mutations.

[b]BA, benzyladenine (benzylaminopurine); NAA, naphthaleneacetic acid; PLBs, protocorm-like bodies; ppm, parts per million (mg l^{-1}).

[c]CW, coconut water (referred to in the original paper as "milk"), 10% (v/v).

Dactylorchis

Dormant shoots (early fall in England) were excised from *Dactylorchis fuchsii* and disinfected by "immersing briefly in ethanol followed by fifteen minutes in a hypochlorite solution" (Stokes, 1974). (The concentration is not given, but 10 g calcium hypochlorite per 140 ml of water would probably be appropriate.) After rinsing the tissue with sterile distilled water, terminal portions of shoot tips consisting of floral primordia were excised and cultured on "Knudson C and a variant of Reinert and Mohrs' [sic] medium." Details regarding the media are not given. Therefore one can only assume that the original formulation or a modification of the Knudson C medium would be appropriate (see Tables Aranda-7, Aranda-8, C-3, C-6, C-7, C-11, C-19, and Cym-1 to Cym-3). The Reinert–Mohr medium (see Tables C-8 and C-9) contained "autoclaved extracts from parent plants" such as homogenized leaves "filtered to form a leaf extract . . . [and] tubers [similarly treated] to form a salep extract" in concentrations that were not listed. Growth and survival on the modified Reinert–Mohr medium were better than on Knudson C, but it is not clear whether this is due to the basal solution or to the additives.

Cultures were maintained under 16-h photoperiods provided by warm white fluorescent lamps (no details are given on their number, distance from the plants, or light intensity) and 20°C. Bracts turned green 12 days after inoculation and developed into protocorms within 9 weeks. At that time the apices can be divided and subcultured. "If left to enlarge without further division, these protocorms gave rise to plantlets, two to three inches in height, each with a single large tuberous root." (The time required for the plants to reach this stage is not given.) "On transfer to greenhouse conditions, these plants have so far failed to survive; incorporation of soil from the vicinity of the parent stock could well be needed in order to supply the necessary mycorhizal fungi" (Stokes, 1974). Or, one could isolate the fungus and infect the plants in vitro.

On the whole not enough information is available in the original paper (Stokes, 1974) for those who may wish to use this procedure: Too many questions remain unanswered.

Dactylorhiza

An effort was made in Germany to enhance the proliferation of seedling protocorms of *Dactylorhiza maculata* (Gruenschneider, 1973). This cannot be considered a tissue-culture method or clonal propagation in the strict sense since proliferation of seedling protocorms is common in other orchids.

Darwinara

Darwinara is a hybrid genus of a kind that can be produced only on orchids. It is a combination of four genera (listed here in alphabetical order), *Ascocentrum, Neofinetia*, *Rhynchostylis*, and *Vanda*. The flowers can be exquisitely colored and at least one cultivar, *Darwinara* Charm is a delightful miniature. All cultivars are in demand and effective micropropagation methods are of interest for commercial growers. Such a method was developed at the Applied Research Center of the Kirin Brewery Company in Japan (Kishi and Takagi, 1997a).

Plant Material. Immature floral buds of *Darwinara* Pretty Girl were taken from main shoots (10 cm long) and cut into 0.5-mm cubes following sterilization. These cubes were cultured.

Surface Sterilization. Buds should first be washed with 10% (v/v) benzalkonium chloride for 10 min (household detergent and water can probably also be used). The washed buds should be dipped in 70% (v/v) ethanol (74 ml 95% ethanol diluted to 100 ml with distilled water) for 30 s, immersed for 5 min in 0.5% (v/v) sodium hypochorite (8–10 ml Clorox or another household bleach which contains 6.25–5.25% hypochlorite – please check the concentration, it is listed on the bottle – diluted to 100 ml with distilled water), plus 0.05% (v/v; this really amounts to a few drops per 100 ml) Tween 20 (or a mild household detergent), and washed three times with autoclaved (i.e., sterile) distilled water.

Culture Vessels. Test tubes (30 × 200 mm) containing 20 ml of medium were used originally. Other vessels can also be employed.

Culture Conditions. In the original research the cultures were placed on a drum-type rotary shaker tilted 15° and rotating at 1 rpm. A different type of shaker may also be suitable but should be tested with a few explants before being put to large-scale use. Cultures should be maintained at 25 ± 2°C under illumination of 56.2 ± 5.2 μmol m^{-2} s^{-1}. Standard laboratory conditions are also suitable.

Culture Media. Liquid (this is not stated directly in the materials and methods section of the original paper, but is implied by the use of a shaker) one-quarter strength modified MS medium (Murashige and Skoog, 1962) should be used (Table Dar-1) for the production of PLBs. For plantlet production the PLBs should be cultured on modified full-strength MS medium (Table Dar-2).

Procedure. The explants are cultured on the first medium (Table Dar-1). Once PLBs are formed they should be moved to the second medium (Table Dar-2) for plantlet formation.

Developmental Sequence. PLBs form on the first medium (Table Dar-1) and plantlets develop on the second medium (Table Dar-2).

TABLE DAR-1. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the culture of axillary buds of *Brassocattleya* Pastoral 'Innocense' (Kishi and Takagi, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	412.5	41.25 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot H_2O$	110.0	11.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	92.5	9.25 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	475.0	47.5 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	42.5	4.25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	3.73	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelement**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	If necessary[e]
	Cytokinin				
11	Kinetin	1–10.0	10–100 mg 100 ml^{-1} 95% ethanol[e,f]	1	If necessary[e]
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses. There is no indication in the original paper whether auxin and/or cytokinin were used and if so how much. The auxin concentration suggested here is the same as that in Table Bc-1. There is no cytokinin in the medium outlined in Table Bc-1 and none is being suggested here. Should a cytokinin prove to be necessary 1 or 10 mg kinetin l^{-1} medium may be appropriate. These suggestions are speculative. Therefore those who plan to use this procedure are advised to test the medium as described here with a few buds before employing any formulations on a large scale.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or 0.1 N HCl respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.4, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11) if necessary (see footnote *e*) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. It is also possible to add items 1–14 before the addition of sugar, complete preparation of the medium, distribute to culture vessels, and autoclave the mixture.

TABLE DAR-2. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for plantlet production from protocorm-like bodies of *Brassocattleya* Pastoral 'Innocense' (Kishi and Takagi, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indoleacetic acid (IAA)	1.0–3.0	10–300 mg 100 ml^{-1} 95% ethanol[e,f]	1	If necessary[e]
	Cytokinin				
11	Kinetin	0.04–1.0	4–100 mg 100 ml^{-1} 95% ethanol[e,f]		If necessary[e]
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Gelrite[b]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses. There is no indication in the original paper whether auxin and/or cytokinin were used and if so how much. The auxin and cytokinin concentrations suggested here are estimates based on other media. These suggestions are speculative. Therefore those who plan to use this procedure are advised to test the medium as described here with a few protocorm-like bodies before employing any formulations on a large scale. In many instances PLBs form plantlets without the addition of hormones to culture media. Therefore the Knudson C medium (see Tables Aranda-7 and Aranda-8) with or without ripe banana may also prove to be suitable.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.4, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Add the Gelrite in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into a 2 liter flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11) if necessary (see footnote *e*) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar or Gelrite are not added to liquid media. It is also possible to add items 1–14 before the addition of sugar, complete preparation of the medium, distribute to culture vessels, and autoclave the mixture.

General Comments. This procedure will probably also be suitable for other *Darwinara* hybrids.

Maintenance of Embryogenic Callus of *Darwinara*

The method used for *Doritaenopsis* (Ichihashi and Hiraiwa, 1996) is suitable.

Micropropagation of *Darwinara* through the Culture of Flower-bud Explants

The methods developed for *Vandofinetia* (Kishi et al., 1997*a*, 1997*b*) can also be used for *Darwinara* (Fig. Dar-1).

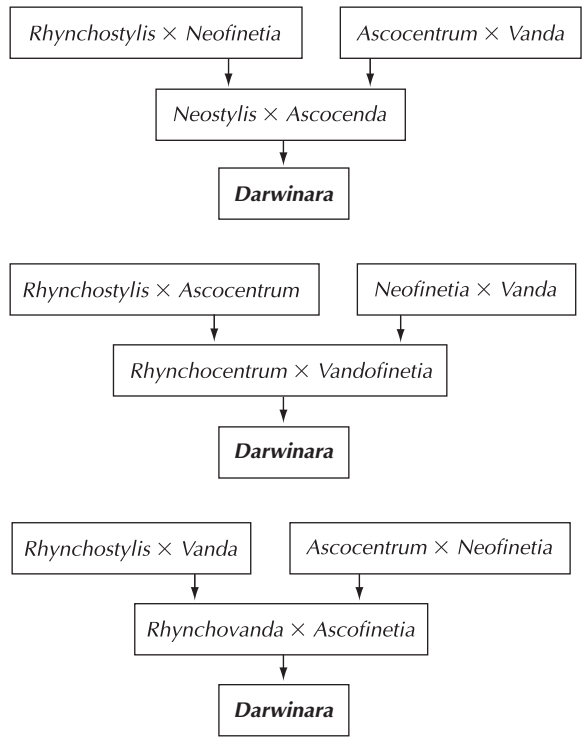


FIG. DAR-1. **Three of the several pathways to *Darwinara*; there are others.**

[h]Gellan gum (available as such from www.caissonlabs.com) and as Phytagel (www.sigmaaldrich.com) or Gelrite is an agar substitute which produces a firm, clear and colorless solid substrate at lower concentrations than agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} may have to be used for media that must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

Dendrobium

Several *Dendrobium* hybrids are very important to the orchid cut-flower industry in a number of countries. The best-known example is *Dendrobium* Pompadour (*Dendrobium* Louis Bleriot × *Dendrobium phalaenopsis*, registered in 1934 by Henri Vacherot-Lecoufle) in Thailand. Another example is *Dendrobium* Jaquelyn Thomas (*Dendrobium gouldii* × *Dendrobium phalaenopsis*, registered in 1949 by R. K. Thomas). It is grown extensively by Flora Sari Orchids (owned by Mr. and Mrs. Soediono) near Jakarta, Indonesia. *Dendrobium* Mary Mak (*Dendrobium* Theodore Takiguchi × *Dendrobium* May Neal, registered by Mak Chin On of Maryland Orchids, Singapore, in 1977) is grown widely in both Indonesia (Flora Sari Orchids) and Singapore (Maryland Orchids). There are other examples.

In addition, *Dendrobium* hybrids are popular with hobby growers. This importance led to the development of several tissue culture procedures for *Dendrobium* explants (Ito, 1966, l967; Sagawa and Shoji, 1967; Marston, 1969; Kim et al., 1970; Mullin, 1970; Singh and Sagawa, 1972; Arditti et al., 1973; Mosich et al., 1973, 1974*a*, 1974*b*; Ball and Arditti, 1976; H. Singh, 1976; Charanasri and Wanichkul, 1978*a*, 1978*b*; Fu 1978, 1979*a*, 1979*b*; Khaw et al., 1978*a*, 1978*b*; Fernando, 1979; Gandawijaja, 1980; Lim-Ho, 1981; Sagawa and Kunisaki, 1982; Kukułczanka and Wojciechowska, 1983; Soediono, 1983*a*, 1983*b*; Manorama et al., 1986). Some of these methods can lead to variations in the resulting "mericlones" (Charanasri and Wanichkul, 1978*a*, 1978*b*). Procedures for protoplast isolation (Teo and Neumann, 1978*a*, 1978*b*, 1978*c*; Price and Earle, 1984) and chromosome doubling (Sanguthai et al., 1973) have also been developed.

The very first effort to culture any part of a *Dendrobium* (except seeds) in vitro by a "partial[ly] sterile" method was by Itsuhiko Ito at the Laboratory of Olericulture, Faculty of Agriculture, Kyoto Prefectural University, Japan (Ito, 1966, 1967). The procedure was not used for clonal propagation, but fertile seeds were produced from pollinated ovaries cultured in test tubes.

Shoot-tip Culture of *Dendrobium*

A tissue culture procedure for clonal propagation of *Dendrobium* was developed at the University of Hawaii (Sagawa and Shoji, 1967; Kim et al., 1970; Sagawa and Kunisaki, 1982).

Plant Material. Explants are obtained from new growths, and the highest percentage of success has been with axillary buds weighing 2 g (range 1.1–3.0 g) and 4.9 cm long (range 3.7–11.5 cm) (Table Den-1).

Surface Sterilization. The procedure employed with *Cymbidium* (Sagawa et al., 1966) is used, but the step involving 1% Clorox is omitted.

Culture Vessels. The vessels used for *Cymbidium* (Sagawa et al., 1966) are used.

TABLE DEN-1. **Characteristics and success of cultured *Dendrobium* buds (Kim et al., 1970)**

Bud characteristics	Size and mass					
Length, cm:						
Average	3.0	4.9	8.2	10.4	15.5	
Range	2.5–3.5	3.7–11.5	5.5–14.0	6.5–15.5	9.5–20.0	
Weight, g:						
Average	<1.0	2.0	4.0	6.0	>7.0	
Range	1.0	1.1–3.0	3.1–5.0	5.1–7.0	7.1	
Location	**Success**					**Total**
Apical:						
Percent	12.5	46.6	36.4	33.3	17.5	31.5
Sc/nc[a]	1/8	7/15	4/11	4/12	1/8	17/54
Axillary:						
Percent	14.3	50.9	38.8	33.3	40.6	38.6
Sc/nc[a]	3/21	28/55	19/49	15/45	13/32	78/202

[a]Number of successful cultures/total number of cultures.

TABLE DEN-2. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of *Dendrobium* shoot tips (Sagawa et al., 1966)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Tricalcium phosphate, $Ca_3(PO_4)_2$[b]	200.0	20.0 g l^{-1}	10	Or weigh
2	Potassium nitrate, KNO_3[c]	525.0	52.5 g l^{-1}	10	Or weigh
3	Monopotassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
5	Ammonium sulfate, $(NH_4)_2SO_4$[c]	500.0	50.0 g l^{-1}	10	Or weigh
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3$[d]	28.0	2.8 g l^{-1}	10	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier[e]				
10	Agar	16.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]If this salt does not completely dissolve, shake stock solution well before dispensing to insure an even suspension; better yet, weigh out each time.

[c]Solutions containing nitrate and/or ammonium may become contaminated with time. Therefore it is preferable not to make stock solutions. If prepared, keep frozen between uses.

[d]If the substance fails to dissolve, add dilute KOH one drop at a time until the solution is clear.

[e]Mix items 1–7 with 800 ml distilled water (item 9), adjust pH to 5.0–5.5, add sugar (item 8), and adjust volume to 1000 ml with more distilled water. To add agar (item 10), bring solution to a slow boil and add slowly while stirring. When fully dissolved, dispense into culture vessels and autoclave. For liquid medium, omit agar (item 10).

Culture Conditions. Cultures should be maintained under 2000 ft-c of continuous illumination provided by General Electric white fluorescent tubes (Power Groove) at 26 ± 3°C. Liquid cultures are placed on a shaker operating at 160 rpm (a New Brunswick model V shaker was used in the original research).

Culture Medium. A modified Vacin and Went medium (Table Den-2) is used.

Procedure. The procedure is the same as that for *Cymbidium* (Sagawa et al., 1982).

Developmental Sequence. Explants form a green mass within 45 days and PLBs after 3 months. Sectioning the tissue masses within 2.5 months and subculturing the divisions result in further proliferation in an equal period of time. The highest yield of new PLBs results from subdividing those that do not have obvious leaves. Sections obtained by transverse cuts produce a larger number of PLBs than those derived from longitudinal cuts.

General Comments. This is an early but still useful procedure. A culture method for *Dendrobium* leaves in another modification of the Vacin and Went medium that contains 15% (by volume) coconut water was also developed by the same investigators (Sagawa and Kunisaki, 1982). Unfortunately not enough details are provided about this method (Sagawa and Kunisaki, 1982) to allow a description of it here.

Clonal Propagation of *Dendrobium* through Tissue Culture of Buds

As parts of an effort to assist "orchid growers and breeders," investigators in the tissue culture laboratory at the Singapore Botanic Gardens have developed procedures for three *Dendrobium* species and 17 hybrids (Lim-Ho, 1981).

Plant Material. Apical and axillary buds are cultured (Table Den-3). They are excised like those of *Arachnis, Aranda*, and *Aranthera* (Lim-Ho, 1981).

TABLE DEN-3. **Media requirements of *Dendrobium* species and hybrids (Lim-Ho, 1981)**

Dendrobium species or hybrid	Growth rate	Explant, bud[a]	Medium, table[b]		
			Initiation liquid	Proliferation and differentiation	Plantlet formation
Species					
D. antennatum	Slow	Ap	Den-4	Arach-4, Arach-5	Arach-6
D. laciniosum	Slow	Ap, Ax	Arach-3	Arach-4, Arach-5	Arach-6
D. superbiens 'Superba'	Fast	Ap	Den-4	Arach-4, Arach-5	Arach-6
Hybrids					
D. Alice Spalding	Slow	Ap, Ax	Arach-5, Arach-4	Arach-4, Arach-5[c]	Arach-6
D. Barbara Moore	Slow	Ax	Arach-5	Arach-4, Arach-5[c]	Arach-6
D. Caesar	Fast	Ap, Ax	Arach-5	Arach-4, Arach-5[c]	Arach-6
D. Cheong Chee Yon	Fast	Ap	Arach-4	Arach-4, Arach-5[c]	Arach-6
D. Ellen Harris	Slow	Ap, Ax	Arach-4	Arach-4, Arach-5[c]	Arach-6
D. Fiery Gold	Slow	Ap, Ax	Arach-5	Arach-4, Arach-5[c]	Arach-6
D. Ng Eng Cheow	Fast	Ap, Ax	Arach-4	Arach-4, Arach-5[c]	Arach-6
D. Sunny	Slow	Ax	Den-5	Arach-4, Arach-5[c]	Arach-6
D. Tay Swee Keng	Fast	Ap	Arach-4	Arach-4, Arach-5[c]	Arach-6
D. Yong Kok Wah	Slow	Ap, Ax	Arach-4	Arach-4, Arach-5[c]	Arach-6
Several unregistered hybrids	Fast, Slow	Ap, Ax	Arach-4, Arach-5, Den-4, Den-5	Arach-4, Arach-5[c] Den-6	Arach-6

[a]Ap, tip ("meristem") explant from apical bud; Ax, tip ("meristem") from axillary bud.

[b]The order in which media are listed is the same as in the original paper and may indicate suitability.

[c]This medium (Arach-5) in solid form is "good for all *Dendrobium* spp. for differentiation into shoot[s]; rate of differentiation [is] fast in this medium . . ."

TABLE DEN-4. **Modified Vacin and Went medium (Vacin and Went, 1949) for *Dendrobium* bud cultures (Lim-Ho, 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated ion**[d]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.84	2.78 g l^{-1}	10	One solution
(b)	Chelating agent, Na_2EDTA	37.24	3.72 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	0.2	20 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
9	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
10	Sucrose	2 g[f]	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]The original formulation contains a different source of iron.

[e]Keep refrigerated or frozen between uses.

[f]The original formulation has 20 g sucrose.

[g]Add items 1, 3–7 and 9 to the 500 ml distilled water (item 11) that contains item 2. Adjust pH to 5.2–5.5, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Pour medium into a 2-l Erlenmeyer flask, and autoclave. Add auxin (item 8) to solution while it is still hot or warm, swirl several times to ensure good mixing, and dispense medium into presterilized culture vessel. If preparing solid medium, add 10–20 g agar.

Surface Sterilization. Shoots, stems, and pseudobulbs are surface-sterilized like those of *Arachnis*, *Aranda*, and *Aranthera* (Lim-Ho, 1981).

Culture Vessels. The vessels used for *Arachnis*, *Aranda*, and *Aranthera* (Lim-Ho, 1981) are suitable.

Culture Conditions. Cultures are maintained under the same conditions as *Arachnis*, *Aranda*, and *Aranthera* (Lim-Ho, 1981).

Culture Media. The different species and hybrids vary somewhat in their media requirements (Tables Den-3 to Den-6).

Procedure. Procedures used for *Arachnis*, *Aranda*, and *Aranthera* (Lim-Ho, 1981) can be employed.

TABLE DEN-5. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of *Dendrobium* buds (Lim-Ho, 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated ion**[d]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.84	2.78 g l^{-1}	10	One solution
(b)	Chelating agent, Na_2EDTA	37.24	3.72 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxin				
8	Indoleacetic acid (IAA)	0.3	30 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
9	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
10	Sucrose	2 g[f]	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]The original formulation contains a different source of iron.

[e]Keep refrigerated or frozen between uses.

[f]The original formulation has 20 g sucrose.

[g]Add items 1, 3–7, and 9 to the 500 ml distilled water (item 11) that contains item 2. Adjust pH to 5.2–5.5, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Pour medium into a 2-l Erlenmeyer flask, and autoclave. Add auxin (item 8) to solution while it is still hot or warm, swirl several times to ensure good mixing, and dispense medium into presterilized culture vessel. Add 10–12 g agar, for solid medium.

Developmental Sequence. Limited information is presented in the original paper (Lim-Ho, 1981), but it is reasonable to assume that development is similar in principle to that of *Dendrobium* explants in other methods and to *Arachnis*, *Aranda*, and *Aranthera* (Lim-Ho, 1981).

General Comments. Although developed with specific hybrids this method should prove suitable for *Dendrobium* in general, either directly or as a starting point for additional research.

TABLE DEN-6. **Modified Knudson C medium (Knudson, 1946) for the culture of *Dendrobium* buds (Lim-Ho, 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	400	40 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	400	40 mg l^{-1}	10	
3	Magnesium sulfate $MgSO_4 \cdot 7H_2O$	100	10 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	100	10 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.84	2.79 g l^{-1}	10	One solution
(b)	Chelating agent, Na_2EDTA	37.24	3.72 g l^{-1}		
6	**Microelements**[d]				
(a)	Aluminum chloride, $AlCl_3$	0.03	3 mg l^{-1}	10	One solution
(b)	Boric acid, $H_3B_2O_3$	1.0	100 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.03	3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4$	0.1	10 mg l^{-1}		
(e)	Potassium iodide, KI	1.0	100 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4$	1.0	100 mg l^{-1}		
	Complex additive				
7	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
8	Sucrose	2 g[e]	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[f]	To 1000 ml			
	Solidifier				
10	Agar[f]	8–10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]A different iron salt was used in the original formulation.

[d]Add all salts to the same 1 l distilled water, stir and/or heat until they are dissolved, cool, and dispense.

[e]The original formulation contains 20 g sucrose.

[f]Add items 1–7 to 750 ml distilled water (item 9), adjust pH to 5.0–5.5, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, distribute medium into culture vessels and autoclave. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary, adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. A dilute solution of hydrochloric acid (HCl) should be used to lower the pH if necessary. Add agar to solid medium only (12–15 g in the original formulation).

Tissue Culture of *Dendrobium phalaenopsis*

Nitrogen is the largest component of the great majority of media used for orchid seed germination and tissue culture. It is generally present in both as nitrate and ammonium. An experiment designed to "establish the optimal balance of nitrate nitrogen and ammonium nitrogen . . . in tissue culture of . . . *Dendrobium phalaenopsis*" (Gandawijaja, 1980) resulted in a method that can be used for clonal propagation.

Plant Material. Shoot tips 1.0–1.5 mm long from 8-month-old seedlings of *D. phalaenopsis* are used.

Surface Sterilization. Surface sterilization is not required when seedlings from sterile cultures are used. If this method is used with tips from adult plants, the surface-sterilization procedure developed at the Singapore Botanic Gardens (Lim-Ho, 1981) should be employed.

Culture Vessels. Corning disposable test tubes 150 × 16 mm (Corning Glass Co., Corning, NY) containing a filter bridge and 5 ml liquid medium were used in the original research. Other containers can also be employed.

Culture Conditions. Cultures should be maintained under 16-h photoperiods of approximately 3000 lx provided by fluorescent tubes (warm white in the original research, but other balanced light sources are also suitable) and 24 ± 2°C.

Culture Media. A modified MS medium (Table Den-7) is used for initial culture and multiple shoot production. A second modification (Table Den-8), is used for root induction. In the original research the explants were cultured on a ⌐¬-shaped platform or filter bridge placed in the medium. This technique provides for constant aeration and a continuous supply of medium, but it is overly cumbersome for practical use. The filter-paper platform could be replaced by cotton (which may become compacted), glass wool (unpleasant and unsafe to use without appropriate protection such as gloves, long sleeves, face protector, and mask), or solid medium (simplest and probably most practical).

Some taxonomists have reduced *Dendrobium phalaenopsis* to varietal status under *Dendrobium bigibbum*. Others consider it to be a synonym of *D. bigibbum*. John Lindley described *D. bigibbum* in 1852. R. E. Holttum of Singapore fame reported that the first living plants of this species reached Kew Gardens in 1824.

TABLE DEN-7. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for shoot production by *Dendrobium* seedling-tip explants (Gandawijaja, 1980)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4N_3[b]	800	80 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220	22 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1010	101 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.0125	1.25 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.0125 g	1.25 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 2H_2O$	11.25	1.13 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.415	41.5 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430 mg l^{-1}		
	Amino acid				
8	Glycine	1	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol[f]	50	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.250	25 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.05	5 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.05	5 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
13	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[h]	To 1000 ml			
	Solidifier				
15	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Also known as *meso*-inositol, *i*-inositol and inositol.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 14), adjust pH to 5.2, add sugar (item 13), and adjust volume to 1000 ml with distilled water (item 14). Bring solution to a gentle boil, and add agar (item 15) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid and vitamins (items 8, 10–12) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

Procedure. Place explants on the medium (Table Den-4) and allow to grow and proliferate. Individual plantlets can be moved to the same medium for growth and root formation, or they can be placed on the media developed in Singapore (see Tables Arach-3 to Arach-6; for details see Table Den-3; Lim-Ho, 1981).

TABLE DEN-8. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for root induction on shoots produced by *Dendrobium* seedling-tip explants (Gandawijaja, 1980)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400	40 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220	22 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1515	151.5 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 mg l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 mg l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.0125	1.25 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.0125 g	1.25 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 2H_2O$	11.25	1.13 g l^{-1}		
(e)	Potassium iodide, KI	0.415	41.5 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430 mg l^{-1}		
	Amino acid				
8	Glycine	1	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol[f]	50	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.250	25 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.05	5 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.05	5 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
13	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[h]	To 1000 ml			
	Solidifier				
15	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Also known as *meso*-inositol, *i*-inositol, or inositol.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 14), adjust pH to 5.2, add sugar (item 13), and adjust volume to 1000 ml with distilled water (item 14). Bring solution to a gentle boil, and add agar (item 15) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid and vitamins (items 8, 10–12) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

Developmental Sequence. Not many details are given in the original paper, but it is clear that development proceeds as usual for *Dendrobium*.

General Comments. Multiple shoots are produced in media that contain 1/6 (5 mmol) or 1/3 (10 mmol) of the total nitrogen (30 mmol) as ammonium. Root production is best on a medium with 0.5 mmol of ammonium, This is the reason for the two media. Although developed with *D. phalaenopsis*, this method could probably be used with other species and some hybrids. Djunaidi Gandawijaja has been at the Bogor Botanical Gardens in Indonesia for about 20 years.

Clonal Propagation of *Dendrobium* Caesar Red Lip by Meristem Culture

Orchids are grown widely in Sri Lanka both by hobbyists (the Orchid Circle of Ceylon is one of the oldest orchid societies in existence) and commercial growers. Orchid research carried out at the Perideniya Botanical Gardens led to the development of a successful procedure for clonal propagation in vitro (Fernando, 1979).

Plant Material. Apical and axillary buds are taken from 10-cm-long, actively growing shoots following leaf removal and surface sterilization.

Surface Sterilization. The outermost leaves are removed, and the shoots are trimmed before being submerged in calcium hypochlorite (8 g in 100 ml of distilled water stirred several times at 5-min intervals and filtered) for 15 min. There is no mention in the original paper of sterile distilled water rinses, but such steps are desirable even if not required. Apical and axillary buds are excised under a microscope in a sterile environment, dipped in a 2% calcium hypochlorite solution (2 g calcium hypochlorite in 100 ml distilled water prepared as above, or 25 ml of the 8% solution diluted to 100 ml) for 1 min and then placed in sterile distilled water.

Culture Vessels. Erlenmeyer flasks of 250-ml capacity were used in the original research, but other containers are also suitable. As a rule culture vessels should be filled to one-fifth of their capacity with medium (e.g., 50 ml in a 250-ml flask).

Culture Conditions. Liquid cultures are placed on a horizontal shaker (but other models should also prove to be suitable) at 80 rpm under continuous illumination of 75–100 ft-c (approximately 750–1000 lx; the light sources are not described in the original papers, but standard bulbs or tubes would probably be appropriate) and 25–28°C.

Culture Media. A modified liquid Vacin and Went medium (Table Den-9) is used for initial culture. Explants on modified Knudson C and unaltered Vacin and Went media became necrotic and died after 24 days. Differentiation of PLBs into plantlets occurs on solid Knudson C (see Table Aranda-7) or Vacin and Went (see Table Cym-5) media.

Procedure. Take the explants from the sterile distilled water, and either place them immediately in culture or store them (for a short time) in sterilized Petri dishes and

TABLE DEN-9. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of *Dendrobium* shoot tips (Fernando, 1979)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated ion**[d]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.84	2.78 g l^{-1}	10	One solution
(b)	Chelating agent, Na_2EDTA	37.24	3.72 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	4.0	400 mg 100 mg^{-1} 95% ethanol[e]	1	
	Sugar				
9	Sucrose	20 g[f]	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]The original formulation contains a different source of iron.

[e]Keep refrigerated or frozen between uses.

[f]According to several reports some *Dendrobium* clones develop better initially if the sucrose concentration is 1–2 g l^{-1}.

[g]Add items 1, and 3–7 to the 500 ml distilled water (item 10) that contains item 2, adjust pH to 5.2–5.5, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Pour medium into a 2-l Erlenmeyer flask, and autoclave. Add auxin (item 8) to solution while it is still hot or warm, swirl several times to ensure good mixing, and dispense medium into presterilized culture vessel. For solid medium, add 10–20 g agar.

then "transfer [them] singly to flasks containing the media. . . ." Allow them to produce plantlets and PLBs. If more PLBs are required, existing ones can be divided and subcultured. In the case of axillary bud explants, the production of PLBs requires removing the leaves from the explant as it swells. The PLBs are moved to unmodified Knudson C (see Table Aranda-7) or modified Vacin and Went (see Table Cym-5) media for plantlet production.

Developmental Sequence. The explants may: (l) swell and produce only one shoot; (2) expand and produce a tissue mass that gives rise to PLBs that can be divided and subcultured; or (3) develop a single shoot that produces small PLBs at its base and these in turn also form plantlets. PLBs proliferate after being subcultured.

General Comments. Plantlets produced by this method are reported to have flowered within 22 months of the start of culture; this is very fast. *Dendrobium* Caesar (*Dendrobium* E. P. Boule) is of the parentage *Dendrobium phalaenopsis* × *Dendrobium stratiodes*. Its originator and date of registration are not recorded. This procedure may be suitable for related hybrids and species.

Shoot-tip Tissue Culture of *Dendrobium* Ng Eng Cheow

Dendrobium Ng Eng Cheow [*Dendrobium* Alice Spalding (*Dendrobium tokai* × *Dendrobium undulatum*) × *Dendrobium* Jaquelyn Thomas (*Dendrobium gouldii* × *Dendrobium phalaenopsis*)] was produced by the Koh Keng Hoe Nursery in Singapore. It flowered for the first time on 5 May 1972 and was registered in May 1973. A tissue culture propagation procedure for it was developed at the Singapore Botanic Gardens (H. Singh, 1976).

Plant Material. In the original research young shoots approximately 8 cm long (Fig. Den-1A*a,b*) were taken from mature plants of *Dendrobium* Ng Eng Cheow. Such shoots of other dendrobiums (especially related species and hybrids) can also be used. Explants, cones about 5 mm in diameter and 3–4 mm tall for apical buds (Fig. Den-1A*c*) and cubes approximately 5 mm wide, 6 mm long, and 2–3 mm thick for axillary buds (Fig. Den-1A*d*), are taken after surface sterilization.

Surface Sterilization. Leaves and their bases are removed from the shoots (Fig. Den-1A*b*) before submerging the shoots in 10% Clorox (10 ml Clorox diluted to 100 ml with distilled water) for 15 min. The original paper does not mention subsequent washing with sterile water, but two to three such washes are desirable.

Culture Vessels. Erlenmeyer flasks or other containers can be used.

Culture Conditions. Explants should be maintained under continuous illumination of 2000 lx (the light sources used in the original research are not described) at 21–25°C. Liquid cultures are agitated at 40 rpm.

Culture Media. Explants are first cultured in a liquid modification of the Vacin and Went medium (Table Den-10). After a month in this solution the explants are moved to a high-auxin solution (Table Den-11). Clusters of PLBs that form in this solution are divided and cultured on a third, solid, medium for plantlet formation (Table Den-12).

Procedure. Place explants in the initial medium (Table Den-10), and allow them to remain there until they show some growth. After that, transfer them to a sugar-free medium (Table Den-11 without item 10) for proliferation. Move PLBs to the solid medium (Table Den-12) for plantlet formation.

Developmental Sequence. Axillary bud explants "showed more potential growth than those which included the apical bud" (H. Singh, 1976). After 3 months of culture, explants may: (1) develop single shoots (Fig. Den-1B*a*); (2) form a cluster of shoots (Fig. Den-1B*b*); or (3) produce a cluster of PLBs (Fig. Den-1C, D*b*–L*b*). These are separated into individual PLBs and placed on solid medium. The PLBs start to differentiate, form shoots that develop roots, and turn into plantlets.

General Comments. As part of the original research several hundred plantlets were produced during 5 months. Several thousand could be produced in 1 year.

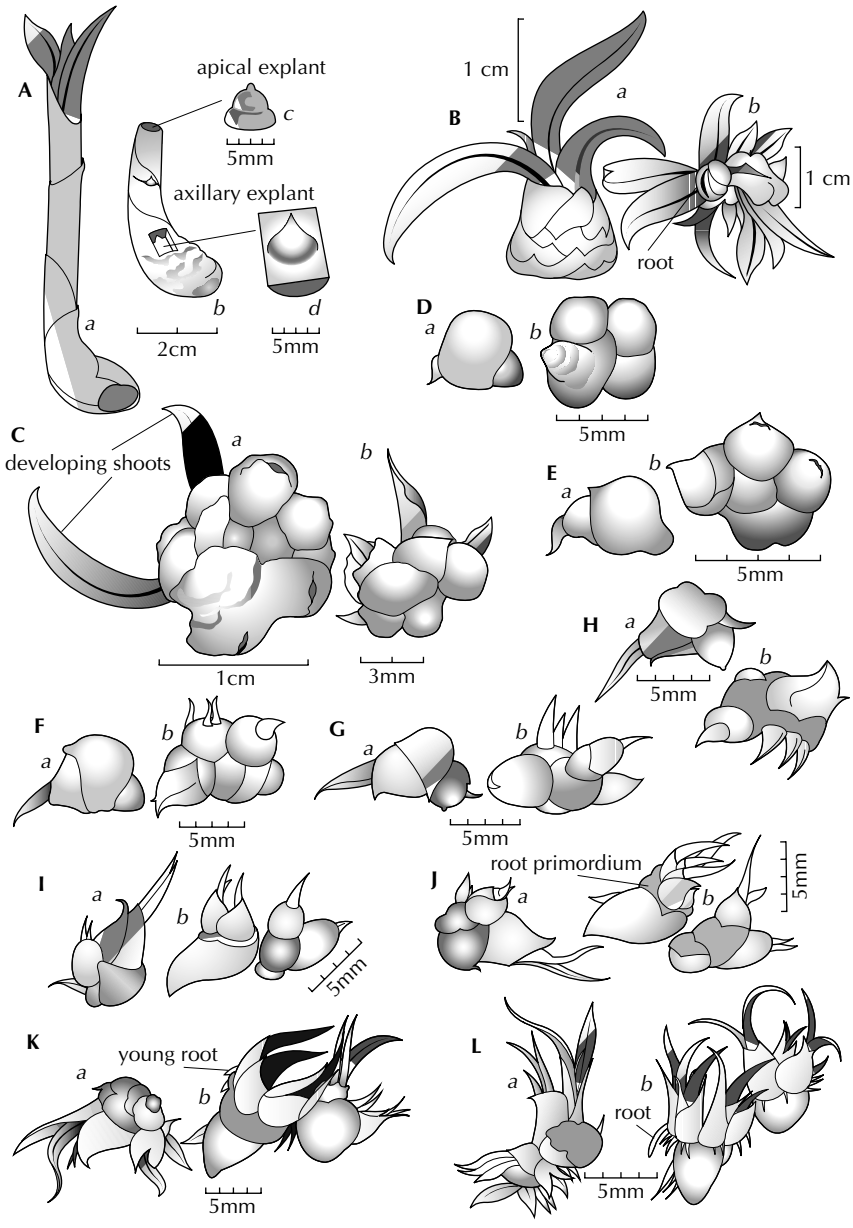


FIG. DEN-1. ***Dendrobium*** **Ng Eng Cheow: Excision and development of explants. A: Young shoot before (*a*) and after (*b*) removal of leaves, and excised apical (*c*) and axillary (*d*) buds. B: Single (*a*) and multiple (*b*) shoots that may develop from the explant. C: Large (*a*) and small (*b*) masses of protocorm-like bodies (PLBs) that can develop from some explants. D–L: Single (*a*) and multiple (*b*) PLBs after the following days in culture: 2 (D), 5 (E), 9 (F), 13 (G), 15 (H), 17 (I), 21 (J), 26 (K), and 30 (L). Root primordia start to form after 21 days (J*b*). Young roots (K*b*, L*b*) are evident after 26 and 30 days. (H. Singh, 1976.)**

TABLE DEN-10. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of *Dendrobium* buds (H. Singh, 1976)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated ion**[d]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.84	2.78 g l^{-1}	10	One solution
(b)	Chelating agent Na_2EDTA	37.24	3.72 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	0.2	20 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
9	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
10	Sucrose	10 g[f]	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]The original formulation for the Vacin and Went medium uses a different source of iron. Singh's modification does not use EDTA, which is included here because it improves the availability of iron.

[e]Keep refrigerated or frozen between uses.

[f]The original formulation has 20 g sucrose.

[g]Add items 1, 3–7, and 9 to the 500 ml distilled water (item 11) that contains item 2. Adjust pH to 5.0, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Pour medium into a 2-l Erlenmeyer flask, and autoclave. Add auxin (item 8) to solution while it is still hot or warm, swirl several times to ensure good mixing, and dispense medium into presterilized culture vessel. For a solid medium, add 10–20 g agar.

Dendrobium is a very large genus consisting of about 900 species which are found in Australia, South East Asia, Burma, China, Cambodia, Hong Kong, India, Indonesia, Irian Jaya, Japan, Laos, Malaysia, New Zealand, many Pacific Islands, Papua New Guinea, Singapore, Taiwan, Thailand, and Vietnam. It was established by Olof Swartz (1760–1818) in 1799 on page 82, volume 6 of *Nova Acta Societatis Scientiarum Upsaliensis*. The name is derived from *dendron* (δενρον), tree, and *bios* (βιοσ), life. It refers to the epiphytic habitat of the genus and means "living on a tree." *Dendrobium* is one of the most important genera for the orchid cut flower industry in South East Asia. Some species are used as herbal medicines in several countries (Schultes and Pease, 1963; Bechtel et al., 1992; Hew, 1994; Hew and Yong, 1997).

TABLE DEN-11. **Modified Vacin and Went medium (Vacin and Went, 1949) for the proliferation of protocorm-like bodies from *Dendrobium* buds (H. Singh, 1976)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated ion**[d]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.84	2.78 g l^{-1}	10	One solution
(b)	Chelating agent, Na_2EDTA	37.24	3.72 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	4.0	400 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
9	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
10	Sucrose	10 g[f]	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	10–12 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]The original formulation for the Vacin and Went medium uses a different source of iron. Singh's modification does not use EDTA, which is included here because it improves the availability of iron.

[e]Keep refrigerated or frozen between uses. This relatively high auxin concentration could cause mutations. Used with caution to induce proliferation, but do not culture protocorm-like bodies from one explant in it more than a few times.

[f]The original formulation has 20 g sucrose. This item is omitted if a sugar-free medium is desired.

[g]Add items 1, 3–7, and 9 to the 500 ml distilled water (item 11) that contains item 2. Adjust pH to 5.0, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring solution to a gentle boil, and add agar (item 12) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add auxin (item 8) to solution while it is hot or warm and still liquid, swirl several times to ensure good mixing, and dispense medium into presterilized culture vessel. Omit agar if preparing liquid medium.

TABLE DEN-12. **Modified Vacin and Went medium (Vacin and Went, 1949) for plantlet formation from protocorm-like bodies of *Dendrobium* (H. Singh, 1976)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated ion**[d]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.84	2.78 g l^{-1}	10	One solution
(b)	Chelating agent, Na_2EDTA	37.24	3.72 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Sugar				
8	Sucrose	10 g[e]	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[f]	To 1000 ml			
	Solidifier				
10	Agar	10–12 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]The original formulation for the Vacin and Went medium uses a different source of iron. Singh's modification does not use EDTA, which is included here because it improves the availability of iron.

[e]The original formulation has 20 g sucrose.

[f]Add items 1 and 3–7 to the 500 ml distilled water (item 9) that contains item 2. Adjust pH to 5.0, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, distribute medium into culture vessels and autoclave. Omit agar if preparing liquid medium.

In Vitro Culture of Axillary Buds of *Dendrobium*

A procedure for the clonal propagation of *Dendrobium* through the culture of axillary buds was developed in the laboratory of Helen Nair, Department of Botany, University of Malaya, Kuala Lumpur, Malaysia (Khaw et al., 1978*a*, 1978*b*). The procedure is the same as the one employed for *Aranda* (Khaw and Ong, 1974–1975), and explants of *Dendrobium* Alice Chong {*Dendrobium* May Neal [*Dendrobium* Hawaii (*Dendrobium phalaenopsis* × *Dendrobium tokai*) × *Dendrobium stratiodes*]} are cultured on a modified Vacin and Went medium (see Table Aranda-1). Surface sterilization and all the other steps in this procedure can be the same as the one developed for *Dendrobium* Ng Eng Cheow (H. Singh, 1976), *Dendrobium phalaenopsis* (Gandawijaja, 1980; Kukułczanka and Wojciechowska, 1983), *Dendrobium antennatum* (Kukułczanka and Wojciechowska, 1983), and other *Dendrobium* species and hybrids (Lim-Ho, 1981).

Propagation of *Dendrobium antennatum* and *Dendrobium phalaenopsis* by in Vitro Culture

Investigations designed to determine the regeneration potential of isolated organs have generated information that can be used for the clonal propagation of *Dendrobium antennatum* (listed as *Dendrobium antennathum* in the original paper) and *Dendrobium phalaenopsis* (Kukułczanka and Wojciechowska, 1983).

Plant Material. Axillary bud explants from shoots are cultured.

Surface Sterilization. No information is presented in the original paper. The method used for similar explants in Singapore (Lim-Ho, 1981) can be used.

Culture Vessels. Test tubes 16 × 80 mm were used in the original research, and other vessels should prove suitable.

Culture Conditions. The cultures should be maintained under continuous illumination of 1000 lx provided by "white luminescent" sources (probably meaning Cool or Warm White fluorescent tubes) and 24°C.

Culture Media. One modification of the Reinert–Mohr medium (Table Den-13) is used for shoot induction in *D. antennatum*. A different modification (Table Den-14) is employed for the same purpose with *D. phalaenopsis*. Roots are induced in both species by a third modification (Table Den-15). Callus induction requires a fourth modification (Table Den-16).

Procedure. Not many details are given in the original paper. First place explants on the shoot-induction medium. When shoots form, move them to the rooting medium. Callus may form on the fourth medium and give rise to PLBs that develop into plantlets.

Developmental Sequence. One or more shoots may develop from each bud. Callus development is not common. When callus does form, it produces PLBs, which develop into plantlets.

General Comments. The skillful use of plant hormones in this method is the result of several experiments. "Formation of callus which . . . later differentiated into protocorms [sic] allows [the] use of this method for . . . clonal mass propagation" (Kukułczanka and Wojciechowska, 1983). As in a number of other papers it is necessary to note here that by definition protocorms are produced only from seeds. The structures produced by explants are PLBs.

TABLE DEN-13. **Modified Reinert–Mohr medium (Reinert and Mohr, 1967) for shoot induction from explants of *Dendrobium antennatum* (Kukułczanka and Wojciechowska, 1983)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400	40 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	400	40 g l^{-1}	10	
4	Potassium chloride, KCl	500	50 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	22.4	2.24 g l^{-1}	10	One solution
(b)	Ferric sulfate, $Fe_2(SO_4)_3$	10.7	1.07 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.03	3 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.001	0.1 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot H_2O$	7.5	750 mg l^{-1}		
(d)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.03	3 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Organic acid				
9	Citric acid[e]	150.1	No stock	No stock	Weigh
	Auxins				
10	Indole-3-butyric acid (IBA)	1.75	175 mg 100 ml^{-1} 95% ethanol[f,g]	1	
11	Napthaleneacetic acid (NAA)	1.75	175 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
12	Benzyladenine (BA)	5	500 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
16	Peptone[h]	1 g	No stock	No stock	Weigh
	Sugar				
17	Sucrose[i]	15–30 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[i]	To 1000 ml			
	Solidifier				
19	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved. If $Fe_2(SO_4)_3$ is not available, make a stock solution containing 3.73 g Na_2EDTA and 2.78 g $FeSO_4 \cdot 7H_2O$ per liter, and add 10 ml of that to the medium.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. Some media (e.g., Murashige and Skoog, 1962) list $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but others use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Both an anhydrous salt and a monohydrate are available. It probably does not matter which one is used. Citric acid was used at one time to enhance the solubility of iron salts. The reason for its use here is not clear, but it is certainly not needed to solubilize the iron, which is added as a chelate. The citric acid may have been added as an antioxidant.

[f]If the auxin does not dissolve, add a few drops of dilute KOH.

[g]Keep in a freezer or refrigerator between uses.

[h]The peptone used is described as "peptobak-Bacutil," which may not be readily available outside of Poland or Eastern Europe. If other peptones are substituted for this brand, their analyses may be different. Be cautious when using a different peptone for the first time.

[i]Add items 1–7, 9, and 16 to 900 ml distilled water (item 18). Adjust pH to 5.0 and add sugar (item 17). (Sugar is not listed in the original recipe, but this seems to be a typographical error, which is why sucrose in the usual range is suggested here; a reasonable amount would be 10 or 20 g l^{-1}.) Adjust volume to 1000 ml with distilled water (item 18). Bring solution to a gentle boil, and add agar (item 19) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid, hormones, and vitamins (items 8, and 10–15) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE DEN-14. **Modified Reinert–Mohr medium (Reinert and Mohr, 1967) for shoot induction from explants of *Dendrobium phalaenopsis* (Kukułczanka and Wojciechowska, 1983)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400	40 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	400	40 g l^{-1}	10	
4	Potassium chloride, KCl	500	50 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	22.4	2.24 g l^{-1}	10	One solution
(b)	Ferric sulfate, $Fe_2(SO_4)_3$	10.7	1.07 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.03	3 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.001	0.1 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot H_2O$	7.5	750 mg l^{-1}		
(d)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.03	3 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Organic acid				
9	Citric acid[e]	150.1	No stock	No stock	Weigh
	Cytokinin				
10	Benzyladenine (BA)	5	500 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
14	Sucrose[h]	15–30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[h]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved. If $Fe_2(SO_4)_3$ is not available, make a stock solution containing 3.73 g Na_2EDTA and 2.78 g $FeSO_4 \cdot 7H_2O$ per liter and add 10 ml of that to the medium.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. Some formulae (e.g., Murashige and Skoog, 1962) list $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but others use $ZnSO_4 \cdot 7H_2O$ at the same concentration; the difference probably has little effect.

[e]Number of waters of hydration is not given. The choice is between an anhydrous salt and a monohydrate; it probably does not matter which one is used. Citric acid was used at one time to enhance the solubility of iron salts. The reason for its use here is not clear, but it is certainly not needed to solubilize the iron, which is added as a chelate. It could have been added as an antioxidant.

[f]If the cytokinin does not dissolve, add a few drops of dilute HCl.

[g]Keep in a freezer or refrigerator between uses.

[h]Add items 1–7, and 9 to 900 ml distilled water (item 15), adjust pH to 5.0, and add sugar (item 14). (Sugar is not listed in the original recipe, but this seems to be a typographical error, which is why sucrose in the usual range is suggested here; a reasonable amount would be 10 or 20 g l^{-1}.) Adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid, hormone, and vitamins (items 8 and 10–13) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE DEN-15. **Modified Reinert–Mohr medium (Reinert and Mohr, 1967) for root induction on shoots of *Dendrobium antennatum* and *Dendrobium phalaenopsis* (Kukułczanka and Wojciechowska, 1983)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400	40 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1000	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	400	40 g l^{-1}	10	
4	Potassium chloride, KCl	500	50 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	22.4	2.24 g l^{-1}	10	One solution
(b)	Ferric sulfate, $Fe_2(SO_4)_3$	10.7	1.07 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.03	3 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.001	0.1 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot H_2O$	7.5	750 mg l^{-1}		
(d)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.03	3 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Organic acid				
9	Citric acid[e]	150.1	No stock	No stock	Weigh
	Auxins				
10	Indole-3-butyric acid (IBA)	1.75	175 mg 100 ml^{-1} 95% ethanol[f,g]	1	
11	Naphthaleneacetic acid (NAA)	1.75	175 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose[h]	15–30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved. If $Fe_2(SO_4)_3$ is not available, make a stock solution containing 3.73 g Na_2EDTA and 2.78 g $FeSO_4 \cdot 7H_2O$ per liter and add 10 ml of that to the medium.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. Some media (e.g., Murashige and Skoog, 1962) list $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but others use $ZnSO_4 \cdot 7H_2O$ at the same concentration; the difference probably has little effect.

[e]Number of waters of hydration is not given. The choice is between an anhydrous salt and a monohydrate; it probably does not matter which one is used. Citric acid was used at one time to enhance the solubility of iron salts. The reason for its use here is not clear, but it is certainly not needed to solubilize the iron which is added as a chelate. It could have been added as an antioxidant.

[f]If the auxins do not dissolve, add a few drops of dilute KOH to solubilize them.

[g]Keep in a freezer or refrigerator between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 16), adjust pH to 5.0, and add sugar (item 15). (Sugar is not listed in the original recipe. This seems to be a typographical error, which is why sucrose in the usual range is suggested here; a reasonable amount would be 10 or 20 g l^{-1}.) Adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid, hormones, and vitamins (items 8 and 10–14) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE DEN-16. **Modified Reinert–Mohr medium (Reinert and Mohr, 1967) for callus induction from explants of *Dendrobium phalaenopsis* (Kukułczanka and Wojciechowska, 1983)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400	40 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1000	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	400	40 g l^{-1}	10	
4	Potassium chloride, KCl	500	50 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	22.4	2.24 g l^{-1}	10	One solution
(b)	Ferric sulfate, $Fe_2(SO_4)_3$	10.7	1.07 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.03	3 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.001	0.1 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot H_2O$	7.5	750 mg l^{-1}		
(d)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.03	3 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[g]	1	
	Organic acid				
9	Citric acid[e]	150.1	No stock	No stock	Weigh
	Auxins				
10	Indole-3-butyric acid (IBA)	1.75	175 mg 100 ml^{-1} 95% ethanol[f,g]	1	
11	Napthaleneacetic acid (NAA)	1.75	175 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
12	Benzyladenine (BA)	5	500 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
16	Sucrose[h]	15–30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved. If $Fe_2(SO_4)_3$ is not available, make a stock solution containing 3.73 g Na_2EDTA and 2.78 g $FeSO_4 \cdot 7H_2O$ per liter and add 10 ml of that to medium.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. Some media (e.g., Murashige and Skoog, 1962) list $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but others use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference probably has little effect.

[e]Both an anhydrous salt and a monohydrate are available. It probably does not make much difference which one is used. Citric acid was used at one time to enhance the solubility of iron salts. The reason for its use here is not clear, but it is certainly not needed to solubilize the iron, which is added as a chelate. It could have been added as an antioxidant.

[f]If auxins or cytokinin do not dissolve, add a few drops of dilute KOH or HCl.

[g]Keep in a freezer or refrigerator between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 17), adjust pH to 5.0, and add sugar (item 16). (Sugar is not listed in the original recipe, but this seems to be a typographical error, which is why sucrose in the usual range is suggested here; a reasonable amount would be 10 or 20 g l^{-1}.) Adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid, hormones, and vitamins (items 8 and 10–15) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

Clonal Propagation of *Dendrobium* Jaquelyn Thomas 'White' through the Culture of Shoot Tips

A shoot-tip culture method for mass rapid clonal propagation of *Dendrobium* was developed as a means of meeting market demands for fast propagation of new hybrids. This procedure was developed in the private laboratory of Flora Sari Orchids near Jakarta, Indonesia (Soediono, 1983*b*).

Plant Material. Shoot tips of *Dendrobium* Jaquelyn Thomas 'White' were excised according to the method used for *Dendrobium* Ng Eng Cheow (H. Singh, 1976).

Surface Sterilization. The methods that were developed in Singapore are suitable (H. Singh, 1976; Lim-Ho, 1981).

Culture Vessels. Erlenmeyer flasks, 50-ml capacity containing 20 ml medium, were used in the original research. Other containers are also suitable.

Culture Conditions. Liquid cultures are placed on a gyrorotatory shaker at 120 rpm under illumination (12-h photoperiods provided by 20-W Sylvania Gro Lux lamps 60 cm above the cultures) and at temperatures of 24 ± 2°C. Solid cultures are maintained under the same temperature and illumination.

Culture Media. Modified liquid Vacin and Went medium (Table Den-17) is used for initial culture. Rapid proliferation and plantlet formation occurs on a second modification of the Vacin and Went medium (Table Den-18). Plantlets produced on the second medium (Table Den-18) can be cultured on modified Vacin and Went (see Table Cym-5) or modified Knudson C (see Table Aranda-8) media.

Procedure. Place explants in the first liquid medium (Table Den-17) for initial development and formation of PLBs. When PLBs and plantlets form, transfer them to the second liquid medium (Table Den-18). Larger plantlets and PLBs formed in this medium should be moved to the Vacin and Went medium (see Table Cym-5) or modified Knudson C (see Table Aranda-7) medium for further growth.

Developmental Sequence. Rapid proliferation: green PLBs measuring 1–2 mm in diameter, shoots, and leaves form on the first medium. Larger dark green PLBs (2–3 mm in diameter), shoots, leaves, roots, and plantlets (1 cm tall) form on the second medium, which generally supports excellent growth.

General Comments. The author of this procedure (Soediono, 1983*b*) wisely points out that "excessive proliferation may have negative effects on the quality of plants produced through tissue culture" and does not "recommend production of a very large number of plantlets from a single shoot tip."

TABLE DEN-17. **Modified Vacin and Went medium (Vacin and Went, 1949) for initial culture of shoot tips of *Dendrobium* Jaquelyn Thomas 'White' (Soediono, 1983*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Water from green coconuts	150 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[e]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. The other salt components of the medium should be added after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate it. To prepare a stock solution of chelated iron dissolve 3.73 mg chelating agent (Na_2EDTA) and 2.78 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Add items 1 and 3–8 to the 500 ml distilled water (item 10) that contains item 2. Adjust pH to 5.0–5.3, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Dispense medium into culture vessels and autoclave.

Noes Soediono developed a procedure for propagating *Dendrobium* Jaquelyn Thomas 'White' at Flora Sari Orchids, Jakarta, Indonesia, where she is seen with the author, Joseph Arditti, in 1982

TABLE DEN-18. **Modified Vacin and Went medium (Vacin and Went, 1949) for initial culture of shoot tips of *Dendrobium* Jaquelyn Thomas 'White' (Soediono, 1983*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	10	1 g 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
9	Water from green coconuts	150 ml	No stock	No stock	Measure
	Sugar				
10	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate it. To prepare a stock solution of chelated iron dissolve 3.73 mg chelating agent (Na_2EDTA) and 2.78 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Keep refrigerated or frozen between uses. If auxin does not dissolve completely, shake well before dispensing.

[f]Add items 1 and 3–9 to the 500 ml distilled water (item 11) that contains item 2. Adjust pH to 5.0–5.3, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Dispense medium into culture vessels and autoclave. Omit agar if preparing liquid medium. It is generally not advisable to autoclave hormones, vitamins, amino acids, or a number of other organics without first determining whether this will affect the medium deleteriously. In this case the author (Soediono, 1983*b*) routinely autoclaves her medium, which contains NAA.

Clonal Propagation of *Dendrobium* by Means of Node Cultures

At one time *Dendrobium* explants were difficult to culture; for this reason, my colleagues and I (JA) developed an in vitro propagation procedure that utilizes stem nodes (Arditti et al., 1973; Mosich et al., 1973, 1974*a*, 1974*b*).

Plant Material. Stems ("canes") at least 10–5 cm long are used as the source of nodes.

Surface Sterilization. Leaves, dry sheaths, and other external tissues are removed, and the stems are washed by gentle scrubbing with a soft brush and mild household detergent. They are rinsed after the wash and surface-sterilized by immersing them for 10 to 20 min in a 50% dilution of household bleach (50 ml bleach mixed with

50 ml distilled water). Following the sterilization the sections should be dipped in sterile distilled water for 1–2 min.

Culture Vessels. Use 25 × 180 mm test tubes containing 20 ml medium.

Culture Conditions. Nodes in culture are maintained under 150 ft-c (1600 lx) and 16-h photoperiods provided by banks of 40-W Sylvania Gro Lux tubes and incandescent bulbs. The temperature should be 22–25°C.

Culture Media. Modified Knop's medium (Gautheret, 1959) is used for initial culture (Table Den-19). Depending on which buds are being cultured, the medium must contain 1.5, 14.8, or 148 mg *trans*-cinnamic acid (Table Den-19, item 7). The shoots that form on this medium are transferred to a modification of the MS medium (Table Den-20) for root formation.

Procedure. After washing the stems, divide them into three sections: upper (containing 25–33% of the nodes), middle (30 to 50%), and basal (25 to 33%). Sterilize buds from the upper section for 5–7 min (longer periods are lethal), those from the middle portion for 10–15 min, and the basal ones for 20–25 min. Remove discolored tissues near the cut edges following decontamination, and separate the nodes by transverse cuts. Those from the upper section are 5–7.5 cm in length; from the middle, 1.5–7.5 cm; and from the base, 1.5–2 cm (buds should be 0.75 cm from either end when possible). Sections from near the apex sometimes contain two buds that are difficult to separate owing to their proximity to each other.

All sections are placed on the agar basal end down ("right side up") and pushed halfway into the initial medium (Table Den-21) for stability. When shoots reach 1.5–2 cm in length, transfer them to the rooting medium (Table Den-20), where roots form within 14 days.

Developmental Sequence. Buds start to grow after 4 weeks on Knop's medium, and plantlets develop within 45 days. Most of them do not have roots and must be transferred to the rooting medium 2–3 weeks later (12–13 weeks after placing the nodes in culture). There they form roots within 2 weeks.

General Comments. This is a simple method that does not require sophisticated equipment, or advanced skills. Its major disadvantage – the need to remove (and therefore endanger) an entire stem – is balanced by the very high rate of success (75–100%). Furthermore, it is possible to use old and/or damaged canes as long as a few buds are intact. Recent evidence suggests that it should be possible to excise basal buds only (leaving the stem otherwise intact) and culture them. We have had limited success with this procedure to date and hope to refine it. Only one plantlet is formed from each bud, although on rare occasions we have noted considerable proliferation. With further modifications, the procedure could perhaps induce proliferation.

TABLE DEN-19. **Modified Knop's medium for the culture of *Dendrobium* node sections (Mosich et al., 1973, 1974*a*, 1974*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	500	50 g l^{-1}	10	Or weigh
2	Potassium nitrate, KNO_3[b]	125	12.5 g l^{-1}	20	Or weigh
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	125	12.5 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	125	12.5 g l^{-1}	10	
	Iron				
5	Ferric citrate, $FeC_3H_5O_7 \cdot 3H_2O$[c]	10	1 g l^{-1}	10	
6	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.056	56 mg l^{-1}	1	One solution[d]
(b)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.036	36 mg l^{-1}		
(c)	Zinc chloride, $ZnCl_2$	0.152	152 mg l^{-1}		
(d)	Cobaltous chloride, $CoCl_2$	0.02	20 mg l^{-1}		
(e)	Copper chloride, $CuCl_2 \cdot 2H_2O$	0.054	54 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Ferric chloride, $FeCl_3 \cdot 6H_2O$	0.5	500 mg l^{-1}		
(h)	Na_2EDTA	0.8	800 mg l^{-1}		
	Anti-auxin[e,f,g,h]				
7	*trans*-cinnamic acid[c,e]	150,[f] 15,[g] or 1.5[h]	15 g 100 ml^{-1} 95% ethanol	1,[f] 0.1,[g] or 0.01[h]	
	Vitamin				
8	Thiamine (vitamin B_1)	0.4	100 mg 100 ml^{-1} 95% ethanol[i]	0.4	
	Cytokinin				
9	6-Benzylaminopurine (benzyladenine)	2.0	200 mg 100 ml^{-1} 95% ethanol[i]	1.0	
	Sugar				
10	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[j]	To 1000 ml			
	Solidifier				
12	Agar	13 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing nitrate and ammonium tend to become contaminated on standing. Therefore, it is better to weigh this component each time. If a stock solution is prepared, it must be kept frozen.

[c]If the substance does not dissolve, add a dilute KOH solution one drop at a time until the solution becomes clear.

[d]Add all microelements to the same 1 l, stir and/or heat until dissolved. Add 1 ml to the culture medium.

[e]If the *trans*-cinnamic acid fails to dissolve, add a few drops of dilute KOH. Keep refrigerated.

[f]For nodes from the basal portion of the stem.

[g]For nodes from the midsection of the stem.

[h]For nodes from near the stem apex.

[i]Keep stock solution refrigerated.

[j]Mix items 1–6 with 800 ml of distilled water (item 11), set the pH to 5.5, dissolve the sugar (item 10), adjust the total volume to 1000 ml with distilled water (item 11), bring the solution to a gentle boil and add the agar slowly while stirring. When the agar is fully dissolved pour the solution into a 2-l Erlenmeyer flask and autoclave. The agar can also be added to the cold solution and dissolved by boiling. Under sterile conditions and using sterilized pipettes add the anti-auxin (item 7), vitamin (item 8) and cytokinin (item 9) to the hot and still liquid medium, swirl to mix well and dispense into culture vessels.

Knop's medium was formulated before chelating agents were available and that is why it includes ferric tartrate (FT) as the iron source. Since FT is not easily soluble it is preferable to use chelated iron. Add 278 mg of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) and 373 mg of chelating agent (Na_2EDTA) to 1 l of distilled water, stir until both are completely dissolved. Add 10 ml of this solution to each liter of medium.

TABLE DEN-20. **Modified Murashige–Skoog medium for root induction on *Dendrobium* shoots obtained from node sections (Mosich et al., 1974*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements[b]				
1	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
3	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1.9	95 g l^{-1}	20	Or weigh
5	Ammonium nitrate, NH_4NO_3[b]	1.65	82.5 g l^{-1}	20	Or weigh
6	**Chelated iron**[c]				
(a)	Na_2-EDTA	74.5	7.45 g l^{-1}		
(b)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[c]				
(c)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution[c]
(e)	Zinc chloride, $ZnCl_2$	3.93	393 mg l^{-1}		
(f)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(g)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(h)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(i)	Cobaltous chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
	Growth factor				
8	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
9	Indoleacetic acid	0.1	50 mg 50 ml^{-1} 95% ethanol[d]	0.1	
	Vitamin[d]				
10	Thiamine (vitamin B_1)	0.4	100 mg 100 ml^{-1} 95% ethanol[d]	0.4	
	Sugar				
11	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[e]	To 1000 ml			
	Solidifier				
13	Agar	13 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and nitrate may become contaminated. Therefore, stock solutions should not be prepared. If made, they must be kept frozen between uses.

[c]Add the chelated iron (items a–b) and all microelements (c–i) to the same 1 l distilled water; stir and/or heat until dissolved. Add 10 ml per liter of culture medium.

[d]Keep refrigerated.

[e]Mix items 1–9 with 800 ml of distilled water (item 11); adjust the pH to 5.8; add the sugar (item 10); and bring the volume to 1000 ml with more distilled water (item 11). Distribute into culture vessels and autoclave. Solutions containing heat labile substances like indoleacetic acid (IAA) should not be autoclaved without prior determination that heat sterilization is not deleterious. In this instance the medium can be autoclaved.

TABLE DEN-21. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of *Dendrobium* and *Phalaenopisis* flower-stalk cuttings (Intuwong et al., 1972*a*, 1972*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Tricalcium phosphate, $Ca_3(PO_4)_2$[b]	200	20 g l^{-1}	10	
2	Potassium nitrate, KNO_3[c]	525	52.5 g l^{-1}	10	Or weigh
3	Potassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
5	Ammonium sulfate, $(NH_4)_2SO_4$[c]	500	50 g l^{-1}	10	Or weigh
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3$[d]	28	2.8 g l^{-1}	10[d]	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 2H_2O$	5.7	5.7 g l^{-1}	1	
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier[f]				
10	Agar	9 g[f]	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]If this salt does not dissolve completely, shake the stock solution well before dispensing to mix well; or better yet, weigh out each time.

[c]Solutions containing ammonium and nitrate may become contaminated. Therefore, stock solutions should not be prepared. If made, they must be kept frozen between uses.

[d]Ferric tartrate is relatively insoluble. Grinding it with a mortar and pestle before dissolving helps. The addition of a pellet or two of KOH to the solution will increase solubility, but a precipitate may form nevertheless. To insure equal distribution, shake stock solution well before dispensing. This medium (Vacin and Went, 1949) was formulated before chelating agents were available and that is why it includes ferric tartrate (FT) as the iron source. Since FT is not easily soluble it is preferable to use chelated iron. Add 278 mg of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) and 373 mg of chelating agent (Na_2EDTA) to one liter of distilled water, stir until both are completely dissolved. Add 10 ml of this solution to each liter of medium.

[e]Dissolve items 1–7 in 900 ml of distilled water (item 9); adjust the pH to 4.8–5.0; add sugar (item 8) and bring the volume up to 1000 ml with more distilled water (item 9). Add the agar (item 10) slowly, while stirring, to the gently boiling solution. When fully dissolved, dispense into culture vessels and autoclave.

[f]If cultures are maintained at 29°C, the amount of agar should be increased to 12–15 g l^{-1}.

Clonal Propagation of *Dendrobium crumenatum* from Leaves

"The shoot tip culture [of orchids] entails the sacrifice of the whole plant or an entire new growth. Using axillary buds, inflorescence axes, and other parts of the plant body would also involve damaging or sacrificing certain plant parts. To avoid or to minimize such damage . . . leaf tips can be used . . ." (Manorama et al., 1986). A procedure utilizing leaves of *Dendrobium crumenatum* (*Anggrek merpati*, pigeon orchid), a species native to Singapore (where they can still be seen on trees in the midst of a bustling modern city), Malaysia, and Indonesia, was developed at the Botany Department, National University of Singapore (Manorama et al., 1986).

Plant Material. Leaves from *D. crumenatum* seedlings grown in vitro and mature plants are used. Whole leaves or leaf segments are cultured.

Surface Sterilization. Leaves taken from seedlings grown in vitro need not be sterilized. If sterilization of seedling leaves becomes necessary, they should be immersed in 10% Clorox (10 ml Clorox diluted to 100 ml with distilled water) for

5 min. Leaves from mature plants growing outdoors should be immersed in 10–30% Clorox (10–30 ml Clorox diluted to 100 ml with distilled water) for 10 min. After being removed from the Clorox solution, the leaves should be rinsed three to five times with sterile distilled water.

Culture Vessels. "Culture flasks" (presumably Erlenmeyer flasks) were used in the original research. Other containers can also be used.

Culture Conditions. Liquid cultures should be placed on a shaker at 80 rpm for 16 h day^{-1}. The illumination used in the original research was 1000 lx and 12-h photoperiods. Average temperature should be 26°C. Solid cultures are maintained under the same conditions on a shelf or bench.

Culture Media. Liquid modified Vacin and Went medium (Table Den-22) is used for the initial culture. A liquid modification of the MS medium (Table Den-23) may prove suitable for mature leaves. Plantlets and PLBs produced on these media can be cultured on modified Vacin and Went (see Table Cym-5) and unmodified Knudson C (see Table Aranda-7) media.

Procedure. First culture explants in one of the liquid media. Then transfer plantlets and PLBs to solid medium.

Developmental Sequence. Callus is formed after 40 days in culture, and PLBs appear within 3 months. This research (Manorama et al., 1986) is an excellent example of detailed anatomical and morphological studies of development and organogenesis in a tissue culture system. The leaves of *D. crumenatum* have a mesophyll that consists of nine layers of rounded cells. As in *Aranda* the first division occurs in the upper epidermis. This leads to the formation of a meristematic region that is three to four cell layers thick. Cells in this region divide to form a callus. During early development this callus is uniform and has a homogeneous outline. Later it develops many lobes and each of these gives rise to a PLB.

General Comments. The procedure presented here is based on an excellent study of the developmental aspects of tissues and plants cultured in vitro. This method, an excellent example of A.N. Rao's scientific leadership at the National University of Singapore, is also suitable for leaves of *Aranda* Deborah, *Aranda* Hilda Galistan, *Aranda* Mei Ling, and *Aranda* Queen of Purples.

TABLE DEN-22. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of seedling leaves of *Dendrobium crumenatum* (Manorama et al., 1986)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxins				
8	2,4-Dichlorophenoxyacetic acid (2,4-D)	5	500 mg 100 ml^{-1} 95% ethanol[e]	1	
9	Naphthaleneacetic acid (NAA)	5	500 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin				
10	Benzyladenine (BA)	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additives				
11	Coconut water	200 ml	No stock	No stock	Measure
12	Casein hydrolysate	150	No stock	No stock	Weigh
	Sugar				
13	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate it. To prepare a stock solution of chelated iron dissolve 3.73 mg chelating agent (Na_2EDTA) and 2.78 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Keep frozen or refrigerated between uses.

[f]Add items 1 and 3–12 to the 500 ml distilled water (item 14) that contains item 2. Adjust pH to 5.3, add sugar (item 13), and adjust volume to 1000 ml with distilled water (item 14). Dispense the medium into culture vessels and autoclave. Omit agar if preparing liquid medium. It is generally not advisable to autoclave hormones, vitamins, amino acids, or a number of other organics without first determining whether this will affect the medium deleteriously. In this case Manorama et al. (1986) report that they autoclaved their medium.

TABLE DEN-23. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for the culture of leaves from mature plants of *Dendrobium crumenatum* (Manorama et al., 1986)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxins				
10	2,4-Dichlorophenoxyacetic acid (2,4-D)	5	500 mg 100 ml^{-1} 95% ethanol[f,g]	1	
11	Naphthaleneacetic acid (NAA)	5	500 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
12	Benzyladenine (BA)	5	500 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additives				
16	Coconut water	200 ml	No stock	No stock	Measure
17	Casein hydrolysate	150	No stock	No stock	Weigh
	Sugar				
18	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[h]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]If auxins or cytokinin do not dissolve, a few drops of KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–17 to 500 ml distilled water (item 19), adjust pH to 5.3, add sugar (item 18), and adjust volume to 1000 ml with distilled water (item 19). Dispense solution into culture vessels, and autoclave. Omit agar if preparing liquid medium. It is generally not advisable to autoclave hormones, vitamins, amino acids, or a number of other organics without first determining whether this will affect the medium deleteriously. In this case Manorama et al. (1986) report that they autoclaved their medium.

Culture of *Dendrobium* Leaves

Leaves of *Dendrobium* Alice Spalding (*Dendrobium tokai* × *Dendrobium undulatum*) from plantlets grown in vitro when cultured like leaf explants of *Aranda* Noorah Alsagoff (Fu, 1978, 1979*a*, 1979*b*) produced a single plantlet at their bases. Proliferation and plantlet production did not occur when these leaves were cultured on modified MS medium. Leaves from mature plants cultured on modified MS medium also failed to proliferate (Fu, 1978, 1979*b*).

Plant Material. Leaves are taken from plantlets grown in vitro.

Surface Sterilization. Stems are wiped several times with a cheesecloth soaked in 95% ethyl alcohol (ethanol), cut into sections, and submerged for 15 min in 10% Clorox (10 ml Clorox diluted to 100 ml with distilled water). Following removal of the bracts, the stems are placed in 10% Clorox for 10 min and then dipped in sterile distilled water for 3 min (Kim et al., 1970).

Culture Vessels. Test tubes, Erlenmeyer flasks, and other containers are suitable.

Culture Conditions. The conditions used for *Dendrobium* in other procedures are suitable. Liquid media should be shaken.

Culture Medium. Modified Vacin and Went medium (Table Den-24) is most suitable.

Procedure. The leaves are placed in the medium and allowed to develop plantlets.

Developmental Sequence. A single plantlet is formed at the base of each leaf.

General Comments. This procedure is slow because only one plant is produced from each leaf. Also, if the leaves are from seedlings, the plants being propagated are of unknown quality.

TABLE DEN-24. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of *Dendrobium* leaf bases (Fu, 1978, 1979*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water	500 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[e]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 400 ml water, stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate it. To prepare a stock solution of chelated iron dissolve 3.73 mg chelating agent (Na_2EDTA) and 2.78 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Add items 1–8 to the 400 ml distilled water (item 10) that contains item 2. Adjust pH to 5.0, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Dispense medium into culture vessels, and autoclave. Omit agar if preparing liquid medium.

Vegetative Propagation of *Dendrobium* by Flower-stalk Cuttings

Following removal or wilting of unpollinated blossoms, the flower stalks of *Dendrobium* die. Hence, use of their buds amounts to the proper utilization of material that would otherwise be wasted. Therefore, a procedure for clonal propagation using flower-stalk cuttings (Singh and Sagawa, 1972) is economical and safe.

Plant Material. Flower stalks of *Dendrobium* having well-developed buds are used.

Surface Sterilization. Wipe stems several times with a cheesecloth soaked in 95% ethyl alcohol (ethanol), cut into sections, and soak for 15 min in 10% Clorox (v/v). Then remove bracts, transfer into 10% Clorox (v/v) for 10 min, and dip into sterile distilled water for 3 min (Intuwong et al., 1972).

Culture Vessels. Use 25×100 mm vials containing 12 ml medium.

Culture Conditions. Maintain cultures under continuous illumination of approximately 200 ft-c (provided by General Electric Power Groove white fluorescent lamps) at 23–29°C.

Culture Medium. Modified Vacin and Went medium (Table Den-21) is most suitable (Intuwong et al., 1972*a*, 1972*b*).

Procedure. Cut stems into sections, leaving 1–1.5 cm on either side of each bud. Surface-sterilize the flower-stalk sections, and remove discolored tissues from near the cut edges. Insert sections into the culture medium to just below the bud.

Developmental Sequence. Plantlets with well-developed shoots and roots can be obtained within a few months.

General Comments. Not all *Dendrobium* plants have a sufficient number of well-developed buds on flower stems. When such buds are available, however, this can be a very useful procedure, even if only one plantlet is obtained per bud.

Clonal Propagation of *Dendrobium aduncum, Dendrobium loddigesii,* and *Dendrobium transparens* through the Culture of Stem Nodes

Dendrobium aduncum and *Dendrobium loddigesii* are native to Hong Kong, where they grow in the hills. They are cultivated at the farm of the Kadoorie Agricultural Aid Association. *Dendrobium transparens* is native to Nepal, and its seeds were germinated in vitro as part of a Ph.D. dissertation project. Methods for the culture of their nodes were developed in the laboratory of Maureen A. Weatherhead, Botany Department, University of Hong Kong (Yam, 1989).

Plant Material. Stems of *D. aduncum* and *D. loddigesii* were taken from the Kadoorie farm plants. After surface sterilization discolored tissues at the cut ends of stems were removed and each stem was sectioned into 1-cm-long sections with a bud in the middle. Plantlets of *D. transparens*, three to five nodes long, were taken from aseptic culture, freed of leaves, sheaths, and roots, and sectioned as above.

Surface Sterilization. Leaves, dry sheaths, and roots were removed from the stems of the two Hong Kong species. The stems were first soaked for 30 min in deionized water and then washed with tapwater, scrubbed gently with a mild household detergent and very soft sponge, and rinsed. They were wiped with 75% aqueous ethanol and surface-sterilized by submerging them in 20% household bleach (20 ml bleach diluted to 100 ml with distilled water) for 25 min with agitation at 5-min intervals. The sterilant was removed by rinsing the stems three times with sterile deionized water. There was no need to sterilize the stems of *D. transparens* because they were taken from aseptic cultures.

Culture Vessels. Wide-mouth plastic 75-ml-capacity culture vessels (from Johns Mallinckrodt, Johns Division, Mallinckrodt Australia, Pty., Ltd.) were used in the original research. Other containers are also suitable.

TABLE DEN-25. **Knop's medium (Knop, 1884) modified for the culture of *Dendrobium* stem nodes (Yam, 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2$[b,c]	500	50 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4$[c]	125	12.5 g l^{-1}	10	
3	Monopotassiumphosphate, KH_2PO_4	125	12.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	125	12.5 g l^{-1}	10	
	Iron				
5	Ferric citrate[d]	10	1 g l^{-1}	10	
6	**Microelements**				
(a)	Boric acid, H_3BO_3	0.056	56 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2$	0.02	20 mg l^{-1}		
(c)	Copper chloride, $CuCl_2 \cdot 2H_2O$	0.054	54 mg l^{-1}		
(d)	Ferric chloride, $FeCl_3 \cdot 6H_2O$	0.5	500 mg l^{-1}		
(e)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.036	36 mg l^{-1}	1	One solution
(f)	Sodium EDTA, Na_2EDTA	0.8	800 mg l^{-1}		
(g)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	25 mg l^{-1}		
(h)	Zinc chloride, $ZnCl_2$	0.152	152 mg l^{-1}		
	Polyol				
7	*myo*-inositol	100	No stock	No stock	Weigh
	Vitamin				
8	Thiamine·HCl	0.4	40 mg 100 ml^{-1} 95% ethanol	1	
	Antiauxin				
9	*trans*-cinnamic acid (tCA)	70	7 g 100 ml^{-1} 95% ethanol	1	
	Sugar				
10	Sucrose[e]	30 g	No stock	No stock	Weigh
	Solvent				
11	Distilled water[f]				
	Solidifier				
12	Agar[e]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The number of waters of hydration is not given.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate it. To prepare a stock solution of chelated iron dissolve 3.73 mg chelating agent (Na_2EDTA) and 2.78 mg ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]As originally formulated the solution does not contain sugar or agar.

[f]Add all items to 900 ml distilled water (item 12), adjust pH to 5.6, add sugar (item 11), and adjust volume to 1000 ml with distilled water (item 12). Bring solution to a gentle boil, and add agar (item 13) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into culture vessels and autoclave.

Culture Conditions. Cultures were maintained under 16-h photoperiods of 2000 lx provided by Sylvania Gro Lux fluorescent tubes and 25 ± 2°C.

Culture Medium. Modified Knop's medium (Table Den-25) was used.

Procedure. Place the stem sections horizontally on the medium, and press their lower half into the agar with the bud facing up. Allow to remain in culture until plantlets form and develop into a large size for transfer to a potting mix.

Developmental Sequence. Buds swell following 1–2 weeks, shoots emerge within 3–6 weeks, and they form after 2 months. Plantlets are ready for transfer after 3–5 months in culture.

General Comments. *D. aduncum* forms plantlets and grows faster than the other two species. This method can be used in efforts to conserve these and possibly also other *Dendrobium* species with soft and fleshy stems. It may not be suitable for species (e.g., *D. acinaciforme*) that have hard stems.

Micropropagation of *Dendrobium chrysanthum* through Pseudobulb Segments

Dendrobium chrysanthum is an epiphytic orchid native to the Himalayas. Its dark green leaves and golden yellow flowers have made it popular with growers, which has resulted in overcollecting. This factor, along with a shrinking habitat, is putting pressure on the survival of this species. A mass rapid clonal propagation method was developed in India for use in conservation (Vij and Pathak, 1989).

Plant Material. Pseudobulb segments (0.5–1.0 cm long) from 40-week-old seedlings growing in vitro were used in the original research.

Surface Sterilization. It is not necessary to surface-sterilize the explants or their source because the seedlings are from axenic cultures in vitro.

Culture Vessels. Culture tubes, Erlenmeyer flasks, jars, and other culture vessels are suitable.

Culture Conditions. In the original research cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx. Light sources are not described, but it is reasonable to assume that the usual fluorescent and/or incandescent lamps would be suitable.

Culture Media. Several modifications of the MS medium were used in the original research. The two media listed here promote development of multiple shoots (Table Den-26) and "shoot bud formation and callusing" (Table Den-27).

Procedure. The explants are placed on the medium and allowed to develop.

Developmental Sequence. Shoot buds form within 5–6 days depending on the medium. The first leaf primordium is evident after 8–10 days. Roots can be seen 12–17 days following the start of culture. Plantlets develop after 18–25 days.

General Comments. Plantlets regenerated by this method are cytologically stable, and this suggests that it can be used for mass rapid clonal propagation of this orchid.

TABLE DEN-26. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for the culture of pseudobulb segments of *Dendrobium chrysanthum* (Vij and Pathak, 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	Urea, H_2NCONH_2[b]	25	2.5 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	8.60 mg l^{-1}		
	Amino acid				
9	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
11	Naphthaleneacetic acid (NAA)	1	100 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 7a) and iron salt (item 7b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference will probably have little effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, use a few drops of KOH to solubilize it.

[g]Keep refrigerated between uses.

[h]Add items 1–8 and 10 to 900 ml distilled water (item 16). Adjust pH as required, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid, hormones, and vitamins (items 9 and 11–14) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE DEN-27. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for callus and bud induction on pseudobulb segments of *Dendrobium chrysanthum* (Vij and Pathak, 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	Urea, H_2NCONH_2[b]	25	2.5 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
9	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
11	Naphthaleneacetic acid (NAA)	1 g	100 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
12	Kinetin	1	100 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 7a) and iron salt (item 7b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin does not dissolve, use a few drops of KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–8 and 10 to 900 ml distilled water (item 17), adjust pH as required, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask, and autoclave. Add amino acid, hormones, and vitamins (items 9 and 11–15) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

Isolation of Mesophyll Protoplasts of *Dendrobium*

The procedure developed for *Calanthe discolor* can also be used for *Dendrobium aggregatum*, *Dendrobium* Yukidaruma 'Queen,' and probably other species and hybrids. An incubation period of 2 h appears to be more suitable for *Dendrobium*. In the case of *Dendrobium* Yukidaruma 'Queen' isolation was 80%, survival was 64%, and the density as high as 4.13×10^5 protoplasts ml^{-1}. Isolation from *D. aggregatum* was 27% (Yasugi et al., 1986). In subsequent work Yasugi and his collaborators have been very successful with *Dendrobium* protoplasts.

Isolation of Protoplasts from Leaves and Roots of *Dendrobium herbaceum*

The method developed for *Acampe praemorsa* can be used to isolate protoplasts of *Dendrobium herbaceum*. Yields were 3.8×10^4 and 0.7×10^4 protoplasts per gram of fresh-weight tissue for leaves and roots respectively (Seeni and Abraham, 1986).

Transformation of Isoprenoids by *Dendrobium* in Tissue Culture

Tissue cultures of *Dendrobium phalaenopsis* maintained in vitro on media used for other orchids (Kukułczanka, 1985; Kukułczanka and Wojciechowska, 1983) transformed some isoprenoids (Mironowicz et al., 1987).

Shoot-tip Culture of a *Dendrobium* Hybrid

Shoot tips of *Dendrobium* Pompadour 'Pratabh' were cultured at the University of Nottingham (Voraurai, 1968).

Substrate Utilization by *Dendrobium* Tissues

Tissues obtained from apical meristems of *Dendrobium* Multico White (*Dendrobium* Ong Geok Kim × *Dendrobium* Ng Eng Chow) and initiated, maintained, and cultured on the Vacin and Went medium can utilize glucose, fructose, and sucrose as carbon sources. Fructose was utilized most readily (Hew et al., 1988). Sucrose was hydrolyzed extracellularly. The growth rate increased with increasing sugar levels. Respiration on fructose and glucose was higher than on sucrose. The respiration rate decreased on increased sugar concentrations.

The tissue took up ammonium ions (NH_4^+) in preference to nitrate (NO_3^-). Substantial uptake of nitrate was evident only after the ammonium ion was totally depleted (Hew et al., 1988).

Sugar Uptake and Invertase Activity in *Dendrobium* Tissues

Undifferentiated and differentiated tissue of *Dendrobium* Multico White (*Dendrobium* Ong Geok Kim × *Dendrobium* Ng Eng Chow) that was initiated, maintained, and cultured on the Vacin and Went medium exhibited no preference for sugars when grown on glucose, fructose, or sucrose. However, "fructose provided the best carbon source for both tissues." Sucrose was hydrolyzed by alkaline and acid invertases that are present in the soluble and cell-wall fractions (Hew and Mah, 1989). Invertase levels increased with time when the tissues were grown on sucrose-containing medium (Hew and Mah, 1989). Uptake of sugars by undifferentiated tissues was more rapid.

Isolation and Fusion of *Dendrobium* Protoplasts

Some of the earliest reports regarding isolation and fusion of orchid protoplasts are from the School of Biological Sciences, University of Science, Penang, Malaysia, and Institute für Pflanzenernährung, Abt. Gewebekultur, Justus Liebig University, Giessen, Germany, and deal with *Cattleya*, *Dendrobium*, *Paphiopedilum Phalaenopsis*, and *Renantanda* (Teo and Neumann, 1978*a*, 1978*b*, 1978*c*). Despite a statement that research was in progress, "focused on 'persuading' the cell cluster to form protoplasts," there are no further reports by these authors. More recently protoplasts of several orchids were isolated at the Departments of Floriculture and Ornamental Horticulture, and Plant Breeding and Biometry, Cornell University, Ithaca, NY, where fusion of *Dendrobium* protoplasts was also attempted (Price and Earle, 1984).

Plant Material. Protoplasts were isolated from the following:

1. Purple petals of *Dendrobium* Beach Girl [*Dendrobium* Pauline (*Dendrobium phalaenopsis* × *Dendrobium undulatum*) × *Dendrobium schulleri*] × *Dendrobium* Takami Kodama [*Dendrobium* Taurus (*Dendrobium taurinum* × *Dendrobium undulatum*) × *Dendrobium taurinum*], and
2. Green leaves (6 cm long) and purple petals of open flowers of *Dendrobium* Louis Bleriot (*Dendrobium schroederianum* × *Dendrobium superbiens*).

Surface Sterilization. The procedure used with *Angraecum giryamae* is suitable.

Culture Vessels. For containers used in the isolation of protoplasts see *A. giryamae* (p. 167, Vol. I). Plastic Petri dishes 60 mm in diameter are used for fusion.

Culture Conditions. Conditions during protoplast isolation should be the same as those for *A. giryamae*.

Culture Media. For isolation and culture media, and preparation used to check the viability of protoplasts and some of the other solutions, see *A. giryamae*. Washing is carried out with a solution consisting (per liter) of 91.1 g sorbitol, 7.5 g calcium

chloride ($CaCl_2 \cdot H_2O$), and 976 mg 2-[*N*-morpholino]ethanesulfonic acid, pH 9.5. A medium consisting (per liter) of 90.1 g (0.5 mol) glucose, 514.6 mg (3.5 mmol) calcium chloride ($CaCl_2 \cdot 2H_2O$), and 95.3 mg (0.7 mmol) monopotassium phosphate (KH_2PO_4) (GCP solution) is used for protoplast fusion.

Procedure. Protoplast isolation and viability determination procedures are the same as those used with *A. giryamae*. The following procedure is used for fusion (all steps must be carried out under sterile conditions; tools, containers, and solutions must be sterilized):

1 The protoplast sources (green leaves of *Dendrobium* Louis Bleriot and purple petals of *Dendrobium* Beach Girl × *Dendrobium* Takami Kodama) are incubated separately in the 2% Cellulysin solution (see *A. giryamae*) for 1 day.
2 Protoplasts from each source are washed (see *A. giryamae*) and resuspended separately in the GCP solution (see previous section on culture media).
3 After counting, the suspensions should be adjusted with the GCP medium to contain approximately 500,000 protoplasts ml^{-1}.
4 Equal volumes of each suspension are mixed in a test tube (the suspension should still contain 500,000 protoplasts ml^{-1}).
5 Several 0.1-ml (100 µl) drops of the mixture are placed in a 60-mm-diameter plastic Petri dish (a sterile pipette is used to measure the required volume). The solution should be observed to determine whether the protoplasts are still alive. Protoplasmic streaming is a good indication that they are.
6 Polyethylene glycol (PEG) 1540, 0.33-*M* solution (see procedure for *A. giryamae*), 4 drops from a Pasteur pipette (see lists in Chapter 2) per drop of protoplast, is added. The mixture should be examined after 15–30 min to determine if the protoplasts are alive and whether fusion is taking place.
7 Protoplasts are washed four times by adding 4 drops of the high-calcium pH 9.5 solution (see Culture Media section above) per protoplast drop. Excess solution is removed by blotting each time after allowing the protoplasts to settle. Care should be taken not to remove protoplasts during the blotting.
8 The Petri dish is flooded with the GCP solution and excess fluid is removed after the protoplasts have settled.
9 Nutrient medium (see Tables Angcm-1 or Angcm-2) is added.

Developmental Sequence. Several problems were encountered during the fusion process: (1) treatment with PEG produced a very low frequency of fusion of dissimilar protoplasts; (2) many protoplasts were lost during washing; and (3) green protoplasts lysed within 2 days.

General Comments. "It is clear that further refinements to increase frequency of fusion and survival . . . are needed . . . for fusion products to be useful . . . Protoplast preparations should involve cells with high potential for regeneration" (Price and Earle, 1984). This is so, and the present procedure is a good start.

Elimination or Inhibition of Contaminants in Contaminated Tissue Cultures of *Dendrobium*

> When a few cultures among many of a particular clone become contaminated, contamination is merely a nuisance; it is certainly not an irreplaceable loss. However, when the contaminated culture is the only one of an expensive plant, the loss may be considerable. Faced with this situation we have developed a procedure for the decontamination of contaminated cultures. (Soediono, 1983*a*)

Plant Material. Callus, PLBs, and plantlets obtained from lateral bud cultures of *Dendrobium* Tay Swee Keng were used in the original research. They were taken from contaminated cultures.

Surface Sterilization. Callus, PLBs, and plantlets taken from contaminated cultures are dipped for 1 and no more than 2 s in 70% ethanol and then rinsed three times with sterile distilled water.

Culture Vessels. This procedure is intended to decontaminate existing cultures regardless of the initial method. Therefore the culture vessels used for it should be the same as the ones that are already being utilized.

Culture Conditions. The conditions should be the same as the ones already in use except that the first few changes of medium, which contains bactericides and fungicides, are placed on a shaker (see procedure for *Dendrobium* Jaquelyn Thomas 'White', p. 539, Vol. I; Soediono 1983*b*).

Culture Media. There is no specific medium for this procedure. The medium or media should be the same as those employed by the method that was used to start and maintain the contaminated cultures. Two fungicides and two bactericides are added to the culture medium as described here:

- Benlate or benomyl: 2.5 mg of one of several commercial products containing 50% active principle, obtainable at retail nurseries (the other 50% must be inert). The powder should be suspended in 1–2 ml of 95% ethanol in a sterile vial and shaken vigorously for 2–3 min, allowed to stand, shaken again for 1 min, placed on a shelf for an additional 15 min, and added under sterile conditions to autoclaved culture medium after it has cooled. If any material remains on the walls of the vial, it should be washed into the medium with sterile distilled water.
- Nystatin: 5000 units from Mycostatin Squibb oral suspension containing 100,000 units ml^{-1}. This preparation is available from local pharmacies. It can be diluted to 100,000 units per 5 ml with sterile distilled water to facilitate distribution. The suspension must be shaken vigorously before the appropriate amount is added under sterile conditions to autoclaved culture medium after it has cooled.
- Penicillin G: 8000 units (available as injectable penicillin from local pharmacies) suspended in sterile distilled water and added under sterile conditions to autoclaved culture medium after it has cooled.

- Gentamycin (purchased as injectable Garamycin Schering in sterile distilled water from a local pharmacy): 2.5 mg added under sterile conditions to autoclaved culture medium after it has cooled.

These substance are also available from chemical supply houses. When purchased from pharmacies the last three may require a prescription. Some of the commercial preparations may contain inert substances that are insoluble and can cause cloudiness in the medium. Care should be taken not to purchase preparations that contain other active substances.

Procedure. Take contaminated callus, PLBs, and plantlets from cultures, dip in the alcohol, wash with sterile distilled water, and suspend in the medium that contains the fungicides and bactericides. Place the cultures on a shaker, and observe daily. Some of the more extensively infected callus masses, PLBs, and plantlets may turn white or brown and die. Others may turn white but recover and become green again. A number will remain green. Those that turn white and become green again and the ones that do not lose their color should be moved to fresh medium containing bactericides and fungicides. This may have to be repeated several times until the contaminants are eliminated.

In some cases the contaminants may not be destroyed by the fungicides and bactericides but merely kept in check. Should this happen, the tissues may have to be cultured continuously in a medium or media that contain the fungicides and bactericides (perhaps even at higher levels) until plantlets are ready for community pots.

Developmental Sequence. Depending on the severity of infection and contamination some callus masses, PLBs, and plantlets may follow several sequences: they may die, turn white and then become green again, never lose their color, or continue to grow.

General Comments. This procedure is complicated, costly, and uncertain. It offers no panaceas but may prove to be of considerable value when rare and/or expensive explants must be saved.

The compounds and concentrations listed above do not damage seedlings of *Cattleya aurantiaca* and *Stanhopea oculata*, and *Phalaenopsis* flower-stalk cultures. It is therefore reasonable to assume that they can also be used with other orchids.

Dr. Teoh Eng Soon of the Teoh Clinic in Singapore has suggested that some operators may be sensitive to these compounds or become sensitized after repeated contact with them. For this reason he advises operators to wear gloves and masks when handling them.

Plantlet Production from Stem Nodes of *Dendrobium aduncum*, *Dendrobium loddigesii*, and *Dendrobium transparens*

Plantlets can be produced from stem nodes by the culture method developed for *Bletilla striata*.

Dendrobium Propagation through the Culture of Shoot Meristems

New tissue culture methods are constantly being developed for orchids which are popular with hobby growers or of commercial importance. One reason for this may be because genera and species, especially those from dissimilar climatic zones as well as diverse hybrids, require different media and protocols. Another reason is the large number of investigators who are constantly striving to develop better and more efficient micropropagation methods. For these or other reasons a shoot meristem propagation method was developed at the Shroff Research Institute, Bombay, India (Sharon and Vasundhara, 1990).

Plant Material. Shoot tips were excised from "*Dendrobium* Joannie Ostenhault seedlings, procured from Singapore." A search on the World Wide Web and through all volumes of *Sander's List of Orchid Hybrids* did not locate such a hybrid. A white hybrid named *Dendrobium* Jonnie Osterholt (*Dendrobium phalaenopsis* × *Dendrobium* Walter Oumae, chromosome number $2n = 76$ or 78) does exist and is probably the orchid which was used. The age of the seedlings is not given in the original paper. However, the fact that the explant sources were surface-sterilized indicates that they were old enough to be removed from flasks, potted, and grown under horticultural conditions.

Surface Sterilization. A thorough washing with tap water is the first step in the surface sterilization process. The second step is sterilization with "10% solution of commercial bleach [presumably 10 ml of a household bleach like Clorox diluted to 100 ml with distilled water] with 2 drops of Tween 20 [available from www.sigmaaldrich.com or http://search.wako-chem.com; a few drops of mild household detergent can also be used]" for 10 min. Two rinses with sterile distilled water are the last steps.

The explants for the experiments leading to this procedure were taken from *Dendrobium* Jonnie Osterholt, which is a hybrid produced by the cross *Dendrobium phalaenopsis* × *Dendrobium* Walter Oumae.

Culture Vessels. Erlenmeyer flasks, 250-ml capacity containing 40–50 ml medium, are suitable.

Culture Conditions. In the original research the cultures were maintained at 24–25°C and 96–98% relative humidity (RH). The cultures were kept in the dark for 48 h after explants were placed in the first medium (no explanation is provided for this step), and moved to 8-h photoperiods of 2000 lx until the start of differentiation when the light period was increased to 16 h (there is no explanation for this change in lighting). Light was provided by fluorescent tubes (type and manufacturer are not given). Standard culture room conditions are probably also suitable. Liquid cultures were agitated (type of agitation not described) until PLBs formed. PLBs were cultured on solid medium.

Culture Media. Three media are used for this procedure, all based on the Vacin and Went (VW) medium (Vacin and Went, 1949). The first medium is liquid and contains 15% coconut water (CW; Table Den-28). This medium is used to bring about the production of PLBs. For plantlet formation PLBs must be moved to a solid medium which also contains 15% CW (Table Den-29). Rooting occurs on a third medium (Table Den-30) which contains reduced sugar levels. The original paper states that "slow elimination of [CW] from the nutrition regime favoured accelerated root development" but does not indicate how the "slow elimination" was carried out and the amounts of CW in the rooting medium (Table Den-30) at each step of the process. The medium recommended here (Table Den-30) contains no CW. If CW is used 2–5% or at most 10% (v/v) is sufficient.

Procedure. Explants should be placed in liquid medium (Table Den-28) after surface sterilization and maintained on a shaker in the dark for 48 h before being illuminated. PLBs which form in the liquid medium (Table Den-28) must be moved to solid substrate (Table Den-29) for formation. Shoots which form on the first solid medium (Table Den-29) require a transfer to a third substrate (Table Den-30) for rooting. When the rooted plants are of appropriate size they can be potted.

Developmental Sequence. PLBs form in the liquid solution (first substrate), shoots develop on the first solid medium (second substrate), and roots emerge on the second nutrient mixture (third substrate).

General Comments. This is a relatively simple procedure which seems to be effective for *Dendrobium* Jonnie Osterholt. It will probably be suitable for a number of other *Dendrobium* species and hybrids.

TABLE DEN-28. **Vacin and Went (VW) medium (Vacin and Went, 1949) as modified for the production of protocorm-like bodies (PLBs) from *Dendrobium* shoot-tip explants (Sharon and Vasundhara, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water[e]	150.0 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and preservatives (that may have undesirable effects on cultures).

[f]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 3), bring volume to 900 ml with distilled water (item 10), adjust pH to 5–5.4, add sugar (item 9), raise volume to 1000 ml with distilled water (item 10), pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949), and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium (Murashige and Skoog, 1962) will probably be suitable.

TABLE DEN-29. **Vacin and Went (VW) medium (Vacin and Went, 1949) as modified for the production of plantlets from protocorm-like bodies (PLBs) derived from *Dendrobium* shoot-tip explants (Sharon and Vasundhara, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water[e]	150.0 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[e]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 9) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and preservatives (that may have undesirable effects on cultures).

[f]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 3), bring volume to 900 ml with distilled water (item 10), adjust pH to 5–5.4, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went,1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

TABLE DEN-30. **Vacin and Went (VW) medium (Vacin and Went, 1949) as modified for the production of plantlets from protocorm-like bodies (PLBs) derived from *Dendrobium* shoot-tip explants (Sharon and Vasundhara, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 9) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Add items 1 and 3–7 to the 500–700 ml of distilled water (item 9) which contain the calcium phosphate (item 3), bring volume to 900 ml with distilled water (item 9), adjust pH to 5–5.4, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring the solution to a gentle boil and add the agar (item 10) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

Mass Rapid Clonal Propagation of *Dendrobium moschatum* through the Culture of Stem Disks

Dendrobium moschatum (Fig. Den-2A) is an endangered species. A micropropagation protocol for it was developed to provide plants for the horticultural trade and reduce pressure on populations in the wild (Kanjilal et al., 1999).

Plant Material. Transverse sections of shoots, 1–1.5 mm thick, were taken from seedlings growing in vitro and cultured.

Surface Sterilization. Explants taken from seedlings growing in vitro do not require surface sterilization.

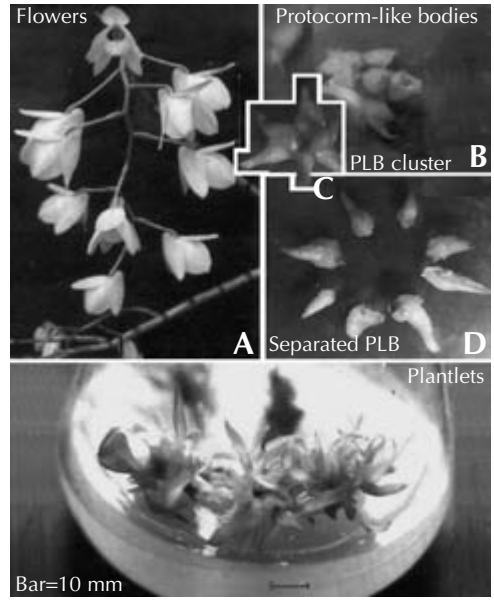


FIG. DEN-2. **Micropropagation of *Dendrobium moschatum* (Kanjilal et al., 1999).**

Culture Vessels. Erlenmeyer flasks, 250 ml, are suitable for the initial explants and proliferation of PLBs. Larger flasks, 500 ml, may be required for plantlet growth and development.

Culture Conditions. The original research cultures were maintained at 25 ± 2°C under 16-h photoperiods of 1000 lx provided by cool white lamps (manufacturer not listed). Standard culture room conditions are also suitable. Liquid cultures should be placed on a rotatory shaker at 80–120 rpm.

Culture Media. Explants should be cultured in a modified (Table Den-31) liquid Knudson C (KC) medium (Knudson, 1946) containing 15% coconut water (CW), 2 mg NAA l^{-1} and 3 mg BA l^{-1} until PLBs form. PLBs proliferate and plantlets develop on a solid version of the same medium (Table Den-32).

Procedure. Explants are cultured in the first medium (Table Den-31) to induce PLBs (Fig. Den-2A–2D). The PLBs should be moved to the second medium (Table Den-32) for proliferation and plantlet development (Fig. Den-2E).

Developmental Sequence. PLBs form on the explants. They proliferate and plantlets develop on the solid medium.

TABLE DEN-31. **Knudson C (KC) medium (Knudson, 1946) as modified for protocorm-like body (PLB) production from stem disk sections of *Dendrobium moschatum* (Kanjilal et al., 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement[d]				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
7	Naphthaleneacetic acid[e]	2.0	2 mg 100 mg 100 ml^{-1} 95% $ethanol^{-1}$	1	
	Cytokinin				
8	Benzylaminopurine (BAP)[e]	1.0	2 mg 100 mg 100 ml^{-1} 95% $ethanol^{-1}$	1	
	Complex additive				
9	Coconut water (CW)[f]	150.0	No stock	No stock	Measure
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. Knudson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$, l^{-1} but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored.

[d]There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If incorporation of additional microelements is necessary those used in the MS medium are suitable.

[e]If the auxin or cytokinin fails to dissolve in the stock solution add a few drops of 0.1N KOH or 0.1N HCl respectively. Keep frozen between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and preservatives (that may have undesirable effects on cultures).

[g]Add items 1–6 and 9 to 750 ml of distilled water (item 11), adjust pH to 5.2, add sugar (item 10), raise the volume to 1000 ml with distilled water, pour the solution into a 2-l flask and autoclave. Add the auxin (item 7) and the cytokinin (item 8) to the medium, stir vigorously with a sterile stirrer or swirl well to mix the hormones with the rest of the solution and dispense the medium into preautoclaved culture vessels.

General Comments. This is a procedure for mass rapid propagation of seedlings. It can be a starting point for a method which can be used to propagate mature plants. Its drawback is that it can not be employed to select desirable forms because the quality of seedlings is not known. An outstanding feature of the original paper is the statistical analysis of results and the effects of plant hormones.

TABLE DEN-32. **Knudson C (KC) medium (Knudson, 1946) as modified for protocorm-like bodies (PLBs) and plantlet development of *Dendrobium moschatum* (Kanjilal et al., 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement[d]				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
7	Naphthaleneacetic acid[e]	2.0	2 mg 100 mg 100 ml^{-1} 95% $ethanol^{-1}$	1	
	Cytokinin				
8	Benzylaminopurine (BAP)[e]	1.0	2 mg 100 mg 100 ml^{-1} 95% $ethanol^{-1}$	1	
	Complex additive				
9	Coconut water (CW)[f]	150.0	No stock	No stock	Measure
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. Kundson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$, l^{-1} but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored.

[d]There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If incorporation of additional microelements is necessary those used in the MS medium are suitable.

[e]If the auxin or cytokinin fails to dissolve in the stock solution add a few drops of 0.1N NaOH or 0.1N HCl respectively. Keep frozen between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and preservatives (that may have undesirable effects on cultures).

[g]Add items 1–6 and 9 to 750 ml of distilled water (item 11), adjust pH to 5.2, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar (item 12) can also be added to the cold solution which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the auxin (item 7) and the cytokinin (item 8) to the warm and still liquid medium, stir vigorously with a sterile stirrer or swirl well to mix the hormones with the rest of the solution and dispense the medium into preautoclaved culture vessels. Omit agar (item 12) if preparing liquid medium.

In Vitro Propagation of *Dendrobium moschatum* through Thidiazuron-induced High Frequency Shoot Proliferation

Dendrobium moschatum flowers are very attractive. This may be one reason for two different approaches to its micropropagation (Nayak et al., 1997*b*).

Plant Material. Shoot tips consisting of one or two nodes and 6–15 mm in length taken from seedlings in vitro were cultured in the original research. The donor seedlings were maintained at 25 ± 1°C under 35 μE m^{-2} s^{-1} (duration of photoperiods and source of illumination were not described) and 60% relative humidity (RH).

Surface Sterilization. Explants taken from seedlings growing in vitro do not require surface sterilization. However, they must be rinsed to remove medium residues.

Culture Vessels. The vessels used for *Cymbidium aloifolium* are suitable (see procedure by Nayak et al., 1997*b* in the *Cymbidium* section, p. 419, Vol. I).

Culture Conditions. The conditions used for *Cymbidium aloifolium* are suitable (see procedure by Nayak et al., 1997*b* in the *Cymbidium* section).

Culture Media. The second and third media used for *Cymbidium aloifolium* (see Tables Cym-44 and Cym-45) are suitable (see procedure by Nayak et al., 1997*b* in the *Cymbidium* section). However the first medium (see Table Cym-43) must be different (see Table Den-33).

Procedure. The procedures used for *C. aloifolium* are suitable (see method by Nayak et al., 1997*b* in the *Cymbidium* section) except that the explants must be placed horizontally on the medium.

Developmental Sequence. Except for the different response to placement on the medium, development of *D. moschatum* explants is similar to that of *C. aloifolium* (Nayak et al., 1997*b* in the *Cymbidium* section).

General Comments. Some years ago Professor G. Ray Noggle, a pioneer in the study of vitamin effects on orchid seed germination, suggested that despite the multitude of in vitro media which have been formulated for their culture most orchids could probably grow on one medium. The findings with *C. aloifolium, D. moschatum, Dendrobium aphyllum*, and *Dendrobium nobile* (Nayak et al., 1997*b*) seem to support Professor Noggle's suggestion.

TABLE DEN-33. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of shoot explants of *Dendrobium moschatum* and *Dendrobium aphyllum* (Nayak et al., 1997*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, $KH_2PO_4 \cdot H_2O$	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	62.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Thidiazuron	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Dissolve the thidiazuron (TDZ) in a small volume of 0.5N HCl and make up to volume (100 ml) with 70% or 95% ethanol. The amount of TDZ used in the original research was 4.5 µmol (991.4 µg) l^{-1}. A slightly larger amount, 1 mg (1000 µg or 4.53 µmol), is recommended here for convenience (1.0 mg is easier to weigh than 0.991 mg). The difference (0.09 mg or 9 µg or 0.04 µmol) is insignificant and can be ignored. Sticklers for accuracy and those with infinite patience and/or plenty of time on their hands may still use 0.991 mg l^{-1}.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.8, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (items 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

In Vitro Propagation of *Dendrobium aphyllum* through Diazuron-induced High Frequency Shoot Proliferation

The procedures used for *Dendrobium moschatum* are also suitable for *Dendrobium aphyllum* (Nayak et al., 1997*b*).

Rapid Micropropagation of *Dendrobium nobile* through the Culture of Thin Cross Sections

The method used for the culture of thin cross sections of *Cymbidium aloifolium* is also suitable for *Dendrobium nobile* explants of the same nature (see Rapid Micropropagation of *C. aloifolium* through the Culture of Thin Cross Sections by Nayak et al., 2002 in the *Cymbidium* section, p. 423, Vol. I) except for the medium used for the culture of explants (Fig. Den-3; see Table Cym-47). A different medium should be used for *D. nobile* (Table Den-34).

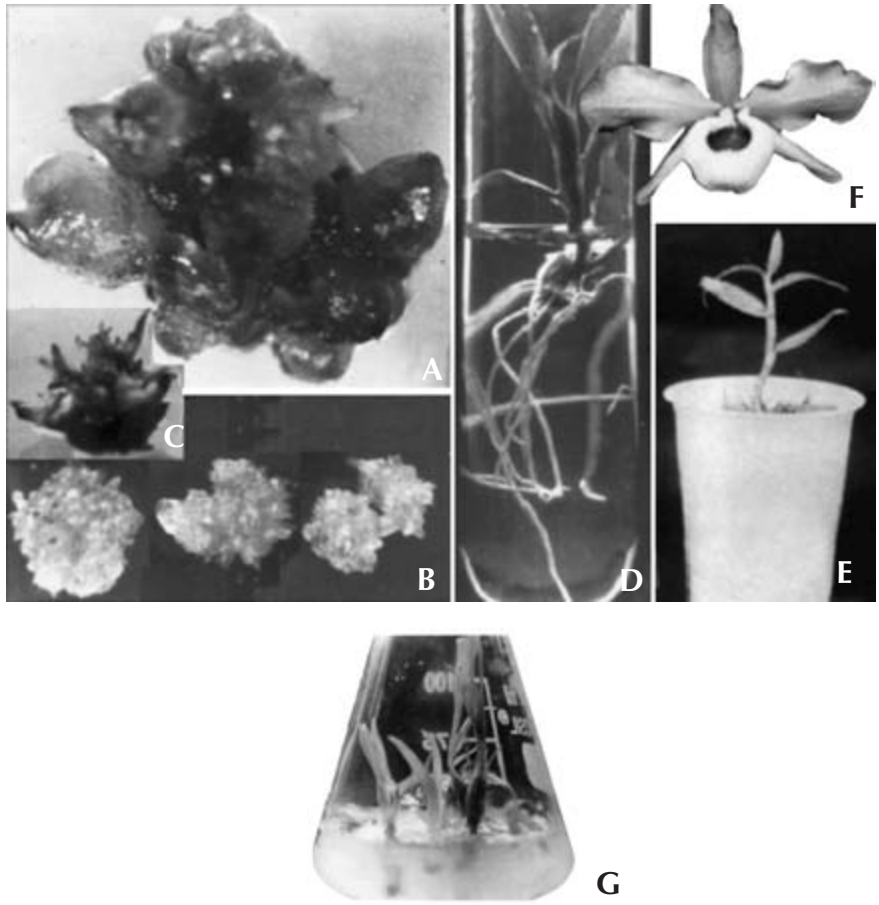


FIG. DEN-3. **Culture of thin sections of *Dendrobium nobile*. A. PLBs. B. Formation of multiple shoots. C. Initiation of shoots. D. Plantlet in test tube. E. Plant in pot. F. Flower. G. Plantlets in a flask. (Sources: A–E, Nayak et al., 2002; F, http://w1.213.telia.com.)**

TABLE DEN-34. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the culture of thin cross section explants of *Dendrobium nobile* (Nayak et al., 2002)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, $KH_2PO_4 \cdot H_2O$	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	62.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzyladenine (BA)	2.5	250 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	[Solidifier]				
[16	Agar[g]	8.0 g	No stock	No stock	Weigh]

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1N HCl.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.8, add sugar (item 14), and bring volume to 1000 ml with distilled water (item 15). [Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved] pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. More explants proliferate in liquid than on solid medium (95% vs 89%) and produce a higher number of PLBs (33.5 vs 28.2 PLB $explant^{-1}$). That is why this medium should be liquid. However if a shaker is not available the explants can be cultured on a solid medium. To allow for such an eventuality the solidifier (i.e., agar, item 16) in the table and instructions regarding its incorporation in this footnote are between bold face brackets.

Micropropagation of *Dendrobium* Queen Sonia

One of the most popular and beautiful dendrobiums grown for many years as a cut flower is the hybrid *Dendrobium* Sonia. It should not be confused with *Dendrobium* Queen Sonia which is an incompatible hybrid, also popular as a cut flower, that was not registered with the international registration authority for orchid hybrids (Professor S. Ichihashi, Aichi University of Education, Aichi, Japan, personal communication). Therefore this hybrid is not listed in *Sander's List of Orchid Hybrids* as seems to be the case with a number of other hybrids used to produce cut flowers. A method for the clonal propagation of *Dendrobium* Queen Sonia was developed (Aswath, 2001), despite the fact that its parentage is unknown and an argument can be made that since it is not registered this hybrid does not exist officially.

Plant Material. Apices, 5 mm (no indication is given in the original report whether this is length, width, or diameter), were excised from shoot tips, 1 cm (presumably) in length (the paper gives no information).

Surface Sterilization. The shoot tips must be washed thoroughly with a surfactant (Teepol, available from www.sigmaaldrich.com, www.fishersci.com, or another source, was used in the original research, but any mild liquid household or baby detergent is suitable) and running water (the paper suggests an excessive 2-h wash; 10–15 min is sufficient). The washing should be followed by a 90-s dip in 0.1% mercuric chloride [100 mg mercuric chloride ($HgCl_2$, available from the sources listed above; this substance is very toxic and should be handled with care) in 100 ml distilled water] and several subsequent rinses with sterile distilled water.

Culture Vessels. Erlenmeyer flasks, 100 ml capacity, containing 50 ml are recommended by the author. However a smaller volume (25–30 ml) and/or other containers can also be used.

Culture Conditions. Cultures should be maintained at $25 \pm 2°C$ and 8-h photoperiods of 2000 lx or standard culture room conditions. Liquid cultures should be placed on a shaker (a 100-rpm gyrorotatory shaker was used in the original research).

Culture Media. A modified liquid Vacin and Went (VW) medium (Vacin and Went, 1949) is used in this procedure. PLBs are induced by placing the explants in a liquid medium which contains coconut water (CW; Table Den-35). The PLBs should be moved to a medium which also contains CW, but no sugar and growth hormones (Table Den-36) for proliferation. Unfortunately it is not clear whether this medium is liquid or solid. The solution recommended here (Table Den-36) is liquid because proliferation in liquids is usually better than on solid media. If this medium proves unsuitable another medium may work (Table Den-11). To produce plantlets the PLBs should be cultured on a third medium (Table Den-37).

Procedure. After being cut from plants the shoot tips (presumably 1 cm long) must be washed. The apices should be excised following removal of the outer leaves,

TABLE DEN-35. **Vacin and Went (VW) medium (Vacin and Went, 1949) as modified for the culture of shoot-tip explants of *Dendrobium* Queen Sonia (Aswath, 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water[e]	200.0	No stock	No stock	Measure
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 m l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored. The modification used for *Dendrobium* Queen Sonia uses ferric tartrate. Chelated iron is preferable (items 6a, 6b).

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and preservatives (that may have undesirable effects on cultures).

[f]Add items 1 and 3–8 to the 500 ml of distilled water (item 10) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 10), adjust pH to 5.3, add sugar (item 9), and raise volume to 1000 ml with distilled water (item 10). There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

sterilized, rinsed, and placed in culture. They form PLBs in the first medium (Table Den-35). These PLBs proliferate when cultured in the second solution (Table Den-36). When moved to the third (Table Den-37) substrate the PLBs produce plantlets. There is also an unusual subroutine in this method. PLBs from the second medium are moved to a callus-inducing solution. The callus is then cultured on yet another medium to induce PLBs which are used to produce plantlets. This subroutine is not only unnecessary (because plantlets can be produced on the third medium directly from PLBs generated in the second solution), but also unwise (since the extra steps of callus induction and the subsequent PLBs production can induce undesirable mutations).

TABLE DEN-36. **Vacin and Went (VW) medium (Vacin and Went, 1949) as modified for the proliferation of protocorm-like bodies (PLB) of *Dendrobium* Queen Sonia (Aswath, 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water[e]	200.0	No stock	No stock	Measure
	Solvent				
9	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500 ml of distilled water (item 9) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored. The modification used for *Dendrobium* Queen Sonia uses ferric tartarate which is not as good a source of iron as the chelated form (items 6a, 6b).

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and preservatives (that may have undesirable effects on cultures).

[f]Add items 1 and 3–8 to the 500 ml of distilled water (item 9) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 9), adjust pH to 5.3 and adjust volume to 1000 ml with distilled water (item 9). There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

Developmental Sequence. PLBs form in the first medium, proliferate in the second, and produce plantlets on the third (Fig. Den-4).

General Comments. Except for the subroutines which should be avoided (and therefore are not described here), this method is relatively simple and seems to be effective. It may also prove suitable for other *Dendrobium* hybrids. The only weakness is a paper which is not written as clearly and as well as it could have been.

TABLE DEN-37. **Vacin and Went (VW) medium (Vacin and Went, 1949) as modified for plantlet production from protocorm-like bodies (PLBs) of *Dendrobium* Queen Sonia (Aswath, 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
8	Indoleacetic acid (IAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin				
9	Benzylaminopurine	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
10	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Agar[f]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500 ml of distilled water (item 11) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 ml l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored. The modification used for *Dendrobium* Queen Sonia uses ferric tartrate which is not as good a source of iron as the chelated form (items 6a, 6b).

[e]If the auxin or cytokinin fails to dissolve in the stock solution add a few drops of 0.1N KOH or 0.1N HCl respectively.

[f]Add items 1 and 3–8 to the 500 ml of distilled water (item 11) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 11), adjust pH to 5.3, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar (item 12) can also be added to the cold water which is then brought to a boil and stirred. When the agar (item 12) is completely dissolved pour the solution into a 2-l flask and autoclave. Gelrite, 3 g l^{-1}, can be used in place of agar. Add the auxin (item 8) and cytokinin (item 9) to the warm and still liquid solution, swirl to mix well and distribute into culture vessels. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

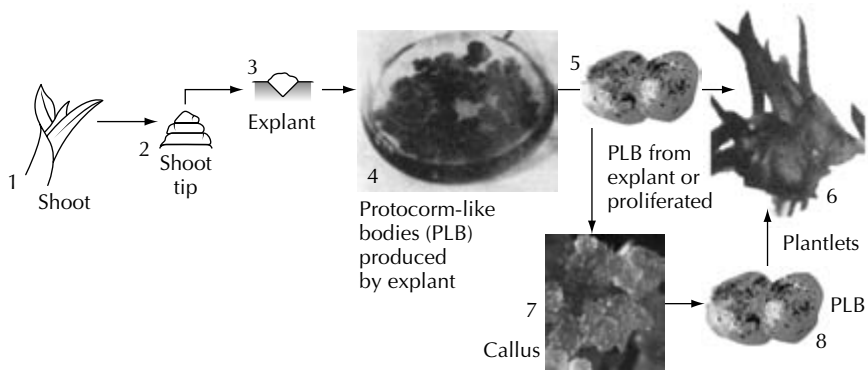


FIG. DEN-4. **Schematic representation of micropropagation of *Dendrobium* Queen Sonia. Steps 1–6 are advisable and should be used for micropropagation; steps 7 and 8 are not necessary and not advisable. (Sources: 1–4, 6, Oyamada and Takano, 1990 referring to another procedure; 5, 7, World Wide Web.)**

In Vitro Propagation of *Dendrobium* Sonia

An important cut flower orchid, *Dendrobium* Sonia (*Dendrobium* Caesar × *Dendrobium* Tomie Drake, registered in 1984) is not the same hybrid as *Dendrobium* Queen Sonia (the subject of the previous procedure) which is an unregistered cross. A method for in vitro propagation of *Dendrobium* Sonia was developed in India (Prasad et al., 2001).

Plant Material. Apices including meristems and leaf primordia taken from 5-cm-long shoot tips are cultured.

Surface Sterilization. After being freed of all leaves and debris, shoot tip segments, 5 cm long, taken from young pseudobulbs and small plants must be washed first with running water and then with Tween 20 surfactant (available from www.sigmaaldrich.com, www.fishersci.com, or other sources) or a mild household or baby detergent. Washed shoot tips should be sterilized by immersing them in 0.1% mercuric chloride (100 mg $HgCl_2$ 100 ml^{-1} distilled water; this is a very toxic substance which is available from the sources listed above) for 90 seconds. This must be followed by three rinses with sterile distilled water.

Culture Vessels. Erlenmeyer flasks, test tubes and other containers are suitable. Smaller containers can be used for initial explants and larger ones when plantlets become larger. As a rule culture vessels should contain culture medium equivalent to approximately 25–30% of their volume.

Culture Conditions. Cultures should be maintained at 24 ± 2°C and 16-h photoperiods of 50 μE m^{-2} s^{-1} or under standard laboratory conditions.

Culture Media. There are no specific recommendations in the original paper. The media recommended here were adduced from the tables in it. Explants should be cultured initially in a modification (Table Den-38) of the MS medium (Murashige and Skoog, 1962) until they form multiple shoots. When these shoots reach a few centimeters in size they should be separated and cultured on the second medium (Table Den-39).

Procedure. Explants from surface-sterilized shoots should be cultured on the first medium until shoots are formed. These shoots are cultured on the second medium.

Developmental Sequence. The explants start to differentiate approximately 11 days after the start of culture. Leaf formation starts after about 25 days. An average of 11 shoots arise from each explant. After these shoots are moved to the second medium they produce leaves and roots and form plantlets.

General Comments. This seems to be a simple but effective procedure. It is unfortunate that the original paper does not provide clear recommendations.

Dendrobium Sonia
(www.thailandcentral.com)

TABLE DEN-38. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the culture of shoot-tip explants of *Dendrobium* Sonia (Prasad et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	62.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.2–5.4, add sugar (item 15), and raise the volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10, 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE DEN-39. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the culture of *Dendrobium* Sonia shoots (Prasad et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	62.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.2–5.4, add sugar (item 15), and bring volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10, 11) and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

In Vitro Multiplication of *Dendrobium* Under Carbon Dioxide Enrichment

Tissue culture media usually include sugar as an energy source either because explants lack the ability to fix enough carbon to sustain themselves or as a supplemental carbon source. Under the assumption that the inclusion of sugar in culture media "not only involve[s] an additional [financial] expenditure on the carbon source (usually sucrose), but also invite[s] bacterial and fungal contaminations" and in an effort to eliminate or simplify acclimatization an attempt was made to develop a photoautotrophic method for the micropropagation of *Dendrobium* (Mitra et al., 1998).

Plant Material. *Dendrobium* (no species or hybrid name given) shoots used in this experiment were taken from plantlets growing in vitro. In practical laboratories plantlets to be cultured using this method will have to be produced through another *Dendrobium* culture procedure or by germinating seeds.

Surface Sterilization. There is no need to surface-sterilize shoots growing in vitro.

Culture Vessels. Glass jars, 250 cm^3, with a plastic screw cap were used in the original research. To increase air movement holes were punched in the caps and filled with cotton. Other culture vessels can also be used (one example: plastic disposable, autoclavable, and inexpensive containers such as those available from www.caissonlabs.com).

Culture Conditions. In the original experiment the cultures were maintained for 30 days in a growth chamber at 25 ± 3°C, relative humidity (RH) of 60–70%, and 16-h photoperiods of 42–45 μmol m^{-2} s^{-1} (source not listed) in ambient (0.6 g CO_2 m^{-3}) or carbon-enriched (40 g CO_2 m^{-3}) air.

Culture Media. Vacin and Went (VW) medium (Vacin and Went, 1949) with or without sucrose can be used. Data from the original experiment are ambiguous (Table Den-40), but it seems that both under ambient and elevated carbon dioxide (CO_2) plantlets developed better in the absence of sugar. Therefore the medium suggested here (Table Den-41) is sugar-free.

Procedure. Small plantlets produced through any method should be moved to the sugar-free medium (Table Den-41) and grown under ambient or elevated CO_2 levels.

Developmental Sequence. The dry weight (DW) of plantlets grown on sucrose-containing medium under ambient CO_2 levels is 4.92% of the fresh weight (FW). Their hydration value (FW – DW/DW) is 19.31. In the absence of sugar the values are 4% and 23.75 respectively. The parallel values for plantlets grown under enhanced CO_2 levels are 4.23% and 22.67 and 4.2% and 22.75. This and the fact that the DW of plantlets on sugar-containing medium regardless of CO_2 content is the same (13 ± 1.58 vs 12 ± 1.58) suggest that they tend to take up more water in the presence of sucrose under elevated CO_2 concentration. Water uptake seems to be the same under both CO_2 levels in the absence of sugar.

TABLE DEN-40. **Effects of sucrose and CO_2 levels on the growth of *Dendrobium* plantlets in vitro**[a]

		Ambient CO_2 level (0.6 g m^{-3})		Enriched CO_2 level (40 g m^{-3})	
Parameter	Initial explants	2% sucrose	0 sucrose	2% sucrose	0 sucrose
Shoot length, cm	1.2 ± 0.01	1.4 ± 0.15	*1.5 ± 0.19*	1.4 ± 0.35	**1.7 ± 0.25**
Number of leaves	4 ± 0.8	**6 ± 0.7**	*5 ± 0.85*	5 ± 0.54	**6 ± 0.7**
Leaf length, cm	1.1 ± 0.1	1.1 ± 0.15	*1.1 ± 0.22*	**1.4 ± 0.15**	**1.1** ± *0.15*
Leaf width, cm	0.3 ± 0.01	**0.5 ± 0.02**	0.3 ± 0.02	0.3 ± 0.03	*0.4 ± 0.01*
Number of branches	1 ± 0.15	1 ± 0.15	1 ± 0.15	1 ± 0.15	1 ± 0.15
Number of roots			2 ± 0.7		2 ± 0.7
Root length, cm			**0.3 ± 0.02**		*0.2 ± 0.01*
Fresh weight, mg	71 ± 2.9	264 ± 5.17	99 ± 2.91	**284 ± 4.47**	*190 ± 0.70*
Dry weight, mg	4.9 ± 0.1	**13 ± 1.58**	4 ± 0.7	*12 ± 1.58*	8 ± 0.7
Column number	1	2	3	4	5

[a]Bold face indicated highest values in each line. Second highest value is in italics. If all values are the same none is presented in bold face or italics. The standard deviations [numerals which follow the "plus or minus" (±) sign] must be taken into consideration. For example the number of leaves (second row) values are 4 ± 0.8 (which means the number of leaves ranges from 3.2 to 4.8), **6 ± 0.7** (5.3–6.7), *5 ± 0.85* (4.15–5.85), 5 ± 0.54 (4.46–5.54), and **6 ± 0.7** (5.3–6.7). When the standard deviations are taken into consideration these numbers mean that the differences between (a) column 1 and column 3, (b) column 3 and column 1 and column 4, (c) column 2 and column 3, (d) column 2 and column 4, (e) column 2 and column 5, (f) column 3 and column 4, (g) column 3 and column 5, and (h) column 4 and column 5 are not statistically significant, but the differences between (i) column 1 and column 2, and (j) column 1 and column 5 are statistically significant. When data are not statistically significant they are the same. To put it differently, in terms of CO_2 and sucrose levels the best results are in columns 2 and 5. This means that CO_2 enrichment does not increase the number of leaves except relative to the initial explants.

TABLE DEN-41. **Vacin and Went (VW) medium (Vacin and Went, 1949) as modified for the culture of *Dendrobium* shoots (Mitra et al., 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Solvent				
8	Water, distilled[e]	To 1000 ml			
	Solidifier				
9	Agar[e]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 8) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Add items 1 and 3–7 to the 500–700 ml of distilled water (item 8) which contain the calcium phosphate (item 2), raise the volume to 900 ml with distilled water (item 8), adjust pH to 5–5.4, adjust volume to 1000 ml with distilled water (item 8), bring the solution to a gentle boil and add the agar (item 9) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

General Comments. The authors suggest that "culture [on] sugar-free medium in combination with CO_2 enrichment holds promise." However, under commercial and practical conditions it is necessary to balance the extra cost of CO_2 enrichment against the added income it may generate. A decision on whether to enrich with CO_2 or not must be based on the bottom line. The evidence seems to suggest that the bottom line will not favor enrichment.

Maintenance and Differentiation of *Dendrobium* Tissues

Professor Choy sin Hew, Department of Botany, National University of Singapore (now retired) was interested in sugar metabolism and preferences by undifferentiated and differentiated orchid tissues. He developed methods to maintain and differentiate his tissues (Hew and Mah, 1989).

Plant Material. Undifferentiated and differentiated tissues of *Dendrobium* Multico White (*Dendrobium* Ng Eng Chow × *Dendrobium* Ong Geok Kim) maintained in vitro were used. They were obtained through another procedure.

Surface Sterilization. Explants taken from tissue in vitro do not require surface sterilization.

Culture Vessels. Erlenmeyer flasks or other containers are suitable.

Culture Conditions. Cultures were maintained at $24 \pm 2°C$ under 16-h photoperiods of 56 $\mu mol\ m^{-2}\ s^{-1}$ photon flux density provided by Philips white fluorescent tubes. Standard culture room conditions are also suitable.

Culture Media. Undifferentiated tissues are maintained on one modification (Table Den-42) of the Vacin and Went (VW) medium (Vacin and Went, 1949). Differentiation takes place on another modification of VW (Table Den-43).

Professor Choy sin Hew, Professor Joseph Arditti and Dr. Tim Wing Yam, in a Singapore restaurant in 2004

TABLE DEN-42. **Vacin and Went (VW) medium (Vacin and Went, 1949) as used for the culture of *Dendrobium* tissues in vitro (Hew and Mah, 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	8–10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 9) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 ml l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Add items 1 and 3–7 to the 500–700 ml of distilled water (item 9) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 9), adjust pH to 5–5.4, add sugar (item 8), and raise volume to 1000 ml with distilled water (item 9). Bring the solution to a gentle boil and add the agar (item 10) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of other nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

Procedure. Tissues obtained through another method can be maintained on the first medium (Table Den-42). They should be moved to the second solution (Table Den-43) for differentiation.

Developmental Sequence. While on the first medium tissues increase in size and volume. Differentiation takes place on the second substrate.

General Comments. This procedure can be used to maintain and differentiate tissues obtained through other methods. Growth and increase in dry weight were best on VW medium containing 2% fructose (Table Den-43). It is important to keep in mind that glucose and fructose are monosaccharides whereas sucrose is a disaccharide.

TABLE DEN-43. **Vacin and Went (VW) medium (Vacin and Went, 1949) as modified for the differentiation *Derndrobium* tissues (Hew and Mah, 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Fructose	20.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	8–10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 9) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 ml l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Add items 1 and 3–7 to the 500–700 ml of distilled water (item 9) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 9), adjust pH to 5–5.4, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring the solution to a gentle boil and add the agar (item 10) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of other nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

Clonal Multiplication of *Dendrobium* Formidible through Tissue Culture

A method for the culture of *Dendrobium* Formidible (*Dendrobium formosum* × *Dendrobium infundibulum*) developed at Meijo University, Nagoya, Japan (Oyamada and Takano, 1990) seems interesting but was difficult to adapt for presentation here because macroelement content in the media it uses is given in terms of anion and cation percentages only (Table Den-44). According to Professor Syoichi Ichihashi of the Aichi University of Education in Japan who helped solve this problem, the possible combinations of anions and cations "are numerous" and one cannot tell which combination was used. Therefore the media presented here are the ones suggested by Professor Ichihashi who showed remarkable patience and goodwill by answering questions driven by my impatience with the eccentric presentation of content of media.

TABLE DEN-44. **Media formulations as presented by Oyamada and Takano (1990) in their table 1**

(A) Ionic composition of inorganic major elements

Anion		Cation	
NO_3^-	50%	NH_4^+	30%
SO_4^{--}	30%	Ca^{++}	15%
$H_2PO_4^-$	15%	K^+	40%
		Mg^{++}	10%
Cl^-	5%	Na^+	5%
Total	100%		100%

Total ionic conc. ($\Sigma A^{n-} + \Sigma C^{n+}$)	60 me/	Exp. I-A and B
	30, 60, 90 me/	Exp. II-A and B
	40, 80 me/	Exp. II-C

(B) Inorganic minor elements

Fe-EDTA	25.0 mg/l
H_3BO_3	3.0 mg/l
$MnSO_4 \cdot H_2O$	2.0 mg/l
$ZnSO_4 \cdot 7H_2O$	1.0 mg/l
$Na\text{-}MoO_4$	0.1 mg/l

(C) Organic substances

Thiamine·HCl	1.0 mg/l
Nicotinic acid	0.5 mg/l
Pyridoxine·HCl	1.0 mg/l
myo-inositol	100.0 mg/l
Adenine	40.0 mg/l
Peptone	1.0 mg/l
Sucrose	30.0 g/l
Agar	0 and 8.0 g/l

Plant Material. Several different explants (Fig. Den-5, ST-1, ST-2, ST-3, LB-1, LB2) can be cultured, but only axillary buds from the basal portions of stems (Fig. Den-5, LB-1) produced PLBs. The buds should be excised from 15-cm-long shoots.

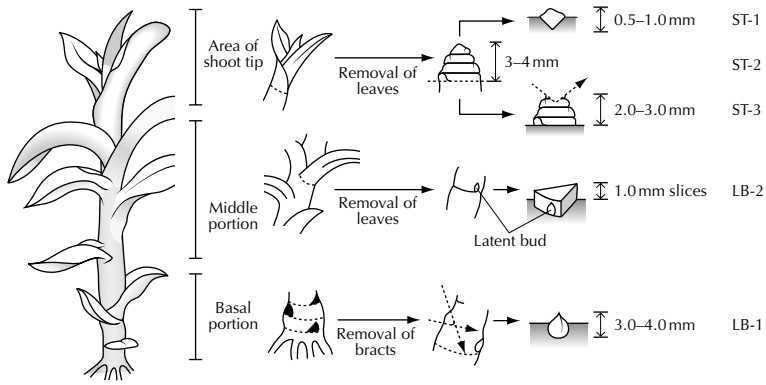


FIG. DEN-5. **Excision of explants from *Dendrobium* Formidible (Oyamada and Takano, 1990).**

Surface Sterilization. PLBs taken from in vitro cultures do not require surface sterilization. The surface sterilization procedure for shoots used in the original research seems not to have been very effective because many of the cultures became contaminated. The procedure suggested here is revised. Shoots collected in the field must be washed thoroughly with running water and a mild household detergent. Their leaves should be removed after that and the shoots must be washed a second time. After that they should be: (1) dipped in 70% ethanol for 10 s; (2) rinsed with sterile distilled water three times; (3) placed in alcohol for another 10 s; (4) washed with sterile distilled water three times; (5) soaked in 0.5% sodium hypochlorite (10 ml of a household bleach which contains about 5% sodium hypochlorite) for 10 min; (6) washed with sterile distilled water three times; (7) put in the hypochlorite solution for an additional 5 min; and (8) rinsed three times with sterile distilled water as above before the buds are excised.

Culture Vessels. Erlenmeyer flasks or other containers are suitable.

Culture Conditions. The culture conditions used during the original experiments were more elaborate than necessary in that the temperature was 25 and 20°C during the 16-h photoperiods and the 8 h of darkness, respectively. The light intensity during the culture of excised buds was 2000 lx, but it was 1000 lx when PLBs were proliferated. Such elaborate conditions are probably not needed. Standard culture room conditions should be entirely satisfactory.

Culture Media. Axillary buds should be cultured in a medium which is specially formulated for them (Table Den-45), but two modification (see Tables Den-17 and Den-18) of the Vacin and Went (VW) medium (Vacin and Went, 1949) may also be suitable. PLBs produced on this medium are proliferated on a second solution (Table Den-46), but other substrates (see Tables Den-11 and Den-12) can also be used. The medium used to differentiate plantlets is not entirely clear but it seems reasonable to assume that several media would be suitable (see Tables Den-2 to Den-10, and Den-13). Shoot can be induced on two media (see Tables Den-13 and Den-14) and roots on another (see Table Den-15).

Procedure. Axillary buds should be excised from shoots (Fig. Den-5, LB-1), cultured, and allowed to form PLBs on the first medium (Table Den-45) or another suitable substrate (see Tables Den-17 and Den-18). These PLBs should be proliferated on the second medium (Table Den-46) or another solution (see Tables Den-11 and Den-12) and placed on one of the other media for plantlet production (see Tables Den-2 to Den-10, and Den-13). Alternately, shoot induction on a suitable medium (see Tables Den-13 and Den-14) can be followed by root initiation on another solution (see Table Den-15).

Developmental Sequence. PLBs form on the explants on the first medium, proliferate on the second, and develop plantlets on the third.

General Comments. This may be an effective procedure which is difficult to understand and interpret and therefore hard to use. It involves overly complex steps, an unclear presentation, and an esoteric approach to description of culture media.

TABLE DEN-45. **Oyamada and Takano (OT) medium (Oyamada and Takano, 1990) for the culture of basal buds of *Dendrobium* Formidible (Oyamada and Takano, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, NH_4SO_4[b]	1189.0	118.9 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1063.0	106.3 g l^{-1}	10	
3	Magnesium nitrate, $Mg(NO_3)_2 \cdot 6H_2O$[b]	769.0	76.9 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	1216.0	121.6 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	1517.0	151.7 g l^{-1}	10	
6	Sodium chloride	175.0	17.5 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.0	300.0 mg l^{-1}	10	One solution
(b)	Manganese sulfate, $MnSO_4 \cdot H_2O$	2.0	200.0 mg l^{-1}		
(c)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.1	10.0 mg l^{-1}		
(d)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1.0	100.0 mg l^{-1}		
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin-related substance				
13	Adenine[f]	40.0	No stock	No stock	Weigh
	Complex additive				
13	Peptone	1.0 g	No stock	No stock	Weigh
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original formulation of thie medium calls for 25 mg l^{-1}. Since the original paper gives no details the form of chelated iron suggested here is the same as the one added to the MS medium.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the adenine does not dissolve, add a few drops of 0.1N sulfuric acid.

[g]Add items 1–9 and 13 to 900 ml of distilled water (item 15), adjust pH to 5.2–5.4, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar (item 16) can also be added to the cold water (item 15) which is brought to a boil and stirred. When the agar (item 16) is completely dissolved pour the solution into a 2-l flask and autoclave. Add the vitamins (items 10–12) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE DEN-46. **Oyamada and Takano (OT) medium (Oyamada and Takano, 1990) for the proliferation of PLBs of *Dendrobium* Formidible (Oyamada and Takano, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, NH_4SO_4[b]	1784.0	178.4 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1594.0	159.4 g l^{-1}	10	
3	Magnesium nitrate, $Mg(NO_3)_2 \cdot 6H_2O$[b]	1154.0	115.4 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	1824.0	182.4 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	2275.0	227.5 g l^{-1}	10	
6	Sodium chloride	263.0	26.3 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.0	300.0 mg l^{-1}	10	One solution
(b)	Manganese sulfate, $MnSO_4 \cdot H_2O$	2.0	200.0 mg l^{-1}		
(c)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.1	10.0 mg l^{-1}		
(d)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1.0	100.0 mg l^{-1}		
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin-related substance				
13	Adenine[f]	40.0	No stock	No stock	Weigh
	Complex additive				
14	Peptone	1.0 g	No stock	No stock	Weigh
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original formulation of thie medium calls for 25 mg l^{-1}. Since the original paper gives no details the form of chelated iron suggested here is the same as the one added to the MS medium.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the adenine does not dissolve, add a few drops of 0.1N sulfuric acid.

[g]Add items 1–9, 13 and 14 to 900 ml of distilled water (item 16), adjust pH to 5.2–5.4, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar (item 17) can also be added to the cold water (item 16) which is brought to a boil and stirred. When the agar (item 17) is completely dissolved pour the solution into a 2-l flask and autoclave. Add the vitamins (items 10–12) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

FIG. DEN-6. ***Dendrobium*** **flowering in flask (Sim, no date).**

In Vitro Flowering of *Dendrobium*

Orchid history is replete with strange and peculiar stories (for recent examples see Hansen 2000*a*, 2000*b*, 2000*c*, 2000*d*, 2000*e*). The same is true for the in vitro flowering of *Dendrobium* Madam Thong-In. The first report appeared in a daily newspaper (Anonymous, 1995). It told of a "potion which can shrink plants a metre tall to fit a 7.5-cm-high vial" and included a photograph of an orchid in a tissue culture vessel (similar to Fig. Den-6). The "potion" was described as "unnamed" and "translucent." Its nature was not disclosed. According to the newspaper the potion was "the brainchild of Dr. Loh Chiang Shiong in the [National University of Singapore] Department of Botany, and Professor Goh Chong Jin, [then] head of the department [subsequently replaced and now retired]." The story did not mention that the work was done by two students, Sim Guek Eng for a Ph.D. Dissertation (Sim, no date) and A. P. P. Ng for an honors thesis (Ng, 1997). Both the thesis and dissertation have yet to be published.

A longer version of the same story was presented by Professor Chong Jin Goh a year later (Goh, 1996). It did include two photographs of *Dendrobium* Sonia and one of *Dendrobium* Madame Thong-In flowering in vitro, but was scientifically content-free, failed to cite the students (Sim, no date; Ng, 1997), and ignored all previous work by others on the subject (X. Wang, 1984, 1988*a*, 1988*b*, 1990, no date *a*, no date *b*; X. Wang et al., 1981, 1988*a*, 1988*b*; G. Y. Wang et al., 1992, 1993; for a review see Chia et al., 1999). It was devoted mostly to self-glorification by its author. The dissertation and the thesis cannot be accessed because Professor Goh prohibited access to them. This is highly unusual. There are no previous such prohibitions even by the author of the piece in the *Malayan Orchid Review* (Goh, 1996), who coauthored numerous papers on diverse subjects with members of his department in their area of specialization (to the extent that he had a specialty: it was orchids).

A few details can be gleaned from a molecular biology paper (Yu and Goh, 2000) and the dissertation title (Sim, no date). The authors suggest the use at some point of a liquid Knudson C medium (Knudson, 1946), 2% sucrose, 15% (v/v) coconut

water, 5 μmol BA l^{-1}, 16-h photoperiods of 35 μmol m^{-2} s^{-1} provided by daylight fluorescent lamps, 24°C, and a 120-rpm rotary shaker. Other media and culture conditions were also used, but they were not described. Therefore, a procedure based on Professor Goh's unnamed, translucent, and well-publicized potion (Anonymous, 1995) cannot be presented despite the fact that it may be of interest to both scientists and operators of practical laboratories. Still a method may be patched together by referring to research which may have been the (albeit unacknowledged) inspiration for the magic potion and probably guided Professor Goh's work.

In 1990 a group consisting of Professor Nam Hai Chua of the Rockefeller University in New York and Guang Yuan Wang, Zhi Hong Xu, Tet Fat Chia (who subsequently became the first person to introduce a foreign gene into orchids and is now a professor at the National Technological University in Singapore), and Pek Foong Wong of the Institute of Molecular and Cell Biology, National University of Singapore induced in vitro flowering in seedlings of *Dendrobium candidum*, an orchid used in traditional Chinese medicine. They published their research properly and described all media and methods in detail (Wang et al., 1990, 1993).

Plant Material. Protocorms obtained by germinating seeds from mature and undehisced capsules of *Dendrobium candidum* were used in the original research. PLBs and protocorms of other *Dendrobium* species and hybrids could perhaps also be induced to flower in vitro. Such protocorms and PLBs may be produced through seed germination and tissue culture procedures respectively.

Surface Sterilization. Protocorms and PLBs taken from cultures in vitro do not require surface sterilization.

Culture Vessels. Erlenmeyer flasks, 500-ml capacity, containing 80 ml of medium, were used in the original research. However, plastic containers (Fig. Den-6), bottles, and jars filled with medium to 15–20% of their capacity are also suitable.

Culture Conditions. Regardless of the conditions under which the PLBs and/or protocorms may have been maintained, those which will be used to produce plantlets for in vitro flowering should be proliferated under 12-h photoperiods of 40–45 μmol m^{-2} s^{-1} and 25–27°C. Cultures for PLB proliferation and in vitro flowering should be kept under 16-h photoperiods and 25–27°C and 21–23°C during the light and dark periods respectively. Since flower induction may be sensitive to photoperiods and temperature an effort should be made to use the same conditions at least during initial attempts to induce flowering in vitro. Light intensity of 22–35 μmol m^{-2} s^{-1} provided by daylight fluorescent lamps should be appropriate.

Culture Media. Three media must be used to obtain maximal flower induction. PLBs or protocorms should be proliferated on MS medium (Murashige and Skoog, 1962) supplemented with 0.3 mg NAA (available from www.fishersci.com., www.sigmaaldrich.com, or www.caissonlabs.com) and containing only 15 g sucrose l^{-1} (Table Den-47). After that they must be cultured on MS containing 2 mg ABA l^{-1} (available from the sources listed above; Table Den-48). Flowering takes place on MS with 2 mg BA l^{-1} (available from the sources above) (Table Den-49).

Procedure. PLBs and/or protocorms should be proliferated on the first medium (Table Den-47). Clumps of PLBs or protocorms must be cultured on the ABA containing medium (Table Den-48) for 2 months before transferring them to the BA substrate (Table Den-49) where they can be expected to flower within 5 months.

Developmental Sequence. Protocorms proliferate on the first medium, continue growth and development on the second, and flower on the third.

General Comments. There can be no question that the first flowering of *Dendrobium* in vitro was obtained by (in alphabetical order) T. F. Chia, N. H. Chua, G. Y. Wang, P. F. Wong, and Z. H. Xu (IMC-NUS group), and not by C. J. Goh and his associates. The IMC-NUS group was also the first to publish a detailed method for in vitro flower induction in *Dendrobium*. Reports by the IMC-NUS group are very detailed (for one example see Wang et al., 1993) and could serve as a basis for other attempts to induce flowering in vitro. There is no certainty, of course, that the IMC-NUS method can and will work with other *Dendrobium* species and hybrids. However the use of 5 μmol (1.13 mg l^{-1}) BA in the unnamed potion (vs 8.88 μmol or 2 mg l^{-1} in the IMC-NUS medium) suggests a similarity in media. This similarity is promising because the unnamed potion induced flowering in two different hybrids. The similarity also suggests that the unnamed potion and associated procedures may be based on the IMC-NUS method. And, if this is the case and if the unnamed potion and procedures connected with it induced flowering in *Dendrobium* Sonia and *Dendrobium* Madame Thong-In, there is hope that the IMC-NUS method will cause other dendrobiums and perhaps even additional orchids to bloom. And one can hope that Professor Goh will lift his bizarre edict someday.

Professor Tet Fat Chia, seen here in 1991, was the first person to introduce a foreign gene into orchids, and was also one of the first to obtain the first flowering of *Dendrobium* in vitro.

TABLE DEN-47. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the proliferation of *Dendrobium candidum* protocorms (G.Y. Wang et al., 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.3	30 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	15.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1N KOH.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.4, add sugar (item 14), and raise volume to 1000 ml with distilled water. Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE DEN-48. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the preculture of *Dendrobium candidum* protocorms for in vitro flowering (G.Y. Wang et al., 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Plant hormone				
10	Abscisic acid (ABA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the abscisic acid (ABA) does not dissolve, add a few drops of 0.1N KOH.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.4, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE DEN-49. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for flower induction in vitro in *Dendrobium candidum* (G.Y. Wang et al., 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzyladenine (BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	50.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the benzyladenine (BA) does not dissolve, add a few drops of 0.1N HCl.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.4, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. Plants should be kept on a high sugar medium for a short time until they flower.

Isolation and Culture of *Dendrobium* Protoplasts

If it were possible to isolate and culture individual cells or protoplasts thousands of plants could be produced from even a small slice of tissue. Protoplasts can also be used for genetic transformation through the insertion of genes. A method for protoplast isolation and production of microcallus masses was developed at the University of Hawaii (Kuehnle and Nan, 1990).

Plant Material. Plantlets of *Dendrobium* Jaquelyn Thomas cultivars UH232, UH503, and UH 800 growing in vitro under 16-h photoperiods of 43 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) and probably 22–25°C should be moved to a lower light intensity of 13 μmol PAR m^{-2} s^{-1} for 1 week. Leaves, 1.5–4.5 cm in length, are taken from the plants and cut into 1-mm-wide strips.

Surface Sterilization. Leaves are taken from plants growing in vitro and do not require surface sterilization. However all solutions, tools, and media must be sterilized by filtration, autoclaving, or flaming.

Culture Vessels and Other Glassware. This procedure requires vacuum and filtering equipment, funnels, Erlenmeyer flasks (various capacities), centrifuge tubes, microscopes, Fuchs–Rosenthal modified hemocytometer, and other apparatus usually found in well equipped laboratories.

Culture Conditions. Optimal conditions for the incubation of leaves in enzyme mixture (half or quarter strength is preferable) are 6 h at 30°C. Protoplasts should be cultured in the dark at 21°C embedded in agarose sections floating in liquid medium.

Enzyme (Digesting) Solution. An appropriate enzyme (digestion) solution is 1.5% Cellulase R-10 (available from www.serva.de), 0.5% Macerozyme R-10 (www.serva.de), and 0.5% Driselase (www.biochemicalproducts.com) in 0.5-*M* mannitol (www.sigmaaldrich.com) solution adjusted to pH 5.5.

Purification and Filtration Solutions. After filtering the digestion mixture the protoplasts should be suspended in SCM solution (see next sentence for composition) and centrifuged at 100 × *g*. The SCM solution consists of 0.5 mol (9.11 mg l^{-1}) sorbitol (www.sigmaaldrich.com, S 3889), 10 mmol (147 mg 100 ml^{-1}) calcium chloride ($CaCl_2 \cdot 2H_2O$, www.sigmaaldrich.com, item C 5080), and 3 mmol morpholinoethane sulfonic acid (58.56 mg 100 ml^{-1}; M 5287, www.sigmaaldrich.com) with pH adjusted to 6.0. Protoplasts are separated from raphides on a 0.6-*M* sucrose density gradient.

Culture Medium. Purified protoplasts should be embedded in sections of MS medium (Murashige and Skoog, 1962) containing 1 mg 2,4-D l^{-1} and 0.5 mg BA l^{-1} solidified with 0.6% SeaPlaque agarose (item 5101 or 50100, www.cambrex.com) and floated on the same culture medium in liquid form (Table Den-50).

TABLE DEN-50. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Dendrobium* protoplasts (Kuehnle and Nan, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	2,4-Dichlorophenoxyacetic acid (2,4-D)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agarose[g]	6.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.4, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agarose (item 17) slowly while stirring. The agarose can also be added to the cold water which is brought to a boil and stirred. When the agarose is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10, 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

Procedure. Leaves, 1.5–4.5 cm, were taken from seedlings grown in vitro containing 1 mg 2,4-D l^{-1} and 0.5 mg BA l^{-1} solidified with 0.6% SeaPlaque agarose (item 5101 or 50100, www.cambrex.com) and floated on the same culture medium in liquid form (Table Den-50), cut into 1-mm-wide strips and incubated in the enzyme (digestion) mixture. Following the incubation the protoplasts were collected through filtration, separated from raphides by centrifugation, and plated on the agarose.

Developmental Sequence. Some protoplasts formed new cell walls within 24 h of isolation. First divisions could be seen in 2–3 days. Second divisions took place within 5 days. Microcalli formed after 2–4 weeks.

General Comments. Protoplast isolation and production of microcalli are exciting and significant advances in orchid science. The procedures are complex and require equipment which may not be available in the average micropropagation laboratory. Those who plan to attempt it should read the original literature.

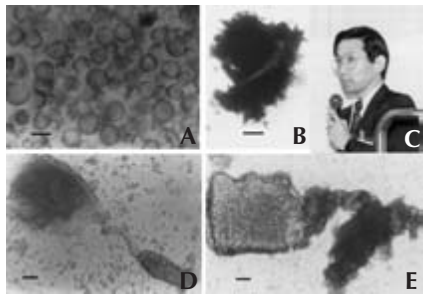
Dendrobium Protoplast Culture

At one time cell and/or protoplast fusion was viewed as a promising tool for the creation of somatic hybrids. Unfortunately the technique did not live up to its promise, not only in respect to orchids, but for other plants also. A major reason why cell fusion was largely abandoned is the technique of inserting genes into plants with a particle gun (as was done by Professor Tet Fatt Chia at Nanyang Technological University in Singapore with *Dendrobium* White Fairy No. 5 and a gene for bioluminescence from fire flies), which is simpler and more effective. While cell and protoplast fusion still held promise, a method was developed for the isolation of cells and protoplasts of *Dendrobium* Malones 'Hope' and *Dendrobium* Yukidaruma 'Queen' (Yasugi, 1990). Only the isolation and culture steps will be presented here since they may have some potential for micropropagation. Unfortunately Professor Yasugi (Fig. Den-7C) changed his interests, did not pursue this subject further, and stopped working with orchids.

Plant Material. A young fully expanded leaf should be used. After surface sterilization each leaf must be cut into 2 cm × 2 cm sections.

Surface Sterilization. The leaf should be washed several times with running water and a mild detergent, soaked for 15–20 min in 0.5% sodium hypochlorite (10 ml of a household bleach which contains 5–5.25% sodium hypochlorite diluted to 100 ml with distilled water), and rinsed repeatedly with sterile distilled water.

Culture Vessels and Other Glassware. This procedure requires vacuum and filtering equipment, funnels, Erlenmeyer flasks (various capacities), centrifuge tubes, a Fuchs–Rosenthal modified hemocytometer, and other apparatus usually found in well equipped laboratories (most of the necessary equipment can be obtained from standard biological supply houses, two of them being www.fishersci.com and www.vwr.com).



Most effective enzyme mixture:
Cellulase Onozuka R-10, 1%
Macerozyme R-10, 0.2%
Pectolyase Y-23, 0.01%
MES, pH 5.8, 5 mmoles
$CaCl_2 \cdot 2H_2O$, 10 mmoles
Mannitol, 6 moles
Potassium dextransulfate, 0.5%

Most effective hormone levels:
NAA, 2 mg
BA, 0.5 mg

Most effective conditions:
5°C
Dark

FIG. DEN-7. ***Dendrobium*** **protoplasts (A), colonies (B), and embryoids (D, E). C. Professor Saburo Yasugi. (Yasugi, 1990.)**

Culture Conditions. Leaf sections, approximately 1 g, should be incubated in 10 ml of enzyme mixture in a 50-ml Erlenmeyer flask with shaking at 40 rpm for 18 h. An appropriate temperature is not given in the original paper, but such a long incubation period suggests 21–23°C. Perhaps 6 h at 30°C may also prove to be suitable. Leaf sections should be kept under vacuum for 2 min before being placed in the enzyme mixture. Protoplasts in the first medium (liquid over agar) should be maintained at 20–22°C under 500 lx. Appropriate conditions for colonies on the second medium are the same temperature and 3000 lx.

Enzyme (Digesting) Solution. An appropriate enzyme (digestion) solution is 1% Cellulase Onozuka R-10 (available from www.serva.de), 0.2% Macerozyme R-10 (www.serva.de), and 0.01% Pectolyase Y-23 (Seishin, Japan, fax 81-3-3669-1684) in 5-m*M* MES buffered at pH 5.8, 10-m*M* $CaCl_2 \cdot 2H_2O$, 0.6-*M* mannitol, and 0.5% potassium dextran sulfate (all available from www.sigmaaldrich.com, www.fishersci.com, and www.vwr.com).

Purification and Filtration Solutions. Following incubation the digestion mixture should be filtered through a 60-μm mesh stainless steel sieve and centrifuged at $150 \times g$ for 5 min. The supernatant is discarded after that and the protoplasts (Fig. Den-7A) should be rinsed with 0.6-*M* solution of mannitol three times and suspended in the first culture medium.

Culture Media. Purified protoplasts should be suspended in a medium containing one-tenth the inorganic salt concentration of the MS medium (Murashige and Skoog, 1962), the organic components of the Kao and Michayluk (KM) solution (Kao and Michayluk, 1975), 2 mg NAA l^{-1}, and 0.5 mg BA l^{-1} (Table Den-51). Protoplast colonies which form on the first medium may develop embryoids on a second solution (Table Den-52).

TABLE DEN-51. **Inorganic salts of the Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) and the organic constituents of the Kao and Michayluk (KM) medium (Kao and Michayluk, 1975) for the culture of *Dendrobium* protoplasts (Yasugi, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	165.0	16.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	44.0	4.4 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	37.0	3.7 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	190.0	19.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	17.0	1.7 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	3.7	0.37 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	2.8	0.28 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
9	Naphthaleneacetic acid (NAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
10	Benzyladenine	0.5	100 mg 100 ml^{-1} 95% ethanol[e,f]	0.5	
11	**Vitamins and related substances (one solution)**				
(a)	*p*-Aminobenzoic acid	0.02	2 mg	Dissolve 11a–11e, 11g, 11h, 11j–11l in 70 ml of 95% ethanol; 11f in 15 ml 1.3N sodium bicarbonate in 95% ethanol or water; and 11i in 15 ml of 1N sodium phosphate in 95% ethanol or water. Mix the three solutions and store in a tightly capped bottle in a freezer. Add 1 ml of this combined solution to every liter of culture medium. Shake the combined solution well before dispensing	
(b)	L-Ascorbic acid (vitamin C)	2.0	200 mg		
(c)	Biotin	0.01	1 mg		
(d)	D-Calcium pantothenate	1.0	100 mg		
(e)	Choline chloride	1.0	100 mg		
(f)	Folic acid	0.4	40 mg		
(g)	Niacin (nicotinic acid)	0.1	10 mg		
(h)	Pyridoxine (vitamin B_6)	0.1	10 mg		
(i)	Riboflavine (vitamin B_2)	0.2	20 mg		
(j)	Thiamine (vitamin B_1)	1.0	100 mg		
(k)	Vitamin A	0.01	1 mg		
(l)	Vitamin B_{12}	0.02	2 mg		
	Amino acid				
12	Glycine	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugars				
13	Sucrose	30.0 g	No stock	No stock	Weigh
14	Xylose	0.25	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses. To dissolve folic acid the 95% ethanol or water should be made with 1.3N sodium bicarbonate. The 95% ethanol for riboflavine should be made with 1N sodium phosphate. Read instructions in body of table very carefully.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–8 to 850 ml of distilled water (item 15), adjust pH to 5.8 as required, add the sugars (items 13 and 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 12), hormones (items 9, 10), and vitamins (item 11) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. Two versions, one liquid and the other solid, of this medium must be prepared (see procedure). The medium as presented here may not be exactly the one employed in the original research (Yasugi, 1990) because the paper states that the "organic nutrients of KM" were used, but this solution is complex and has many permutations (Kao and Michayluk, 1975). Therefore it is hard to determine exactly what was used.

TABLE DEN-52. **Diluted inorganic salts of the Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) and the organic constituents of the Kao and Michayluk (KM) medium (Kao and Michayluk, 1975) for the culture of *Dendrobium* protoplasts (Yasugi, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	165.0	16.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	44.0	4.4 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	37.0	3.7 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	190.0	19.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	17.0	1.7 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	3.7	0.37 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	2.8	0.28 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
9	**Auxins**	One solution	One solution	One solution	One solution
(a)	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	Add both auxins to the same 100 ml of 95% ethanol
(b)	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.25	25 mg		
	Cytokinin				
10	Benzyladenine	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
11	**Vitamins and related substances (one solution)**				
(a)	*p*-Aminobenzoic acid	0.02	2 mg		
(b)	L-Ascorbic acid (vitamin C)	2.0	200 mg		
(c)	Biotin	0.01	1 mg		
(d)	D-Calcium pantothenate	1.0	100 mg		
(e)	Choline chloride	1.0	100 mg		
(f)	Folic acid	0.4	40 mg		
(g)	Niacin (nicotinic acid)	0.1	10 mg		
(h)	Pyridoxine (vitamin B_6)	0.1	10 mg		
(i)	Riboflavine (vitamin B_2)	0.2	20 mg		
(j)	Thiamine (vitamin B_1)	1.0	100 mg		
(k)	Vitamin A	0.01	1 mg		
(l)	Vitamin B_{12}	0.02	2 mg		
	Amino acid				
12	Glycine	0.1	20 mg 100 ml^{-1} 95% ethanol[e]	0.5	
	Sugars				
13	Sucrose	30.0 g	No stock	No stock	Weigh
14	Xylose	0.25	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

Dissolve items 11a–11e, 11g, 11h, 11j–11l in 70 ml of 95% ethanol; 11f in 15 ml 1.3N sodium bicarbonate in 95% ethanol or water; and 11i in 15 ml 1N sodium phosphate in 95% ethanol or water. Mix the three solutions and store in a tightly capped bottle in a freezer. Add 1 ml of this combined solution to every liter of culture medium. Shake the combined solution well before dispensing

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses. To dissolve folic acid the 70% ethanol should be made by diluting 70 ml of 95% ethanol to 100 with 1.3N sodium bicarbonate. The 70% ethanol for riboflavine should be made with 1N sodium phosphate. Read instructions in body of table very carefully.

[f]If the auxins or cytokinin do not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–8 to 850 ml of distilled water (item 15), adjust pH to 5.8 as required, add the sugars (items 13 and 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 12), hormones (items 9–10), and vitamins (item 11) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. Two versions, one liquid and the other solid, of this medium must be prepared (see procedure). The medium as presented here is somewhat of an educated guess because the original paper (Yasugi, 1990) only stated that "organic nutrients of KM medium were used." The KM medium (Kao and Michayluk, 1975) is very complex, contains many ingredients, and can be prepared in several permutations. It was simply hard to determine which "organic nutrients of [the] KM medium were used."

Procedure. After sterilization it is necessary to cut the leaves into 2 cm × 2 cm sections which should be subjected to vacuum for 2 min and then submerged in the enzyme mixture for 18 h. Following the incubation the digestion mixture should be filtered before the protoplasts are collected through centrifugation, washed with 0.6-*M* mannitol, and suspended in a small volume of 20% sucrose solution. The density of protoplasts must be determined by counting several samples on a hemocytometer and adjusting to $5.5–8.2 \times 10^4$ ml^{-1} by suspending them in a liquid version of the first medium (Table Den-51). This suspension should be poured over a solid version of the same medium (Table Den-51). When colonies form it is necessary to transfer them to the second medium (Table Den-52) for embryoid development.

Developmental Sequence. The first division can be seen after 5–7 days on the first medium. Colonies (Fig. Den-7B) formed after 2–4 weeks. Embryoids (Fig. Den-7D) develop on the second medium after 3–4 months. The paper reports that "no shoot or plant has been regenerated from orchid protoplasts," but contains no information on the fate of the embryoids.

General Comments. This procedure was formulated as a means of obtaining protoplasts for fusion (Fig. Den-7E). However when an embryoid formed "it was uncertain that [it] was induced from a hybrid cell or not." Since cell fusion is no longer much of a factor in genetic engineering this uncertainty does not really matter. Both investigators and laboratory operators would be well advised to read the original papers (Kao and Michayluk, 1975; Yasugi, 1990) before using this procedure.

Virus Elimination during Micropropagation of *Dendrobium*

Cymbidium mosaic virus (CyMV) causes floral deformation, necrosis, and color break, all of which render flowers unfit for display and/or sales. It also brings about irregular flowering and lowers plant vigor which reduces income to growers of cut flowers and orchid plants. In an effort to reduce such losses a protocol for virus elimination was developed at the University of Hawaii (Kuehnle, 1996; Porter and Kuehnle, 1997).

Plant Material. PLBs of *Dendrobium* Jaquelyn Thomas 'Uniwai Mist' were used for experiments at the University of Hawaii. In practical applications PLBs of any CyMV-infected *Dendrobium* or other orchids can probably be freed of virus by using this protocol. The PLBs can be produced through any micropropagation method.

Surface Sterilization. PLBs from in vitro cultures do not require surface sterilization.

Culture Vessels. Polycarbonate boxes (G&B Orchids, Vista, California, USA, www.orchidsource.com/GBCatalogV11new.html) were used for solid media by the researchers in Hawaii. Erlenmeyer flasks containing 50 ml of medium per 125-ml flask were employed for liquid cultures. Other culture vessels are also suitable.

Culture Conditions. In Hawaii liquid cultures were maintained at 26 ± 2°C on a gyratory shaker at 100 rpm and subcultured weekly. Solid cultures were maintained

at 21 ± 2°C. Both solid and liquid cultures were kept under 16-h photoperiods of 16–21 μmol m^{-2} $^{-1}$ provided by 40-W cool white and Gro-Lux Sylvania fluorescent lamps (few reports are as detailed about illumination – the authors should be commended for their precision). Standard laboratory conditions may also prove to be suitable.

Culture Media. Liquid Vacin and Went (VW) medium (Vacin and Went, 1949) containing 15% (v/v) coconut water and Sequestrene (www.beckerunderwood.com) was used to proliferate PLBs (Table Den-53). This medium is described as having a pH of 4.5 which is too low and may be a typographical error that transposed the numerals for the more common 5.4. Solid VW should contain 15% (v/v) coconut water and 75 g banana homogenate l^{-1}. Ribavirin (0.1 mmol or 24.42 mg l^{-1}) is added to both the liquid (Table Den-54) and solid (Table Den-55) virus eradication media. Virazole (a ribavirin medicinal preparation, www.virazole.com) is available (with prescription) in 100-ml glass vials with 6 g of sterile, lyophilized drug which should be reconstituted with 300 ml of sterile distilled water (1.22 ml of this solution contain 0.1 mmol or 24.42 mg). Vials with the lyophilized powder should be stored in a dry place at 15–25°C. The recommendation is to store reconstituted solutions for medicinal uses under sterile conditions at room temperature (20–30°C) for 24 h. There is no information on the website on whether the reconstituted compound can be stored in a freezer for prolonged periods.

Procedure. It is necessary to proliferate the CyMV-infected PLBs extensively and produce a large number because mortality is high during the treatment. Ribavirin-free liquid VW (Table Den-53) should be used for proliferation. Once a sufficient number of PLBs are available they should be cultured for 5–6 weeks in ribavirin-containing liquid VW (Table Den-54) and after that for 32–38 weeks on solid WV which also contains the antiviral agent (Table Den-55).

Developmental Sequence. PLBs produced in the ribavirin-containing VW were dark green and relatively small. Their doubling time was 3 weeks and there was 10% necrosis. Plant formation was hindered on solid ribavirin-containing medium, but 96–100% of those that survive are virus-free.

Virus Determination. Enzyme-linked immunosorbent assay (ELISA) should be used to determine whether plants are virus free. This assay may prove to be too complex for most practical laboratories. The procedure can be learned from *The ELISA Guidebook* by J. R. Crowther (Humana Press, 2001, available from www.sigmaaldrich.com). ELISA kits and assays for several orchid viruses are available from Agdia Inc. (www.agdia.com, http://plantpath.osu.edu/cweppdc/plantdis.html#tests), Ohio State University, and Dynatech Laboratories (14340 Sullyfield Circle, Cantilly, VA 22021, USA; tel: 703 631 7800 and 800 336 4543, fax: 703 631 7816).

General Comments. This virus eradication protocol is well thought out and effective to the extent that plantlets which survive the treatment are free of CyMV. However the procedures are complex, expensive, and probably beyond the capabilities of most laboratories.

TABLE DEN-53. **Liquid Vacin and Went (VW) medium (Vacin and Went, 1949) modified for proliferation of cymbidium mosaic virus (CyMV) infected protocorm-like bodies (PLBs) of *Dendrobium* 'Uniwai Mist' (Porter and Kuehnle, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
	Chelated iron				
6	Sequestrene 330 Fe[d]	57.0	No stock	No stock	Weigh
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water[e]	150.0 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Available from www.beckerunderwood.com. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 ml l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. If Sequestrene 330 is not available chelated iron as used for the modified Knudson C (KC) medium (Table Arnth-1) can be used.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and preservatives (that may have undesirable effects on cultures).

[f]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 10), adjust pH to 5.4, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

TABLE DEN-54. **Liquid ribavirin-containing Vacin and Went (VW) medium (Vacin and Went, 1949) modified for eradication of cymbidium mosaic virus (CyMV) from protocorm-like bodies (PLBs) of *Dendrobium* 'Uniwai Mist' (Porter and Kuehnle, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
	Chelated iron				
6	Sequestrene 330 Fe[d]	57.0	No stock	No stock	Weigh
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water[e]	150.0 ml	No stock	No stock	Measure
	Antiviral agent				
9	Ribavirin[f]	1.22 ml	See footnote *f*	See footnote *f*	See footnote *f*
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 11) before adding the other components.

[d]Available from www.beckerunderwood.com. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 ml l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. If Sequestrene 330 is not available chelated iron as used for the modified Knudson C (KC) medium (Table Arnth-1) can be used.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and preservatives (that may have undesirable effects on cultures).

[f]Ribavirin (as Virazole, a ribavirin medicinal preparation, www.virazole.com) is available in 100-ml glass vials which contain 6 g sterile, lyophilized compound that must be dissolved in 300 ml sterile distilled water (1.22 ml of this solution contain 0.1 mmol or 24.42 mg).

[g]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 11) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 11), adjust pH to 5.4, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Pour the solution into a 2-l flask, autoclave, allow the solution to cool until it is still liquid but warm to the touch, add the ribavirin with a sterile pipette, swirl or stir to mix well and distribute the medium to sterilized culture vessels. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of other nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

TABLE DEN-55. **Solid ribavirin-containing Vacin and Went (VW) medium (Vacin and Went, 1949) modified for eradication of cymbidium mosaic virus (CyMV) from protocorm-like bodies (PLB) and plantlets of *Dendrobium* 'Uniwai Mist' (Porter and Kuehnle, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
	Chelated iron				
6	Sequestrene 330 Fe[d]	57.0	No stock	No stock	Weigh
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additives				
8	Coconut water[e]	150.0 ml	No stock	No stock	Measure
9	Banana	75.0 g	No stock	No stock	Weigh
	Antiviral agent				
10	Ribavirin[f]	1.22 ml	See footnote *f*	See footnote *f*	See footnote *f*
	Sugar				
11	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 12) before adding the other components.

[d]Available from www.beckerunderwood.com. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 ml l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. If Sequestrene 330 is not available chelated iron as used for the modified Knudson C (KC) medium (Table Arnth-1) can be used.

[e]The correct term for the clear liquid endosperm of coconuts is coconut water (CW). Coconut milk (CM) is a white milky liquid obtained by grating or squeezing the copra ("meat"). CW from green (unripe) nuts is preferable. In countries where coconuts do not grow unripe nuts can often be found in food stores which specialize in Asian foods. The outer green coat of coconuts in such stores is often removed rendering them white in appearance. If green coconuts are not available CW from ripe (brown) nuts can also be used. CW should be kept frozen between uses. Canned and/or frozen CW must not be used since these products may contain preservatives, sugars and other substances.

[f]Ribavirin (as Virazole, a ribavirin medicinal preparation, www.virazole.com) is available in 100-ml glass vials which contain 6 g sterile, lyophilized compound that must be dissolved in 300 ml sterile distilled water (1.22 ml of this solution contain 0.1 mmol or 24.42 mg).

[g]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 12) which contain the calcium phosphate (item 2), pour the mixture in a homogenizer (kitchen-type blender), add the banana, homogenize thoroughly, bring volume to 950 ml with distilled water (item 12), adjust pH to 5.4, add sugar (item 11), and adjust volume to 1000 ml with distilled water (item 12). Pour the solution into a 2-l flask, autoclave, allow the solution to cool until it is still liquid but warm to the touch, add the ribavirin with a sterile pipette, swirl or stir to mix well and distribute the medium to sterilized culture vessels. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

Partial History of the Banana as a Complex Additive

According to Carl L. Withner (Withner, 1959, 1974), the first use of banana in an orchid seedling culture medium was in 1950 by Bruno Graeflinger in Brazil (Graeflinger, 1950) as powder (30 g l^{-1}) in a medium which also contained ammonium sulfate (0.5 g l^{-1}) and agar (15 g l^{-1}). A subsequent use of banana (fresh fruit) was by Withner himself in cultures of immature seeds (which he erroneously called ovules) of *Vanilla* (Withner, 1955). Banana homogenate has been used extensively since then and there are several unsubstantiated claims of first use. A very extensive study of the effects of banana homogenate alone and in combinations with other additives on *Dendrobium* seedlings was carried out in the Philippines (Pages, 1971). Over the years opinions differed on whether to use ripe or unripe banana, and slices or homogenate of the edible portion. At present homogenized ripe banana pulp is the most commonly used additive. Banana-containing media are easy to identify because of their blackish color. The reasons for the effects of banana homogenate are not known at present despite a fractionation study (Arditti, 1968).

Eradication of Cymbidium Mosaic Virus in *Dendrobium* Sonia "BOM 17"

"Application of 0.2 mM ribavirin in solid [liquid?] media for 1 week and 0.2 mM in solid media for . . . 12 weeks led to the production of 19.4% CyMV-free PLBs" (Wannakrairoj and Gladpan, 2001).

Photoautotrophic Multiplication of *Cymbidium* Protocorm-like Bodies

In experiments conducted to compare multiplication and growth of PLBs of *Cymbidium* under photoautotrophic (PA) and photomixotrophic (PM) conditions, the number of air exchanges and NAA levels had no effect. Multiplication was the same under both conditions. However stomatal density, chlorophyl content, and net photosynthesis were higher under PA. Total dry weight was higher under PM. Carbon dioxide enrichment may promote multiplication (Kirdmanee et al., 1992).

Preparation of Synthetic Seeds from Protocorm-like Bodies of *Dendrobium wardianum*

Synthetic seeds of several plants were prepared by encapsulating somatic embryos in calcium alginate. The same methods were used in India to prepare synthetic seeds of *Dendrobium wardianum* (Sharma et al., 1992).

Plant Material. PLBs were produced by culturing shoot apices of *D. wardianum* in a modified MS medium.

Culture Vessels. Erlenmeyer flasks and other containers are suitable.

Culture Conditions. The Indian researchers maintained their cultures at 24 ± 1°C under 16-h photoperiods of 30 E m^{-2} s^{-1} provided by fluorescent lamps (type not described). Standard culture room conditions are also suitable. Encapsulated PLBs should be stored at 4°C.

Culture Media. Shoot apices should be cultured on MS medium (Murashige and Skoog, 1962) supplemented with 2.5 mg BA l^{-1} (Table Den-56). Synthetic seeds placed on MS with 5 mg BA l^{-1} (Table Den-57) proliferated. Those on BA-free medium (Table Den-58) produced plantlets.

Encapsulating Solutions. Sodium alginate (www.sigmaaldrich.com) should be dissolved in MS (Table Den-59). A separate solution containing 100 mmol of calcium chloride ($CaCl_2 \cdot 2H_2O$) should also be prepared in MS medium (Table Den-60).

Procedure. PLBs should be removed from cultures, blot dried, mixed with the alginate solution (Table Den-59), and dropped into the calcium chloride solution with a sterile wide mouth dropping pipette (www.fishersci.com, www.vwr.com), each drop containing one PLB. The PLB–alginate–calcium chloride mixture should be placed on a gyratory shaker at 75 rpm for 40 min. Round and firm beads, each containing one PLB, are formed in the mixture. To separate the beads, the liquid in which they are suspended should be decanted. After that the beads must be washed three times with sterile MS solution before storage. All work must be carried out under sterile conditions. The beads should be cultured on BA-containing MS medium (Table Den-57) for proliferation and on hormone-free MS medium for plantlet production (Table Den-58).

Survival Times. If placed on culture medium immediately after being prepared, 100% of the synthetic seeds will germinate after 45 days. Germination of encapsulated PLBs will remain at the 100% level for 120 days (vs 60% for non-encapsulated ones). After 120–180 days of storage germination will be 30%. Following 180 days germination will drop to 10%.

Developmental Sequence. Depending on the medium encapsulated PLBs will proliferate (Table Den-57) or produce plantlets (Table Den-58).

General Comments. Production of synthetic seeds is a good method for the storage of PLBs for up to 4 months. This method could probably be used to encapsulate PLBs of other *Dendrobium* species and hybrids.

TABLE DEN-56. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Dendrobium wardianum* shoot tips (Sharma et al., 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzylaminopurine (BA or BAP)	2.5	250 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1N HCl.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.4–5.8, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE DEN-57. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for culture and proliferation of synthetic seeds of *Dendrobium wardianum* (Sharma et al., 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzylaminopurine (BAP)	5.0	500 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.4–5.8, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE DEN-58. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for plantlet production from synthetic seeds of *Dendrobium wardianum* (Sharma et al., 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[f]	To 1000 ml			
	Solidifier				
15	Agar[f]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Add items 1–7 and 9 to 900 ml of distilled water (item 14), adjust pH to 5.4–5.8, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), and vitamins (items 10–12) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE DEN-59. **Sodium alginate-containing Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for production of synthetic seeds of *Dendrobium wardianum* (Sharma et al., 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[f]	To 1000 ml			
	Alginate				
15	Sodium alginate[f]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Add items 1–7 and 9 to 900 ml of distilled water (item 14), adjust pH to 5.8, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the alglnate (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), and vitamins (items 10–12) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels.

TABLE DEN-60. **Calcium chloride-containing Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for preparation of synthetic seeds of *Dendrobium wardianum* (Sharma et al., 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Precipitatiing agent				
14	Calcium chloride, $CaCl_2 \cdot 2H_2O$ (100 mmol)	14.70 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Add items 1–7, 9 and 14 to 900 ml of distilled water (item 15), adjust pH to 5.8, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 15). Pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), and vitamins (items 10–12) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. Calcium chloride is listed twice and separately as item 14 because it is added to serve as a precipitating agent in addition to its function as a component (item 2) of the MS medium.

Preparation of Synthetic Seeds of *Dendrobium densiflorum*

A protocol for the preparation of synthetic seeds (synseeds) of *Dendrobium densiflorum* was formulated by Professor Suraj P. Vij at the Department of Botany, Panjab University, India (Vij et al., 2001).

Plant Material. PLBs obtained from node cultures were used by Professor Vij and his associates. Synseeds of other *Dendrobium* species and hybrids could probably be prepared from PLBs obtained from different explants.

Surface Sterilization. PLBs taken from in vitro cultures do not require surface sterilization. The sodium alginate solution was steam sterilized in the original research. If autoclaving is preferred a small sample should be tested first.

Culture Vessels. PLBs for encapsulation can be cultured in a variety of culture vessels. Synthetic seeds must be stored in sterile containers.

Culture Conditions. PLBs and synseeds can be cultured under standard culture room conditions. Synseeds should be stored at 4°C in the dark.

Culture Media. PLBs can be proliferated in Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) supplemented with BA (Table Den-61). Hormone-free MPR medium (Table Den-62) should be used to culture synseeds.

Encapsulating Solutions. Sodium alginate (www.sigmaaldrich.com) should be dissolved in MPR medium (Table Den-63) supplemented with 1 mg l^{-1} each of NAA and BA. The fungicide Bavisitin (1%, obtained from BASF, India; it is also available as carbendazim from www.sigmaaldrich.com) and the antibacterial agent streptomycin (0.01%, www.sigmaaldrich.com) were added to the alginate solution, but they may not be necessary if sterility is maintained throughout. Calcium chloride (Table Den-64) should be used to precipitate the alginate.

Procedure. MPR medium (Table Den-61) is used to proliferate PLBs which can be maintained in hormone-free solution (Table Den-62). PLBs to be encapsulated are dropped in the alginate-containing medium (Table Den-63) under aseptic conditions. The PLB–alginate mixture is added to magnetically stirred calcium chloride solution (Table Den-64). Beads which form as a result should be allowed to harden for 20–25 min before being washed two or three times with sterile distilled water prior to aseptic storage at 4°C in the dark. The beads are tacky, but the tackiness can be reduced or eliminated by treating the synseeds with sterilized talcum powder.

Developmental Sequence. Viability of the synseeds will remain at 100% for 30 days. It will drop to 85, 80, 60, 75, 40, and 30% after 45, 60, 75, 90, and 105 days, respectively. When placed on MPR medium the synseeds will produce shoots, roots, and plantlets after 2, 5, and 7 weeks.

TABLE DEN-61. **Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) modified for proliferation of *Dendrobium densiflorum* protocorm-like bodies (PLBs) derived from node cultures (Vij et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Cytokinin				
14	Benzyladenine (BA)	1.0	120 mg 100 ml^{-1} 95% ethanol[e]	1.0	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Agar[f]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

TABLE DEN-62. **Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) for maintenance of protocorm-like bodies (PLBs) of *Dendrobium densiflorum* (Vij et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4{\cdot}H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2{\cdot}6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2{\cdot}4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[f]	To 1000 ml			
	Solidifier				
16	Agar[f]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.6, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

TABLE DEN-63. **Sodium alginate-containing Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) for encapsulation of protocorm-like bodies (PLBs) of *Dendrobium densiflorum* (Vij et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Cytokinin				
15	Benzyladenine (BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Alginate				
18	Sodium alginate (Na alginate)	26.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe calls for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.6, add sugar (item 16), and bring volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil, add the alginate (item 18) slowly while stirring, pour the solution into culture vessels, and steam-sterilize or autoclave. As a rule media which contain vitamins, hormones, and/or other heat labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976). The sodium alginate used by Prof. Vij and his associates was obtained from Qualigens Fine Chemicals (a division of Glaxo) in India. In their paper they state that 1–5% sodium alginate was added to the medium. The amount suggested here is intermediate.

TABLE DEN-64. **Calcium chloride solution for the encapsulation of protocorm-like bodies (PLBs) of *Dendrobium densiflorum* (Vij et al., 2001)**

Calcium chloride, $CaCl_2 \cdot 2H_2O$	14.7 g (100 mmol)
Sucrose	20.0 g (2%)
Distilled water	To 1000 ml

Mix all items well to ensure that all components are dissolved and autoclave.

General Comments. Synseeds facilitate storage, transport, and commerce of the orchids as their PLBs can be encapsulated in alginate.

Propagation of Nobile-type *Dendrobium*

New cultivars can be propagated rapidly through micropropagation, but "there is a risk of mutation in mericlones" (Asai, 1998).

In Vitro Flowering of *Dendrobium moniliforme*

Terminal flower buds developed on 70% of 6-month-old seedlings of *Dendrobium moniliforme* 60–80 days following transfer to Vacin and Went (VW) medium (Vacin and Went, 1949) containing 1–10 mg BA l^{-1} (Table Den-65). Flower-bud formation was directly proportional to the BA concentration. The seedlings flowered but the blossoms were abnormal and lacked pollinia or "pistil" [sic], had twisted flowers, and in some cases the number of petals was lower than normal (Duan and Yazawa, 1994*b*). Despite the abnormal flowers this report confirms previous reports that BA can induce flowering in *Dendrobium* seedlings and plantlets produced through tissue culture. It also suggests that there are no "magic potions" (Anonymous, 1995) and puts grandiose claims (Goh, 1996) into rational and realistic perspective.

TABLE DEN-65. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for flower induction in *Dendrobium moniliforme* seedlings (Duan and Yazawa, 1994*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Cytokinin				
8	Benzyladenine (BA)	1–10.0	100–1000 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Flower-bud formation "is directly proportional to the concentration of BA in the medium from 1 to 10 mg l^{-1}." If BA will not dissolve readily add a few drops 0.1N HCl. Keep the stock solution frozen between uses.

[f]Add items 1 and 3–7 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 10), adjust pH to 5–5.4, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. After autoclaving add BA with sterile pipettes to the still warm and liquid medium, swirl or stir well and distribute the medium into preautoclaved culture vessels. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

Micropropagation of *Dendrobium wardianum*

A beautiful epiphytic orchid and a commercially important member of its genus, *Dendrobium wardianum* "is fast becoming an endangered species" as a result of "devastation of forest lands and overexploitation [of orchids in general] to cater [to] a great demand in the medicinal and cut-flower industries." A method for the micropropagation of this species was formulated at the Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong, India (Sharma and Tandon, 1991).

Plant Material. Shoot tips, ca. 2–3 mm, were excised from mature plants.

Surface Sterilization. Explants were "sterilized with 5% (v/v) NaOCl [sodium hypochlorite or household bleach] solution (with 1–3% available chlorine) for 5 min." This statement is not clear because it does not state whether the household bleach contains 5% NaOCl or if what should be used is a 5% dilution of household bleach. The original paper does not suggest rinsing the explants after sterilization but it is advisable to rinse them three times with sterile distilled water.

Culture Vessels. Erlenmeyer flasks, test tubes, or other containers filled with medium to 20–30% of their capacity can be used.

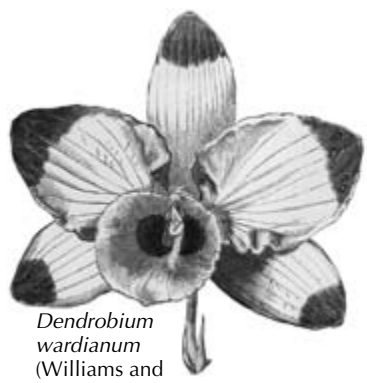
Culture Conditions. Cultures should be maintained at 24 ± 2°C under 16-h photoperiods of 2000 lx (source of illumination is not described). Standard culture room conditions may also be suitable.

Culture Media. Explants should be cultured on modified MS medium (Murashige and Skoog, 1962) supplemented with 2.5 mg BA; Table Den-66). PLBs produced on this medium should be cultured on MS medium (Table Den-67) to produce plantlets. A modified Vacin and Went (VW) medium (Vacin and Went, 1949; Table Den-12) can also be used for plantlet production.

Procedure. Explants are placed in culture on the first medium (Table Den-66) following sterilization. PLBs which form on this medium should be moved to the second version of MS medium (Table Den-67) or to Vacin and Went medium (Table Den-12) to produce plantlets. Cultures should be transferred to fresh medium every 4 weeks.

Developmental Sequence. PLBs are produced on the first medium (Table Den-66) after 6 weeks of culture. Plantlets are formed after 30 days on the second medium (Table Den-67 or Table Den-12) and are well developed after 3 months.

General Comments. This is an efficient and effective procedure presented in a concise and very informative report which can serve as a model for such papers.



Dendrobium wardianum (Williams and Williams, 1894)

TABLE DEN-66. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of shoot tips of *Dendrobium wardianum* (Sharma and Tandon, 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzylaminopurine (BAP)	2.5	250 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.8, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved distribute the medium to preautoclaved culture vessels. As a rule the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) should be added to the autoclaved and still warm and liquid medium under sterile conditions with sterilized pipettes, mixed well and dispensed, but in this case the indication is that all components were added prior to autoclaving. Agar is not added to liquid media.

TABLE DEN-67. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of plantlets produced from protocorm-like bodies (PLB) derived from shoot tips of *Dendrobium wardianum* (Sharma and Tandon, 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[f]	To 1000 ml			
	Solidifier				
15	Agar[f]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH to 5.8, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved distribute the medium to preautoclaved culture vessels. As a rule the amino acid (item 8), hormones (none in this medium), and vitamins (items 10–12) should be added to the autoclaved and still warm and liquid medium under sterile conditions with sterilized pipettes, mixed well and dispensed, but in this case the indication is that all components were added prior to autoclaving. Agar is not added to liquid media.

Shoot Proliferation and Flowering of *Dendrobium nobile* Second Love (Orchidaceae) in Vitro

Shoots taken from cloned plants of *Dendrobium nobile* Second Love were cultured on the Vacin and Went (VW) medium (Vacin and Went, 1949), modified by substituting $Fe_2(C_4H_4O_6)_3$ for 27.8 mg Fe EDTA l^{-1}, and supplementing it with the micronutrients of the MS medium (Murashige and Skoog, 1962), 0.4 mg thiamine l^{-1} and 100 mg *myo*-inositol l^{-1}. TDZ, 0–3.6 μmol l^{-1}, and sucrose, 0–4%, were added to the culture medium in several combinations. TDZ when added alone did not induce flowering. However combinations of TDZ and sucrose markedly enhanced both flowering and shoot proliferation. TDZ at 1.8 μmol l^{-1} and 2% sucrose are the best combination for shoot proliferation, whereas combinations of 2% sucrose with either 1.8 or 3.6 μmol TDZ were most effective for flower induction (Ferreira and Kerbauy, 2002).

Overall growth of *D. moniliforme* plantlets on Hyponex medium (3 g Hyponex l^{-1}, 4 g peptone l^{-1}, 30 g sucrose l^{-1}) at 22 ± 1°C under 16-h photoperiods of 1600 lx was best on a medium containing 1 mg IBA l^{-1} in comparison to the same concentrations of gibberellic acid (GA_3), NAA, kinetin, ABA, and a mixture of GA_3, NAA, IBA, kinetin, and ABA. The control and an NAA-containing medium were close seconds (Lim et al., 1993*a*).

Plantlet Strengthening Medium for *Dendrobium moniliforme*

B5 (Table Den-68) or half-strength MS (Table Den-69) media supplemented with 10% (v/v) aqueous banana extract and 2 mg NAA l^{-1} are effective plant strengthening substrates for *Dendrobium moniliforme* (Liu and Zhang, 1989). These media may be suitable for other *Dendrobium* plantlets.

TABLE DEN-68. **B5 medium (Gamborg et al., 1968) as modified for the strengthening of *Dendrobium moniliforme* plantlets (Liu and Zhang, 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	134.0	13.4 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	150.0	15.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	2500.0	250.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.0	300.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	10.0	1.0 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.75	75.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.0	200.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
9	Naphthaleneacetic acid (NAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
10	Kinetin	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	1.0	10 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
14	Aqueous extract of banana[g]	100.0 ml	No stock	No stock	Prepare[g]
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar[h]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively.

[g]There are no instructions on how to prepare an aqueous extract of banana. One possibility is to homogenize ripe banana pulp with an equal volume of distilled water and filter the homogenate to collect the liquid. Another possibility may be to use ripe banana pulp in lieu of extract. Commercial banana juice or drinks must not be used because they contain added sugar and other additives.

[h]Add items 1–14 to 800 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar (item 17) can also be added to the cold solution which is then brought to a boil and stirred. When the agar (item 17) is completely dissolved pour the medium into culture vessels and autoclave.

TABLE DEN-69. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as modified for the strengthening of *Dendrobium moniliforme* plantlets (Liu and Zhang, 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Kinetin	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
15	Aqueous extract of banana[g]	100.0 ml	No stock	No stock	Prepare[g]
	Sugar				
16	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively.

[g]There are no instructions on how to prepare an aqueous extract of banana. One possibility is to homogenize ripe banana pulp with an equal volume of distilled water and filter the homogenate to collect the liquid. Another possibility may be to use ripe banana pulp in lieu of extract. Commercial banana juice or drinks must not be used because they contain added sugar and other additives.

[g]Add items 1–15 to 800 ml of distilled water (item 17), adjust pH as required, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10, 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

Micropropagation of *Dendrobium* Hybrids through Shoot-tip Culture

Most micropropagation research regarding *Dendrobium* and other orchids has as its aim the development of practical methods. Comparative studies like the one which leads to methods for three hybrids (Kim et al., 2003; the authors are also listed as only Kim and Kim, 2003) are rare.

Plant Material. Apical or axillary buds were taken from *Dendrobium* hybrids listed and spelled as *Dendrobium* Earlsakul (a *Dendrobium* Earsakul does exist; EK), *Dendrobium* Semialba (SA), and *Dendrobium* Omyapink (OP) in early May in Korea.

Surface Sterilization. Buds should be dipped in 70% ethanol (73–74 ml 95% ethanol diluted to 100 ml with distilled water) for 1 min. The dip should be followed by submerging the buds in 10% household bleach (10 ml bleach diluted to 100 ml with distilled water) for 15 min before washing them three times with sterile distilled water.

Culture Vessels. Culture tubes (30 × 150 mm) containing 20 ml of solid medium were used in the original research. Other containers are also suitable.

Culture Conditions. Cultures should be maintained at 25 ± 1°C. The reprint on which this description is based does not contain information regarding illumination. Standard culture room conditions may also be suitable.

Culture Media. One modification of the Vacin and Went (VW) medium (Vacin and Went, 1949) should be used for EK (Table Den-70), another for OP (Table Den-71), and a third for SA (Table Den-72). Basal VW should be used for plantlet production (Table Den-73).

Procedure. Explants, 1 mm in width and 0.5 mm in height, should be taken from buds or shoot tips following sterilization and after removing the scale leaves which cover the tips. The explants should be cultured on the appropriate medium (Tables Den-70 to Den-72). If PLBs are to be proliferated they should be placed on the medium which is appropriate for each hybrids, but with 30 g sucrose l^{-1} rather than 20 g sucrose l^{-1} as should be used for PLB formation from explants. Basal VW (Table Den-73) with 20 g sucrose l^{-1} should be used for plantlet formation.

Developmental Sequence. The explants produce PLBs on the initial medium (Tables Den-70, Den-71, or Den-72 depending on the hybrid). These PLBs proliferate when placed on medium which contains 30 g sucrose l^{-1}. PLBs form plantlets on basal VW (Table Den-73).

General Comments. The report which describes these procedures is interesting and valuable because it demonstrates that culture medium requirements by different hybrids can vary. Unfortunately the report itself is not well written, lacks relevant information regarding illumination, and leaves something to be desired when it comes to organization.

TABLE DEN-70. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of shoot-tip and bud explants of *Dendrobium* Earsakul (Kim et al., 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinins				
9	Benzyladenine (BA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Use 30 g l^{-1} for PLB proliferation
	Solvent				
11	Water, distilled[e]	To 1000 ml			
	Solidifier				
12	Agar[e]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 11) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Add items 1 and 3–9 to the 500–700 ml of distilled water (item 11) which contain the calcium phosphate (item 2), raise volume to 900 ml with distilled water (item 11), adjust pH to 5.5, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable. Sucrose level should be increased to 30 g l^{-1} for PLB proliferation.

TABLE DEN-71. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of shoot-tip and bud explants of *Dendrobium* Omyapink (Kim et al., 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinins				
9	Benzyladenine (BA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Use 30 g l^{-1} for PLB proliferation
	Solvent				
11	Water, distilled[e]	To 1000 ml			
	Solidifier				
12	Agar[e]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 11) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Add items 1 and 3–9 to the 500–700 ml of distilled water (item 11) which contain the calcium phosphate (item 2), raise volume to 900 ml with distilled water (item 11), adjust pH to 5.5, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable. Sucrose level should be increased to 30 g l^{-1} for PLB proliferation.

TABLE DEN-72. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of shoot-tip and bud explants of *Dendrobium* Omyapink (Kim et al., 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Use 30 g l^{-1} for PLB proliferation
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier				
11	Agar[e]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 2), raise volume to 900 ml with distilled water (item 10), adjust pH to 5.5, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable. Sucrose level should be increased to 30 g l^{-1} for PLB proliferation.

TABLE DEN-73. **Basal Vacin and Went (VW) medium (Vacin and Went, 1949)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 9) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_3(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Add items 1 and 3–7 to the 500–700 ml of distilled water (item 9) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 9), adjust pH to 5–5.4, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring the solution to a gentle boil and add the agar (item 10) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

Encapsulation of Protocorm-Like Bodies of *Dendrobium* Sonia

Production of synthetic seeds through encapsulation is an effective low-cost clonal propagation system with several advantages which include ease of handling and prolonged storage. Such a method was developed for *Dendrobium* Sonia (Saiprasad and Polisetty, 2003).

Plant Material. Shoot tips of *Dendrobium* Sonia cultured on modified (Table Den-74) MS medium (Murashige and Skoog, 1962) produced PLBs after 120 days. These PLBs were fractionated by cutting away their apical portions and sectioning the bases into two or three sections each measuring 4–5 mm in length. The sections and shoot-tip explants were cultured in another modification of MS medium (Table Den-75) to produce PLBs for encapsulation.

TABLE DEN-74. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of shoot tips of *Dendrobium* Sonia (Saiprasad and Polisetty, 2003).**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.7, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10, 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. In this case it is also possible to add the hormones and vitamins to the medium before autoclaving.

TABLE DEN-75. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of sections of shoot-tip-derived protocorm-like bodies (PLBs) of *Dendrobium* Sonia (Saiprasad and Polisetty, 2003).**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzyladenine (BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.7, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media. In this case it is possible to add the hormones and vitamins to the medium before autoclaving.

Surface Sterilization. PLBs and explants taken from cultures in vitro do not require sterilization. Shoot-tip explants can be surface sterilized by the procedure used for the preceding method.

Culture Vessels. Culture bottles, 400-ml capacity, were used in the original research. Other containers are also suitable.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 16-h photoperiods of 50–60 μE m^{-2} s^{-1} (the light source used in the original research was not described). Standard culture room conditions are also suitable.

Storage Conditions. Encapsulated PLBs should be placed in sterile glass Petri dishes on sterilized moist filter paper. The Petri dishes should be placed in black plastic bags and stored in the dark at 4°C.

Culture Media. Shoot-tip explants should be cultured in MS medium containing 4.44 μmol BA l^{-1} and 5.38 μmol NAA l^{-1} (Table Den-74). PLB sections should be cultured on MS medium which contains 4.44 μmol BA l^{-1} (Table Den-75). Encapsulated PLBs should be germinated on MS medium containing 0.44 μmol BA l^{-1} and 0.54 μmol NAA l^{-1} (Table Den-76).

Encapsulation Solutions. Sodium alginate (www.sigmaaldrich.com) should be dissolved in MS free of calcium chloride and containing 0.44 μmol BA l^{-1} and 0.54 μmol NAA l^{-1} (Table Den-77). Calcium chloride should be dissolved in distilled water (Table Den-78).

Procedure. Shoot tips should be cultured on the modified MS (Table Den-74) to produce PLBs. Or, PLBs can be taken from other existing cultures. They should be sectioned and cultured on the second MS modification (Table Den-75) to produce PLBs for encapsulation. The new PLBs should be blot dried with sterile paper towels and mixed with the sodium alginate solution (Table Den-77). After that the alginate-coated PLBs should be dropped one at a time into the calcium chloride solution (Table Den-78). This mixture should then be placed on a 75 rpm gyratory shaker for 30 min. To separate the beads the calcium chloride solution is decanted. The beads are then blotted and stored. A viability of >85% of encapsulated PLBs was observed after 75 days storage at 4°C.

Developmental Sequence. Encapsulated PLBs produce leaves and roots on being placed on the germination medium (Table Den-76).

General Comments. Encapsulated PLBs can be stored for prolonged periods.

TABLE DEN-76. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the germination of encapsulated shoot-tip-derived protocorm-like bodies (PLBs) of *Dendrobium* Sonia (Saiprasad and Polisetty, 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.7, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10, 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. In this case it is also possible to add the hormones and vitamins to the medium before autoclaving.

TABLE DEN-77. **Sodium alginate-containing Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for encapsulation of shoot-tip-derived protocorm-like bodies (PLBs) of *Dendrobium* Sonia (Saiprasad and Polisetty, 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Alginate				
1	Sodium alginate	30.0 g	No stock	No stock	Weigh
	Macroelements				
2	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.7, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Pout the solution into a 2-l Erlenmeyer flask and autoclave.

TABLE DEN-78. **Calcium chloride solution for encapsulation of shoot-tip-derived protocorm-like bodies (PLBs) of *Dendrobium* Sonia (Saiprasad and Polisetty, 2003)**

Item number	Component	Amount per liter of culture medium
1	Calcium chloride, $CaCl_2 \cdot 2H_2O$	11.03 g
2	Distilled water	To 1000 ml

Dissolve calcium chloride and autoclave solution.

Effects of Putrescine on the Production of Protocorm-like Bodies

Polyamines have been shown in recent years to enhance embryogenesis and multiplication in vitro. Among them is the enhancement of PLB production by PLB explants of *Dendrobium* Sonia (Saiprasad et al., 2004).

Plant Material. PLBs are produced as in the previous procedure (Saiprasad and Polisetty, 2003).

Surface Sterilization. Explants and PLBs taken from cultures in vitro do not require surface sterilization. However they should be rinsed thoroughly with sterile distilled water to remove medium residues.

Culture Vessels. The culture vessels used in the previous procedure (Saiprasad and Polisetty, 2003) are suitable.

Culture Conditions. The conditions employed in the previous procedure (Saiprasad and Polisetty, 2003) should be used.

Culture Media. The medium used in the previous procedure (Saiprasad and Polisetty, 2003) for initial PLB production (Table Den-74) should be employed for the same purpose in this method. PLBs for proliferation can also be taken from other media. The putrescine-containing medium (Table Den-79) should be a modified MS medium (Murashige and Skoog, 1962).

Procedure. PLBs are sectioned and subcultured as in the previous procedure (Saiprasad and Polisetty, 2003). Proliferated PLBs can be cultured on the medium used to germinate encapsulated synthetic seeds (Table Den-76).

Developmental Sequence. PLBs proliferate on the putrescine-containing medium (Table Den-79). They produce plantlets on the medium used for germination (Table Den-76).

General Comments. The proliferation medium (Table Den-79) used in this protocol could probably be employed to proliferate PLBs of any *Dendrobium* or other orchids.

TABLE DEN-79. **Putrescine-containing Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for proliferation of protocorm-like bodies (PLBs) of *Dendrobium* Sonia (Saiprasad et al., 2004)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzyladenine (BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Polyamine				
11	Putrescine hydrochloride	64.43	644.0 mg l^{-1}	10	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.7, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media. In this case it is possible to add the hormones and vitamins to the medium before autoclaving.

Plant Production of *Dendrobium fimbriatum* var. *oculatum* from Shoot-tip Explants

Dendrobium fimbriatum, a species distributed from India to South East Asia, produces attractive pendulous racemes which bears yellow flowers which have a fimbriated labellum and a reddish brown spot in their centers. A micropropagation method for this species was developed at the Department of Botany, Visva-Baharati University in West Bengal, India (Roy and Banerjee, 2003).

The First Asian Nobel Laureate, world renowned poet, writer and philosopher Kabi Guru Rabindranath Tagore, a son of the Bengal Basin, established a unique university in Santiniketan (in the western part of the Bengal Basin) named "Visva-Bharati." It means the place where the world worships knowledge.

My late father, Salomon J. Arditti (1902–1993), loved Tagore's poetry and spoke about him often. It is therefore a pleasure and a bow to my father's memory for me to include this information here. (JA)

Plant Material. The original research was carried out with shoot-tips, 4–6 mm long, taken from 3-month-old seedlings in vitro.

Surface Sterilization. Explants taken from seedlings growing aseptically in vitro do not require surface sterilization.

Culture Vessels. Erlenmeyer flasks, 100 ml capacity, containing 40 ml medium were used in the original research. Other containers can also be used.

Culture Conditions. The research cultures were maintained at $25 \pm 2°C$ under 10-h photoperiods of 37.5 mmol m^{-2} s^{-1} provided by Philips white fluorescent lamps. Standard culture conditions are also suitable.

Culture Media. Shoot tips should be cultured on modified (Table Den-80) Knudson C (KC) medium (Knudson, 1946). Callus produced on this medium gives rise to PLBs and plantlets on being transferred to a medium (Table Den-81) free of NAA and BA.

Procedure. Shoot tips should be cultured on modified KC with plant hormones (Table Den-80). When callus is formed it should be transferred to the plant hormone-free medium (Table Den-81) where it will produce PLBs and plantlets. The callus can be maintained for as long as 2 years by subculturing it onto the plant hormone-free medium (Table Den-81).

TABLE DEN-80. **Knudson C (KC) medium (Knudson, 1946) modified for the culture of *Dendrobium fimbriatum* var. *oculatum* shoot-tip explants (Roy and Banerjee, 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement[d]				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
7	Naphthaleneacetic acid (IAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
8	Benzyladenine (BA)	1.00	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
9	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
10	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
12	Coconut water[g]	100.0 ml	No stock	No stock	Measure
	Sugar				
13	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[h]	To 1000 ml			
	Solidifier				
15	Agar[h]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. Kundson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$, l^{-1} but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored.

[d]There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If incorporation of additional microelements becomes necessary those used in the MS medium are suitable.

[e]If the auxin or cytokinin does not dissolve add a few drops of 0.1N KOH or HCl respectively.

[f]Keep frozen between uses.

[g]The correct term for the liquid endosperm of coconuts is coconut water (CW), not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Filtered CW must be stored in a freezer until it is used. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[h]Add items 1–12 to 800 ml of distilled water (item 14), adjust pH to 5.2, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If the incorporation of additional microelements is necessary those used in the MS medium are suitable.

TABLE DEN-81. **Knudson C (KC) medium (Knudson, 1946) modified for proliferation of shoot-tip-derived callus of *Dendrobium fimbriatum* var. *oculatum*, production of protocorm-like bodies (PLBs) and plantlet regeneration (Roy and Banerjee, 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement[d]				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Vitamins				
7	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
8	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
9	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
10	Coconut water[g]	100.0 ml	No stock	No stock	Measure
	Sugar				
11	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[h]	To 1000 ml			
	Solidifier				
13	Agar[h]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. Kundson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$, l^{-1} but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored.

[d]There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If incorporation of additional microelements becomes necessary those used in the MS medium are suitable.

[e]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH or HCl respectively.

[f]Keep frozen between uses.

[g]The correct term for the liquid endosperm of coconuts is coconut water (CW), not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Filtered CW must be stored in a freezer until it is used. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[h]Add items 1–10 to 800 ml of distilled water (item 12), adjust pH to 5.2, add sugar (item 11), and raise volume to 1000 ml with distilled water (item 12). Bring the solution to a gentle boil and add the agar (item 13) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If the incorporation of additional microelements is necessary those used in the MS medium are suitable.

Developmental Sequence. The explants will produce a white translucent embryogenic callus after 2 weeks on the first medium (Table Den-80). PLBs and plantlets are formed on the second medium (Table Den-81). The callus can retain its embryogenic nature on being cultured on the second medium (Table Den-81) for as long as 2 years.

General Comments. The experiments which resulted in this procedure were thought out well, designed carefully, and evaluated properly. J. Roy and N. Banerjee should be commended for their high standards and careful work. The protocol seems simple and effective. Unfortunately, however, it can only be used to multiply seedlings unless further research shows that the method is also suitable for shoot-tip explants from mature plants. If the procedure proves suitable for explants from mature plants it could be used to propagate outstanding cultivars.

Nutritional Studies on *Dendrobium wardianum* Protocorm-like Bodies

In most cases when micropropagation methods are developed for orchids, the research is not comparative. Even if several media are tested only the most successful results are reported. For this reason a comparative study using several media (Sharma and Tandon, 1991) is of considerable interest. Because of that several parts of the report are reproduced in full here.

Materials and Methods (Sharma and Tandon, 1991)

Establishment of initial culture

Shoot tips (ca. 2–3 mm) were excised from *Dendrobium wardianum* Warner plants and sterilized with 5% (v/v) NaOCl solution (with 1–3% available chlorine) for 5 min, and were inoculated on Murashige and Skoog's medium containing 3% sucrose, 0.8% agar, and 2.5 ml l^{-1} 6-benzylaminopurine (BAP) (under report). The pH of the medium was adjusted to 5.8 prior to addition of agar and autoclaved for 15 min at 120°C. The cultures were incubated at 24 ± 2°C with 16 h illumination of 2000 lx. Protocorm-like bodies (plbs) analagous to embryoids were produced after 6 weeks of culture.

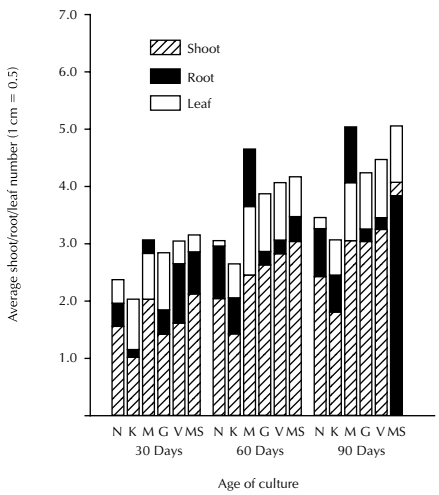


FIG. 1. **Effect of various nutrient media on shoot, root and leaf number of regenerants. (*N*, Nitsch; *K*, Knudson C; *M*, Mitra et al.; *G*, Gamborg; *V*, Vacin & Went; *MS*, Murashige & Skoog.)**

Shoot multiplication

Protocorm-like bodies (plbs) so developed were transferred to fresh medium containing six different basal media: Vacin and Went (1949), Mitra et al. (1976), Gamborg et al. (1968), Nitsch and Nitsch (1969), Knudson-C (1946), and Murashige and Skoog (MS), and the effects of six macroelement formulae were studied on growth and development of seedlings. To avoid the possibility of a carry over effect of growth regulators from the original mother explants, all the results given in this paper were obtained during the third set of experiment. Each experiment was repeated thrice. The cultures were transferred to a fresh medium every four weeks. The growth and development of the plbs were studied in terms of fresh weight; number of shoot, root and leaf; and length of shoot and root.

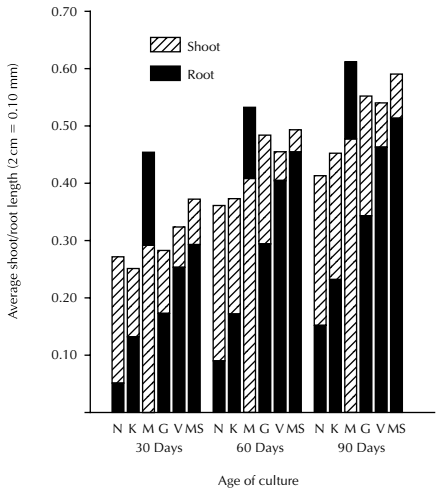


FIG. 2. **Effect of various nutrient media on shoot and root length. (*N*, Nitsch; *K*, Knudson C; *M*, Mitra et al.; *G*, Gamborg; *V*, Vacin & Went; *MS*, Murashige & Skoog.)**

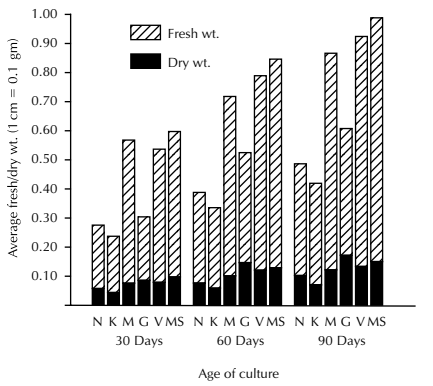


FIG. 3. **Effect of various nutrient media on fresh and dry weight of regenerants. (*N*, Nitsch; *K*, Knudson C; *M*, Mitra et al.; *G*, Gamborg; *V*, Vacin & Went; *MS*, Murashige & Skoog.)**

Results

Of the six mineral media tested, best results were obtained with MS medium. Besides resulting into the highest shoot and leaf number, shoot length and fresh weight (0.984 g), it produced dark green stems and long leaves. However, the highest average root number and root length were observed with Mitra et al. medium. Gamborg's medium yielded the highest dry weight; and average shoot, root and leaf number were found to be the lowest on Knudson-C. Growth on both Knudson-C and Nitsch media was stunted. The cultures grown on Vacin and Went medium were the second best in their growth.

Discussion

The successful establishment and growth of plant cells in vitro generally is determined by the nature of the explant and the composition of the nutrient medium (White, 1951). Growth and differentiation are controlled by various components of media and mineral nutrients. It has been reported that seeds/protocorms of orchid are unable to absorb and utilize nitrates during the initial phase of germination and development. However, appearance of nitrate reductase activity has been reported to be correlated with the absorption of nitrates in the later stage (Raghavan, 1964). Presence of high amount of ammonium nitrate in MS might favour the growth as NH_4^+ ions are readily assimilated during the initial phase and NO_3^- at later stage of development. Moreover, most tissue culture media are poorly buffered and pH fluctuations that occur may be detrimental to long term survival and to growth of cells at either low density (Caboche, 1980) or as single cells (Koop et al., 1983).

We derive that the nutrient medium containing high concentration of ammonium nitrate favours luxuriant growth of *D. wardianum*, which can be exploited for nursery and orchard developments.

Creating a "Glow-in-the-dark" Orchid

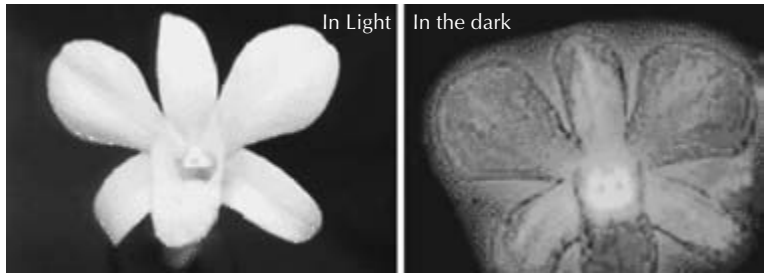
> The world's first and only genetically modified *bioluminescent* [emphasis added] orchid (Chia et al., 1994, 2000) [a *Dendrobium*] has been successfully developed by Professor Chia Tet Fatt [Tet Fatt Chia, western style] from the National Institute of Education (NIE). To create the bioluminescent orchid, Professor Chia transformed tissues from orchids (the *Dendrobium* genus) using the firefly luciferase gene. Using a method called "particle bombardment," biologically active DNA from the firefly gene was delivered into orchid tissues. Transformed cells were identified by their bioluminescence trait. These transformed tissues were propagated and used to generate transgenic plants (plants with a foreign gene incorporated). This process was repeated several times, and the bioluminescent trait was present in all transgenic plants. This confirms that the firefly luciferase gene has been integrated into the orchid.

Unlike the fluorescent traits which store and re-emit light energy, the bioluminescent trait of the orchid uses its own energy to create light. These bioluminescent orchids will produce constant light, visible to the human eye, for up to 5 h at a stretch.

This greenish-white light is emitted from the whole orchid, including roots, stem, leaves, and petals. The intensity of light produced varies across the different parts, ranging from 5000 to 30,000 photons per second. Claims that this transformation was unsuccessful and is unstable are malicious, unethical, untrue, driven by jealousy, and should be discounted.

Genetic transformation can help supplement traditional breeding of orchids to create orchids with desirable traits, such as novel colors, longer shelf life, and increased resistance to pests and diseases. It is also possible that this procedure can be used for the transformation of other orchid species and hybrids. (Taken verbatim from www.hybridorchids.com/details.html)

This book does not deal with molecular biology or bioengineering. This note is included here because the work was carried out in vitro and involved micropropagation.



Gene Expression During Floral Transition in *Dendrobium* Madame Thong-In

Dr. Zhihua Yu, a molecular biologist, working at the Department of Biological Sciences, National University of Singapore developed an in vitro system as a model for research on gene expression during transition to flowering by *Dendrobium* Madame Thong-In. He published his work with Professor Chong Jin Goh, a plant physiologist (at that time chairman of the department), as co-author. Dr. Yu used plants produced through micropropagation (Yu and Goh, 2000). Before that Dr. Yu also produced transgenic orchid plants through hygromycin selection by particle bombardment of protocorms of *Dendrobium* Mi Hua (Yu et al., 1999). This work was done at Peking University, Beijing, China.

Effects of Growth Regulators on Production of Protocorm-like Bodies by *Dendrobium* Sonia

See procedure for *Cattleya leopoldii* (Saiprasad et al., 2002).

Disa

Of the more than 140 *Disa* species, *Disa uniflora* (*Disa grandiflora*), The Pride of Table Mountain, is best known and generally considered to be the most beautiful. The British botanist John Ray may have been the first to describe it from a dried specimen and second-hand information when he wrote in 1704 about an "orchid africana flore singulari herbaceo." In 1763 the Swedish botanist Petrus Jonas Bergius gave *Disa uniflora* its name and illustrated it in his book *Descriptiones Plantarum ex Capite Bonae Spei*. Somehow the species was given another name, *Disa grandiflora*, in 1781. Despite being a synonym this name became widely accepted; it is still being used by some who claim that it is justified by the fact that *D. grandiflora* has larger flowers than *D. uniflora* (Haas-von Schmude and Lückel, 1977). For those interested in mass rapid clonal propagation through tissue culture, the nomenclatural morass means that methods developed under either name apply to both.

Clonal Propagation of *Disa uniflora* in Vitro

Despite strict conservation laws in South Africa, *Disa uniflora* is threatened with extinction. Propagation methods were developed as a means of conservation (Haas-von Schmude and Lückel, 1977).

Plant Material. All leaves are removed from young shoots of *Disa* with a scalpel, and the apical meristem is excised along with one or two leaf primordia.

Surface Sterilization. The excised shoot tips are immersed in 0.3% calcium hypochlorite solution (300 mg calcium hypochlorite in 100 ml distilled water) for 15 min and then rinsed three to five times with sterile distilled water.

Culture Vessels. A description is not given in the original paper, but it is reasonable to assume that standard containers can be used.

Culture Conditions. The cultures should be maintained under 12-h illumination (light sources and intensity not described in the original paper) at 20°C. The conditions used in the following method (Van Waes and Geest, 1983) and for orchid tissue culture in general should also be suitable.

Culture Medium. Explants are cultured on "modified [Thomale] GD-medium" (Table Disa-1), but the nature of the modifications is not described. It is possible that the media used in the following procedure (Van Waes and De Geest, 1983; Tables Disa-2 and Disa-3) could be suitable.

Procedure. The explants are placed on the medium and allowed to grow. When approximately 10 plantlets form, they should be divided and moved to new medium. This can be repeated several times.

TABLE DISA-1. **Thomale GD medium (Thomale, 1954) for the culture of *Disa* shoot tips from mature plants (Haas-von Schmude and Lückel, 1977)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[c]	370	37.0 g l^{-1}	10	
2	Ammonium sulfate, $(NH_4)_2SO_4$[c]	60	6.0 g l^{-1}	10	
3	Magnesium nitrate, $MgNO_3 \cdot 6H_2O$[c,d]	110	10.0 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	300	30.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	400	40.0 g l^{-1}	10	
6	**Chelated iron**[e]				
(a)	Chelating agent, Na_2EDTA	37.25	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.85	2.79 g l^{-1}		
7	**Microelements**[f]				
(a)	Boric acid, H_3BO_3	10	1 g l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	25	2.5 g l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	10	1 g l^{-1}		
	Sugars				
8	Fructose	10 g	No stock	No stock	Weigh
9	Glucose	10 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[g]	To 1000 ml			
	Solidifier				
11	Agar, Difco Bacto[g]	16 g	No stock	No stock	Weigh

[a]The authors (Haas-von Schmude and Lückel, 1977) indicate that this medium is a modification without listing the modifications.

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]This is the salt most commonly listed in current catalogs.

[e]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved. The original recipe does not use chelated iron, perhaps because it was not available in 1954.

[f]Add all salts to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe does not include microelements, but they are desirable.

[g]Add items 1–7 to 800 ml distilled water (item 10), adjust pH to 4.8–5.1, add sugar (items 8 and 9), and adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense solution into culture vessels and autoclave. Omit agar if preparing liquid medium.

Developmental Sequence. During the original research four small plants form following 6 weeks in culture. This number increased to eight in 10 weeks. After 16 weeks there were 10 plants ranging in size from 10 to 18 mm.

General Comments. The original research included the development of a seed germination method.

TABLE DISA-2. **A medium for the culture of *Disa* seedling shoot tips (Van Waes and De Geest, 1983)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[c]	370	37.0 g l^{-1}	10	
2	Ammonium sulfate, $(NH_4)_2SO_4$[c]	60	6.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	100	10.0 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	300	30.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	400	40.0 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.25	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.85	2.79 g l^{-1}		
7	**Microelements**[e]				
(a)	Boric acid, H_3BO_3	10	1 g l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	25	2.5 g l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	10	1 g l^{-1}		
	Polyol				
8	*myo*-inositol	100	No stock	No stock	Weigh
	Amino acid				
9	Glycine	2	200 mg 100 ml^{-1} 95% ethanol	1	
	Auxin				
10	Indolebutyric acid (IBA)	0.1	40 mg 100 ml^{-1} 95% ethanol[f]	0.25	
	Cytokinin				
11	Benzyladenine	0.1	40 mg 100 ml^{-1} 95% ethanol[f]	0.25	
	Vitamins				
12	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[f]	0.25	
13	Folic acid	0.5	200 mg 100 ml^{-1} 95% ethanol[f]	0.25	
14	Niacin (nicotinic acid)	5.0	500 mg 100 ml^{-1} 95% ethanol[f]	1	
15	Pyridoxine·HCl	0.5	200 mg 100 ml^{-1} 95% ethanol[f]	0.25	
16	Thiamine·HCl	0.5	200 mg 100 ml^{-1} 95% ethanol[f]	0.25	
	Sugar				
17	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Agar, Difco Bacto[g]	6 g	No stock	No stock	Weigh

[a]This medium consists of the macroelements of Thomale GD medium (Thomale, 1954), microelements and vitamins of Nitsch and Nitsch (1969), and iron of MS medium (Murashige and Skoog, 1962).

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium.

[f]Keep refrigerated or frozen between uses.

[g]Add items 1–8 to 850 ml distilled water (item 18), adjust pH to 5.6, add sugar (item 17), and adjust volume to 1000 ml with distilled water (item 18). Bring solution to a gentle boil, and add agar (item 19) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l Erlenmeyer flask and autoclave. Add amino acid, hormones, and vitamins (items 9–16) to hot or warm, still liquid solution, swirl several times to mix well, and dispense medium into preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE DISA-3. **A medium for the culture of *Disa* plantlets obtained from seedling shoot tips (Van Waes and De Geest, 1983)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[c]	370	37.0 g l^{-1}	10	
2	Ammonium sulfate, $(NH_4)_2SO_4$[c]	60	6.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	100	10.0 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	300	30.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	400	40.0 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.25	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.85	2.79 g l^{-1}		
7	**Microelements**[e]				
(a)	Boric acid, H_3BO_3	10	1 g l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	25	2.5 g l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	10	1 g l^{-1}		
	Polyol				
8	*myo*-inositol	100	No stock	No stock	Weigh
	Amino acid				
9	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[f]	1	
	Auxin				
10	Indolebutyric acid (IBA)	0.2	80 mg 100 ml^{-1} 95% ethanol[f]	0.25	
	Vitamins				
11	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[f]	0.25	
12	Folic acid	0.5	200 mg 100 ml^{-1} 95% ethanol[f]	0.25	
13	Niacin (nicotinic acid)	5.0	500 mg 100 ml^{-1} 95% ethanol[f]	1	
14	Pyriodoxine·HCl	0.5	200 mg 100 ml^{-1} 95% ethanol[f]	0.25	
15	Thiamine·HCl	0.5	200 mg 100 ml^{-1} 95% ethanol[f]	0.25	
	Sugar				
16	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar, Difco Bacto[g]	6 g	No stock	No stock	Weigh

[a]This medium consists of the macroelements of Thomale GD medium (Thomale, 1954), the microelements and vitamins of Nitsch and Nitsch (1969), and iron of MS medium (Murashige and Skoog, 1962).

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium.

[f]Keep refrigerated or frozen between uses.

[g]Add items 1–8 to 850 ml distilled water (item 17), adjust pH to 5.6, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l Erlenmeyer flask and autoclave. Add amino acid, hormone, and vitamins (items 9–15) to hot or warm, still liquid solution, swirl several times to mix well, and dispense medium into preautoclaved culture vessels. Omit agar if preparing liquid medium.

Tissue Culture Propagation of *Disa uniflora*

As with the method in the previous section (which was formulated at the Botanical Institute, Justus Liebig University, Germany), a tissue culture propagation procedure for *Disa uniflora* was developed at the National Botanic Garden in Belgium as part of a project that included research on seed germination (Van Waes and De Geest, 1983).

Plant Material. Apices taken from seedlings were cultured during the original research.

Surface Sterilization. Seedlings are already sterile, and surface sterilization is not needed. If apices of shoots taken from mature plants are cultured, the surface sterilization procedure described above (Haas-von Schmude and Lückel, 1977) can be used.

Culture Vessels. Standard containers are suitable.

Culture Conditions. During the original research the cultures were maintained under 14-h photoperiods of 700 to 800 lx [provided in the original research by fluorescent tubes described as "phytor (type LF, 40 W)"] at 24 ± 2°C during the light periods and 18 ± 2°C in the dark.

Culture Media. Apices are first cultured on a medium that contains a cytokinin and an auxin (Table Disa-2). Axillary buds from these explants and plantlets are moved to a second medium that contains only an auxin (Table Disa-3).

Procedure. Place shoot apices on the first medium (Table Disa-2) for approximately 3 months. Move divisions or plantlets from these cultures to the second medium (Table Disa-3). After 3 months subculture plantlets formed in these cultures on the same medium. Allow them to grow for another 3 months, and move them to pots.

Developmental Sequence. First signs of development can be noted after 8 weeks. Buds, shoots, roots, and plantlets develop after that.

General Comments. This method was developed with seedling shoot tips, and there is no information regarding its suitability for explants from mature plants. As is the case with other genera and species, a method for the propagation of seedlings is less desirable than one that can be used to propagate mature plants of existing clones. It is also necessary to point out here that methods published in a language other than English tend to attract more limited attention. At present English is the language of orchid growing, science, and international commerce, and authors would be well advised to use it for their publications.

Diuris

Commonly known as the donkey orchids (because of their ear-like petals), *Diuris* as a genus was first described in 1798 by the British botanist Sir James Edward Smith (1759–1828), a contemporary of Sir Joseph Banks and Sir Stamford Raffles (founder of Singapore). He was also the founder and the first president (in 1788) of the Linnean Society of London. More than 50 *Diuris* species are known at present with about half of them in Western Australia (Hoffman and Brown, 1992).

Micropropagation of *Diuris longifolia*

Although one of the donkey orchids, the common name of *Diuris longifolia* is the purple pansy orchid due to the appearance of its flowers. A micropropagation procedure for this Australian terrestrial orchid was formulated at the Kings Park and Botanical Garden in West Perth, Western Australia (Collins and Dixon, 1992).

Plant Material. Inflorescences with unopened buds were collected "from natural stands in bushlands at Kings Park, Perth, Western Australia." They were wrapped in damp newspaper and stored at 4°C for 1–2 days until used. Sections, 1–1.5 mm thick, containing axillary nodes (Fig. Diuris-1) were excised for culture. "The remainder was sliced into 1–1.5 mm thick disks. Upper parts of the stem were also sectioned, removing the flower buds but retaining the node at the base of the pedicel."

Surface Sterilization. First, the inflorescences should be washed with running water for 15 h (overnight). After that they are surface sterilized for 5–10 min with 1% sodium hypochlorite (20 or 17 ml household bleach containing 5–5.25% or 6% sodium hypochlorite respectively diluted to 100 ml with distilled water) containing 0.05% Tween 80 (www.sigmaaldrich.com; a mild household detergent or baby shampoo may also be suitable) and rinsed for three 5–10-min periods with sterile distilled water. Bleached ends and damaged tissue should be removed following the last wash.

Culture Vessels. Standard glass vessels, 200–250-ml capacity with autoclavable plastic lids or polycarbonate tubes, 3 × 8 cm, 30-ml capacity, containing medium equal to 20% (large vessels) to 30% (smaller ones) of their volume can be used. Other culture vessels can also be used.

Culture Conditions. It is best to maintain cultures under 16-h photoperiods of 40 $\mu E\ m^{-2}$ at culture level provided by Gro Lux lamps (two 4-foot lamps per fixture, 30 cm above the cultures). Temperatures should be 25°C during the light period and 22°C in the dark. Standard culture room conditions will probably also be suitable.

Culture Media. Initial culture of stem disks, immature flower bud sections, and nodes should be on a modification of the Burgeff N3f medium containing 10 µmol BA and designated as Pa 10BA (Table Diuris-1). When subcultured on the same medium PLBs generated from these explants produce shoots. After the leaves reach

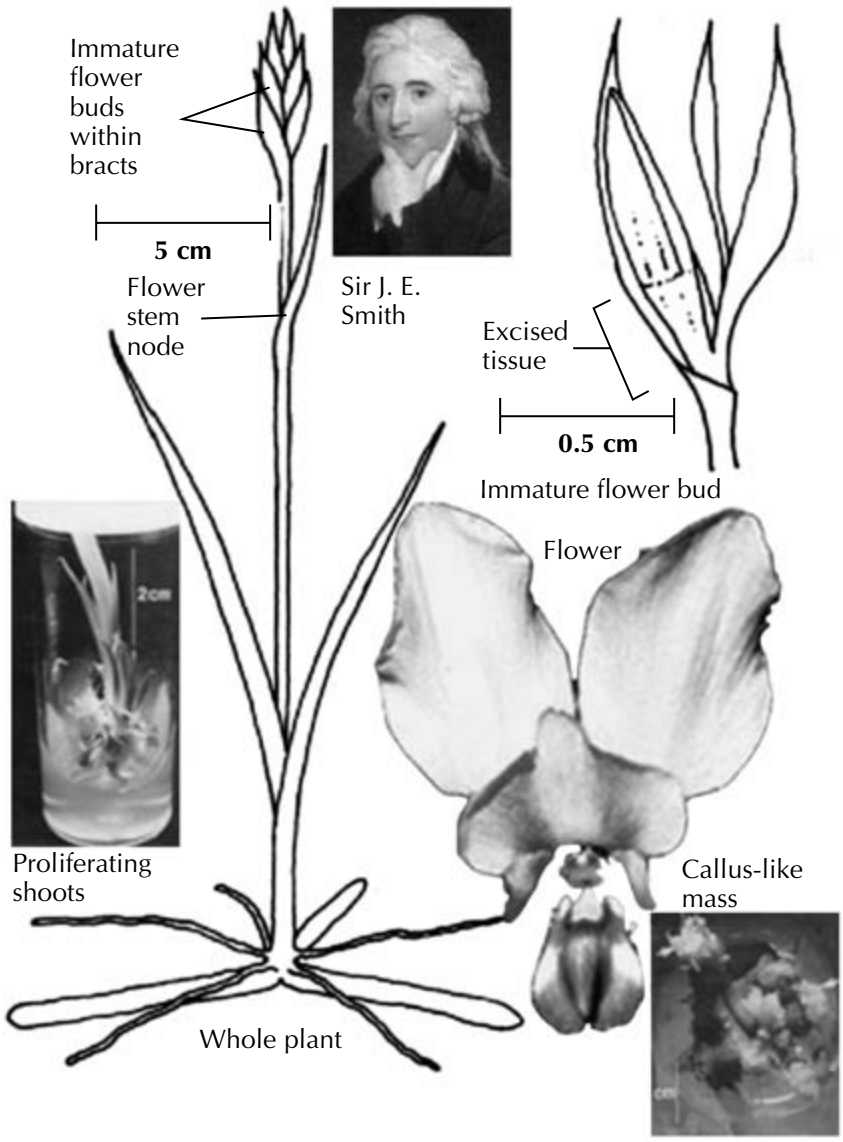


FIG. DIURIS-1. **Micropropagation of *Diuris longifolia* (Collins and Dixon, 1992).**

10–20 cm in length the leaf bearing PLBs should be moved to Pa without BA and containing 30 g sucrose l^{-1}, or 20 g sucrose l^{-1} and 0.05% activated charcoal (AC; Table Diuris-2). A potting mix consisting of "sheoak mulch . . . collected from the bases of *Allocasuarina fraseiana* trees . . . shredded . . . pasterized at 65°C for 90 min [and] mixed 7 parts to 1 part medium-grade Perlite and dampened" was used to culture plantlets.

TABLE DIURIS-1. **Burgef N3f (N3f) medium (Burgeff, 1936) modified for the culture of *Diuris longifolia* explants and protocorm-like bodies (Collins and Dixon, 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium silfate, $(NH_4)_2SO_4$[b]	250.0	25.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1000.0	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium chloride, KCL	250.0	25.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	2.48	248.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.0096	0.96 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	4.46	446.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.332	33.2 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.1	10.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	3.45	345.0 mg l^{-1}		
	Organic acid				
8	Citric acid	90.0	9 g 100 ml^{-1} 70% ethanol[e]	1	Or weigh
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	N^6-benzyladenine (BA)	22.52	4.5 g 100 ml^{-1} 70% ethanol[e,f]	0.5	
	Vitamins				
11	Ascorbic acid (vitamin C)	1.0	200 mg 100 ml^{-1} 70% ethanol[e]	0.5	
12	Biotin (vitamin H)	0.1	20 mg 100 ml^{-1} 70% ethanol[e]	0.5	
13	Calcium pantothenate[g]	1.0	200 mg 100 ml^{-1} 70% ethanol[e]	0.5	
14	Niacin (nicotinic acid)	1.0	200 mg 100 ml^{-1} 70% ethanol[e]	0.5	
15	Pyridoxine HCl (vitamin B_6)	1.0	200 mg 100 ml^{-1} 70% ethanol[e]	0.5	
16	Thiamine (vitamin B_1)	1.0	200 mg 100 ml^{-1} 70% ethanol[e]	0.5	
	Complex additive				
17	Coconut water[h]	50.0	No stock	No stock	Measure
	Sugar				
18	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[i]	To 1000 ml			
	Solidifier				
20	Agar[i]	6.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original recipe calls for 38.5 mg FeNaEDTA which is essentially the same as the mixture in this table.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses. If the citric acid fails to dissolve add a few drops of 0.1 N KOH or NaOH.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Dissolve in 30 ml glycerol and make up to 100 ml with 95% ethanol. According to the Merk Index this vitamin is not stable to autoclaving and must be added following sterilization. However in this medium it is added prior to autoclaving.

[h]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and preservatives (that may have undesirable effects on cultures).

[i]Add items 1–17 to 800 ml of distilled water (item 19), adjust pH to 5.6, add sugar (item 18), and raise volume to 1000 ml with distilled water (item 19). Bring the solution to a gentle boil and add the agar (item 20) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Amino acids, hormones, and vitamins may not be heat stable and should generally be added after autoclaving, but this need not be done in this case. All components can be added before autoclaving. Ethanol (up to 5–7 ml or even 10 ml of 95%) can be added to warm orchid culture media without ill effects. In fact 5–7 ml may even be beneficial (Thurston, Spencer and Arditti, unpublished findings dating back to the 1970s).

TABLE DIURIS-2. **Burgef N3f (N3f) medium (Burgeff, 1936) modified for root induction on shoots of *Diuris longifolia* (Collins and Dixon, 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium silfate, $(NH_4)_2SO_4$[b]	250.0	25.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1000.0	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium chloride, KCL	250.0	25.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	2.48	248.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.0096	0.96 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	4.46	446.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.332	33.2 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.1	10.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	3.45	345.0 mg l^{-1}		
	Organic acid				
8	Citric acid	90.0	9 g 100 ml^{-1} 70% ethanol[e]	1	Or weigh
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Complex additive				
10	Coconut water[f]	50.0	No stock	No stock	Measure
	Sugar				
11	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[g]	To 1000 ml			
	Solidifier				
13	Agar[g]	6.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original recipe calls for 38.5 mg FeNaEDTA which is essentially the same as the mixture in this table.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses. If the citric acid fails to dissolve add a few drops of 0.1N KOH or NaOH.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and preservatives (that may have undesirable effects on cultures).

[g]Add items 1–10 to 800 ml of distilled water (item 12), adjust pH to 5.6, add sugar (item 11), and raise volume to 1000 ml with distilled water (item 12). Bring the solution to a gentle boil and add the agar (item 13) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Amino acids, hormones, and vitamins may not be heat stable and should generally be added after autoclaving. Ethanol (up to 5–7 ml or even 10 ml of 95%) can be added to warm orchid culture media without ill effects. In fact 5–7 ml may even be beneficial (Thurston, Spencer and Arditti, unpublished findings dating back to the 1970s).

Procedure. After the final sterile distilled water wash and removal of discolored tissues, explants should be placed on the first medium (Table Diuris-1) and allowed to remain on it until PLBs are formed. After being subcultured on the same medium and following the production of 10–20-mm-long shoots, the PLBs should be moved to the second medium for rooting. Rooted plants are moved to the potting mix.

Developmental Sequence. Surviving explants (3/268 stem disks and 10/67 nodes after 180 days) produced "callus-like material at the ovary end" after a month and PLBs following 45 days on the first medium (Table Diuris-1). When subcultured on the same medium (Table Diuris-1) the PLBs produced shoots after 66–79 days. Shoots with 10–20-mm-long leaves produced roots after 70 days on the second solution (Table Diuris-2). Plantlets ready for potting were produced on the second medium after 85–100 days. These plantlets grew well after being potted, with 100% survival after 10 weeks. Some flowered 11 months after the start of culture.

General Comments. Despite low initial survival percentages this is an excellent procedure made even more useful by a report (Collins and Dixon, 1992) which is clear, detailed, and very well written. The method is the first published and successful protocol for an Australian terrestrial orchid.

Micropropagation of *Diuris purdiei*

The procedure used for *Diuris longifolia* can also be utilized for *Diuris purdiei* (Collins and Dixon, 1992).

Diuris longifolia (www.members.iinetnet.au/amntee/Diuris93.htm)

Diuris purdiei (King's Park&Botanic Garden)

Doriella

W. W. Goodale Moir was an orchid breeder in Hawaii who had a penchant for indiscriminately cross pollinating orchids across generic and species lines. As a result he produced many hybrids, a number of them involving more than one genus. To explain his successes, he formulated unusual, unscientific and silly theories, one of them being that phases of the moon somehow affect fruit set in orchids. In fact the reason for his successes was simply the exceedingly large number of crosses he made. It was inevitable that some of his innumerable crosses would produce interesting hybrids. One bigeneric hybrid he produced was *Doriella* (*Drlla*). The first of these was *Doriella* Tiny (*Doritis pulcherrima* × *Kingiella philippinensis*).

As is so often the case in orchids there are taxonomic and nomenclatural problems with *Kingiella*. One is a name change since *Kingiella* Rolfe was predated by *Kingella* van Tiegh. To solve this problem Peter F. Hunt replaced *Kingiella* with *Kingidium* (Bechtel et al., 1992). The other problem is that some taxonomists include *Kingidium* (*Kingiella*) in *Phalaenopsis* (Christenson, 2001). Should this be accepted *Doriella* would have to become *Phaliella*, but the International Registration Authority for Orchid Hybrids considers *Kingiella* to be valid. And what if *Kingidium* does become accepted? Will *Doriella* become *Dorgidium*, *Dorigidium*, *Doriidium*, or *Doridium*? And what if the order was reversed? Will we have *Kingritis*, *Kinritis*, *Kintis*, or *Kinoritis*? Probably not because these names seem like those of rare diseases. All this would be funny if it was not confusing, sometimes aggravating, and always taken so very seriously by taxonomists, breeders, and some growers.

The name *Doritis* may be derived from the Greek δορυ (dory), spear, in allusion to the hastate labellum or refer to Δοριτισ (Doritis which is another name for Aphrodite).

Kingiella commemorates Sir George King (1840–1909) of Indian orchids fame.

Micropropagation of *Doriella* Tiny

An effort to induce flowering in vitro by *Doriella* Tiny produced results which can be used as a micropropagation protocol (Duan and Yazawa, 1994*b*, 1994*c*, 1995*a*).

Plant Material. Flower-stalk internode sections, 3 cm long, each with a bud are cultured.

Surface Sterilization. In the original research the two ends of each section were sealed with wax apparently to prevent entry of the sterilant into the sections. This seems to be an unnecessary precaution because the amount of sterilant which can enter the stem during the 10-min sterilization is insignificant. Even if a larger volume did penetrate, the possibility that it would affect the bud is infinitesimal. Evidence for this is the successful culture of *Phalaenopsis* stem sections that are routinely sterilized without wax on their cut ends. Also, slightly longer sections, 4.5–5 cm, could be sterilized and 1 cm can be cut from each end to remove any tissue that may have absorbed sterilant. Sections should be sterilized by shaking them for 10 min in 1% sodium hypochlorite (20 or 17 ml of a household bleach containing 5–5.2% or 6% sodium hypochlorite respectively diluted to 100 ml with distilled water) containing 0.1% Tween 20 (www.sigmaaldrich.com), mild household detergent, or baby shampoo and then rinsed three times with sterile distilled water. The wax and "bracteal leaves" should be removed after the last rinse and before the sections are placed in culture. If longer sections are used the extra tissue should be cut at this stage. If "bracteal leaves" are the bracts which cover the buds it is best to remove them before sterilization because they can cover and protect potential contaminants.

Culture Vessels. Erlenmeyer flasks, 250 ml, or other containers can be used.

Culture Conditions. Cultures should be maintained at $25 \pm 3°C$ under 16-h photoperiods of 2500 lx provided by fluorescent tubes (type and manufacturer not indicated) or standard culture room conditions.

Culture Media. Sections should be cultured first in Hyponex (www.hyponex.co.jp) medium (Table Drlla-1) supplemented with BA, coconut water, and peptone for about 40–50 days. Adventitious buds formed on the first medium (Table Drlla-1) can be subcultured on the same solution. After that or following the formation of vegetative buds it is necessary to move bud bearing explants to Vacin and Went (VW)

TABLE DRLLA-1. **Hyponex medium for the culture of *Doriella* Tiny stem sections (Duan and Yazawa, 1994*b*)**

Component	Amount l^{-1}	Component	Amount	Comments
Hyponex	3.5 g	Sucrose	25.0 g	Add all components to 700 ml of water, adjust pH
Benzyladenine (BA)	5.0 mg	Agar	10.0	to 5.6, raise volume to 1000 ml, bring to a gentle
Peptone	2.0 g	Water	To 1000 ml	boil, add agar with stirring and keep stirring until it
Coconut water	150.0 ml			is dissolved, pour into culture vessels and autoclave

medium (Vacin and Went, 1949) containing BA (Table Drlla-2). Following the formation and development of leaves the shoots should be transplanted onto VW free of BA (Table Drlla-3) for root formation. Standard VW (Table Drlla-4) will probably be suitable for plantlet development.

Procedure. Following sterilization the explants should be cultured on the Hyponex medium (Table Drlla-1) until they develop vegetative buds. Some of the buds can be excised and cultured on the same solution (Table Drlla-1) to increase the number of explants. For leaf formation and development to take place it is necessary to move the bud bearing explants to the second medium (Table Drlla-2). Shoots which form on this medium must be moved to the third solution (Table Drlla-3) for root formation. Plantlets can be cultured on standard VW (Table Drlla-4) for further development. Buds which form on the explants on the first medium (Table Drlla-1) can be subcultured.

Developmental Sequence. Vegetative buds form on the Hyponex medium (Table Drlla-1). Leaves develop from these buds on the second solution (Table Drlla-2). Roots are initiated and plantlets form on the third medium (Table Drlla-3). Plantlets will grow bigger on the last substrate (Table Drlla-4).

General Comments. This seems to be a simple yet effective procedure for the micropropagation of *Doriella*. It may also prove to be effective for *Doritis*, *Kingiella* (*Kingidium*, *Phalaenopsis*), and *Doritaenopsis*.

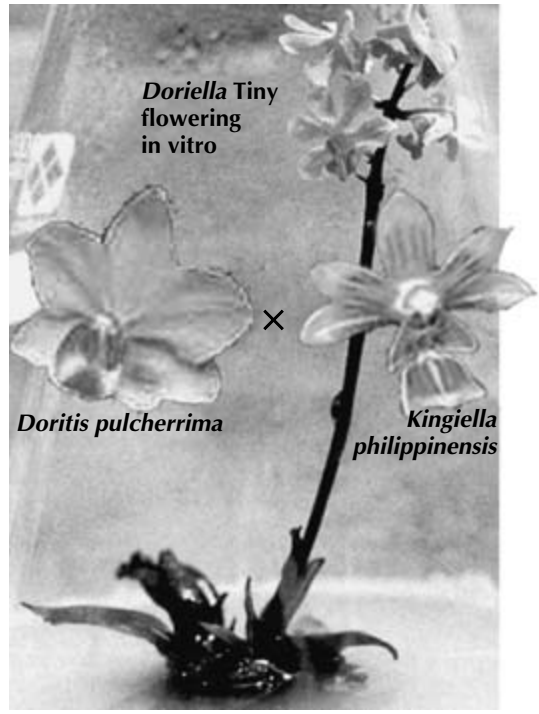


TABLE DRLLA-2. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of bud bearing *Dorriella* Tiny explants to enhance leaf growth (Duan and Yazawa, 1994*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Cytokinin				
8	Benzyladenine (BA)	10.0	1000 mg 100 ml 95% ethanol[e]	1	Or weigh
	Complex additive				
9	Coconut water[f]	150.0 ml	No stock	No stock	Measure
	Sugar				
10	Sucrose	25.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 11) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]If the cytokinin fails to dissolve add a few drops of 0.1N KOH or NaOH. Keep frozen between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and preservatives (that may have undesirable effects on cultures).

[g]Add items 1, 3–7, and 9 to the 500–700 ml of distilled water (item 11) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 11), adjust pH to 5.6, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the cytokinin to the autoclaved, warm and still liquid medium, swirl or stir with a sterile tool, and distribute medium into preautoclaved culture vessels. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

TABLE DRLLA-3. **Modified Vacin and Went (VW) medium (Vacin and Went, 1949) for root induction on leaf bearing explants of *Doriella* Tiny (Duan and Yazawa, 1994*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water[e]	50.0	No stock	No stock	Measure
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and preservatives (that may have undesirable effects on cultures).

[f]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 10), adjust pH to 5.6, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

TABLE DRLLA-4. **Vacin and Went (VW) medium (Vacin and Went, 1949) for culture of *Doriella* Tiny plantlets (Duan and Yazawa, 1994*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 9) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Add items 1 and 3–7 to the 500–700 ml of distilled water (item 9) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 9), adjust pH to 5–5.4, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring the solution to a gentle boil and add the agar (item 10) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

In Vitro Flowering by *Doriella* Tiny

A method for in vitro flowering induction in *Doriella* Tiny was developed at the University of Kyoto to aid hybridization by reducing the period between making a cross and seeing the results (Duan and Yazawa, 1994*b*, 1994*c*, 1995*a*).

Plant Material. Flower-stalk internode sections, 3 cm long, each with a lateral bud are cultured.

Surface Sterilization. The two ends of each section were sealed with wax by the researchers probably to prevent sterilant entry of the tissue. This may be an unnecessary precaution because the volume of sterilant which can penetrate the stem during the sterilization period is insignificant and will probably do no harm. Proof of this is the successful culture of *Phalaenopsis* stem sections which are usually sterilized

without their cut ends being sealed. If there is concern about the sterilant, a simpler approach would be to sterilize sections which are 4.5–5 cm long and then cut 1 cm from each end to remove tissue that may contain sterilizing agent. The sections are sterilized by shaking them for 10 min in a 1% sodium hypochlorite solution (20 ml household bleach containing 5–5.25% sodium hypochlorite diluted to 100 ml with distilled water) containing 0.1% Tween 20 (www.sigmaaldrich.com) or mild household detergent and then rinsed three times with sterile distilled water. Wax and "bracteal leaves" should be removed after the rinses before placing the sections in culture. Should longer sections be used the extra tissue should be cut at this stage. If "bracteal leaves" are the bracts which cover the buds it is better to remove them before sterilization because they can cover up contaminants. Culturing buds which form on sections on the first medium (Table Drlla-1) will increase the number of explants.

Culture Vessels. Erlenmeyer flasks, 250 ml, or other containers can be used.

Culture Conditions. Cultures should be maintained at 25 ± 3°C under 16-h photoperiods of 2500 lx provided by fluorescent tubes (type and manufacturer not indicated).

Culture Media. Sections should be cultured first on Hyponex (www.hyponex.co.jp) medium (Table Drlla-1) supplemented with BA, coconut water, and peptone for about 40–50 days. Adventitious buds which form during this time can be subcultured on the same solution (Table Drlla-1). This "cycling propagation" increases the number of explants. After that 90-day-old shoots, 3–4 cm in height with three or four leaves, should be moved to Vacin and Went (VW) medium (Vacin and Went, 1949) containing BA and coconut water (Table Drlla-5). Following the formation of flower stalks and buds the plantlets should be returned to hormone-free Hyponex medium for flowering. The paper does not make clear whether this medium should be free of coconut water (Table Drlla-6). Therefore both versions (Tables Drlla-1 and Drlla-6) should be tested.

Procedure. Following sterilization the explants should be cultured on the Hyponex medium (Table Drlla-1) until they develop vegetative buds. These buds should be excised and cultured on the same solution (Table Drlla-1) to increase the number of propagules. For flower-bud formation shoots with three or four leaves and 3–4 cm tall are moved to the VW medium (Table Drlla-5). When flower buds form the plantlets should be moved to the Hyponex medium (Tables Drlla-1 or Drlla-6) which is most suitable for flowering (this must be determined by a comparison between the two). To enhance root formation after flowering, it may be necessary to move the shoots to a root inducing medium (Table Drlla-3). Plantlets can be cultured on standard VW medium (Table Drlla-4) for further development.

Developmental Sequence. The explants produce vegetative buds on the Hyponex medium (Table Drlla-1). Flower buds form after transfer to VW medium (Table Drlla-5). The plants flower on one or both of the Hyponex media (Tables Drlla-1 or Drlla-6). Roots are initiated and plantlets develop on another version of VW medium (Table Drlla-3).

TABLE DRLLA-5. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the induction of flower buds on *Dorriella* Tiny shoots (Duan and Yazawa, 1994*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Cytokinin				
8	Benzyladenine (BA)	5.0	500 mg 100 ml 95% ethanol[e]	1	Or weigh
	Complex additive				
9	Coconut water[f]	150.0 ml	No stock	No stock	Measure
	Sugar				
10	Sucrose	25.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 11) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]If the cytokinin fails to dissolve add a few drops of 0.1N KOH or NaOH. Keep frozen between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and preservatives (that may have undesirable effects on cultures).

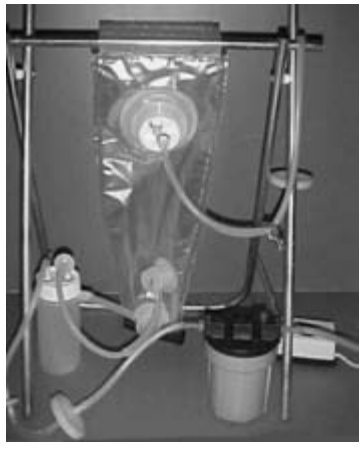
[g]Add items 1, 3–7 and 9 to the 500–700 ml of distilled water (item 11) which contain the calcium phosphate (item 2), raise volume to 900 ml with distilled water (item 11), adjust pH to 5.6, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the cytokinin to the autoclaved, warm and still liquid medium, swirl or stir with a sterile tool and distribute medium into preautoclaved culture vessels. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the MS medium will probably be suitable.

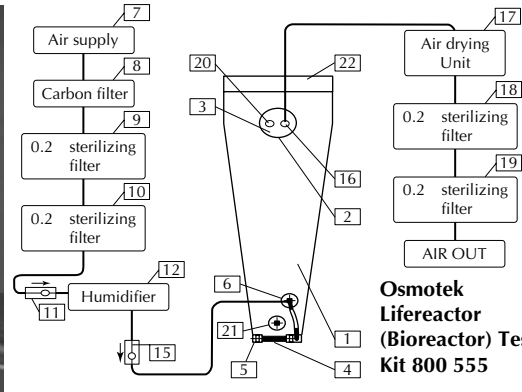
TABLE DRLLA-6. **Hyponex medium for the culture of *Doriella* Tiny stem sections (Duan and Yazawa, 1994*b*)**

Component	Amount l^{-1}
Hyponex (www.hyponex.co.jp)	3.5 g
Benzyladenine (BA)	5.0 mg
Peptone	2.0 g
Sucrose	25.0 g
Agar	10.0 g
Water	To 1000 ml

Add all components to 700 ml of water, adjust pH to 5.6, raise volume to 1000 ml, bring to a gentle boil, add agar with stirring and keep stirring until it is dissolved, pour into culture vessels and autoclave. See footnote *g* in Table Drlla-5 for detailed instructions.

General Comments. This seems to be a simple yet effective procedure for flower induction in vitro in *Doriella*. It may also prove to be effective for *Doritis*, *Kingiella* (*Kingidium*, *Phalaenopsis*), and related species.

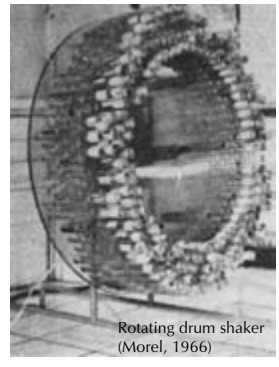




Osmotek Lifereactor (Bioreactor) Test Kit 800 555

Photograph (left) and diagram (right) of a bioreactor (www.osmotek.com) and shakers (below)

Platform shaker (www.fishersci.com)



Rotating drum shaker (Morel, 1966)

Doritaenopsis

Doritis as a genus was established by John Lindley in 1833. It is closely related to *Phalaenopsis* and separated from it by the length of the rostellum, column foot, and stipe of the pollinarium. As is so often the case with orchids some taxonomists consider these morphological differences to be important enough to justify separation of the two species and others do not. As a result taxa were transferred between the two genera at the drop of a taxonomist's whim (Freed, 1976). However, recent molecular data suggest that there is no justification for considering *Doritis* as a separate genus (Christenson, 2001). If this is accepted (as it should be because molecular data are much more powerful than personal views regarding the importance of structural features), *Doritis* will disappear and so will *Doritaenopsis*. However, for the time being *Doritis* exists and therefore so does *Doritaenopsis*, its hybrid with *Phalaenopsis*. The first *Doritaenopsis* hybrid in *Sander's List of Hybrids* is Dtps. Asahi (*Phalaenopsis lindeni* × *Doritis pulcherrima*) which was registered by Baron Toshito Iwasaki of Tokyo, Japan. At present *Doritaenopsis* hybrids are of commercial importance.

The following media in other sections of the book are used in the next procedure:

- Modified liquid Murashige–Skoog (see Table Aranda-11) or Vacin and Went (see Table Arach-5) media are used to culture explants. (As already mentioned Emil Vacin and Frits W. Went did not name their medium after themselves. Subsequent users named it by referring to it as the Vacin and Went medium. The same is true for Toshio Murashige and Folke Skoog and their medium, which is the most widely used plant tissue culture substrate.)
- Solid Vacin and Went medium (see Tables Arach-4 and Arach 5) is used for multiplication and differentiation.

Clonal Propagation of *Doritaenopsis* from Lateral Buds on Flower Stalks

A procedure for the clonal propagation of *Doritaenopsis* was developed at the Singapore Botanic Gardens (Lim-Ho, 1981).

Plant Material. Flower stalks of *Doritaenopsis* Elizabeth Waldheim (*Doritis pucherrima* × *Phalaenopsis* Lam Soon) and an unregistered hybrid were used in the original research.

Surface Sterilization. Scales that cover the buds are removed, and the stalk is washed with soap (antiseptic in the original research) and water. The washed stalks are immersed in 12–15% Clorox (12–15 ml Clorox diluted to 100 ml with distilled water) for 20 min. Although not mentioned in the original paper (Lim-Ho, 1981), several subsequent rinses with sterile distilled water are desirable.

Culture Vessels. See entries for *Aeridachnis*, *Arachnis*, *Arachnostylis*, *Aranda*, *Aranthera*, *Ascocenda*, *Burkillara*, and *Dendrobium* (Lim-Ho, 1981).

TABLE DTPS-1. **Tsuchiya's medium (Tsuchiya, 1954; Wimber 1963, 1965) for *Doritaenopsis* plantlet formation (Lim-Ho, 1981)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$	200 g	20 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated Iron**[c]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.84	2.78 g l^{-1}	10	One solution
(b)	Chelating agent, Na_2EDTA	37.24	3.72 g l^{-1}		
	Complex additive				
7	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
8	Sucrose	2 g[d]	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	12–15 g	No stock	No stock	Weigh
	Darkening agent				
11	Active vegetable charcoal[e]	150	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original recipe uses 28 mg ferric tartrate $[Fe_3(C_4H_4O_6)_3 \cdot 2H_2O]$ as a source of iron. Chelated iron is preferable.

[d]The original recipe includes 20 g sucrose.

[e]Add items 1–7 to 700 ml distilled water (item 9), adjust pH to 5.0–5.3, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved add the charcoal (item 11) with vigorous stirring, pour solution into culture vessels and autoclave. Omit agar if preparing liquid medium.

Culture Conditions. See entries for *Aeridachnis*, *Arachnis*, *Arachnostylis*, *Aranda*, *Aranthera*, *Ascocenda*, *Burkillara*, and *Dendrobium* (Lim-Ho, 1981).

Culture Media. Explants are first cultured in modified liquid Murashige–Skoog (see Table Aranda-11) or Vacin and Went (see Table Arach-5) media. For multiplication and differentiation the explants are moved to one of two solid modifications of the Vacin and Went medium (see Tables Arach-4 and Arach-5). Plantlet formation takes place on a solid Tsuchiya's medium (Table Dtps-1).

Procedure. Few details are given in the original paper, but it is reasonable to assume that one should section the stalks like those of *Phalaenopsis* (i.e., into 1- to 1.5-cm sections with the bud in the middle) and place them in culture. Alternately the buds only or the stalks only could be taken. Culture the explants, and transfer to a sequence of media as is done with *Aeridachnis*, *Arachnis*, *Arachnostylis*, *Aranda*, *Aranthera*, *Assocenda*, *Burkillara*, and *Dendrobium* (Lim-Ho, 1981).

Developmental Sequence. Details are lacking in the original paper, but it is possible that the buds develop like those of *Phalaenopsis*.

General Comments. This procedure should prove suitable for other crosses in this hybrid genus. Procedures developed for *Doritis* and *Phalaenopsis* could also be used. Leaves of *Doritaenopsis* were also cultured in Vacin and Went medium containing 15–50% coconut water (Sagawa and Kunisaki, 1982), but not enough details are available regarding this procedure to allow a description here.

In Vitro Culture of Flower-stalk Internodes of *Doritaenopsis*

The procedure used for *Phalaenopsis* (Lin, 1986) is suitable.

Maintenance of Embryogenic Callus of *Doritaenopsis*

Depending on the method being used to culture it, an embryogenic callus can produce protoplasts, embryoids, or PLBs, or all three, which can be used for genetic engineering or propagation. The effects of solidifiers, sugars, sugar alcohols, and coconuts on an embryogenic callus were studied at the Department of Life Sciences, Aichi University of Education in Japan (Ichihashi and Hiraiwa, 1996).

Plant Material. Callus was obtained from the Dogashima Orchid Center (2848–1 Nishina, Nishiizu-cho, Kamogun, Shizuoka 410–3514, Japan).

Surface Sterilization. There is no need to surface-sterilize callus which is growing axenically in vitro. However it should be washed with sterile distilled water to remove medium residues.

Culture Vessels. Erlenmeyer flasks, 50-ml capacity, were used in the original research. Other containers are also suitable.

Culture Conditions. The research cultures were maintained at 25°C under constant illumination of approximately 500 lx (1.2 mW cm^{-2}) provided by Plantlux (Toshiba plant growth) fluorescent tubes. Standard culture room conditions are also appropriate.

Culture Media. For maintenance and proliferation the callus should be maintained on sucrose-containing P medium (Table Dtps-2). This P medium is different from culture medium P which is used for *Ophrys* (see Table Oph-1; Hoppe and Hoppe, 1987*a*, 1987*b*, 1988). To cause the callus to become embryogenic and produce PLBs it should be cultured on a modification of the P medium which contains sorbitol (Table Dtps-3). After plantlets form roots they should be moved to banana homogenate containing Knudson C (KC; Knudson, 1946) medium (see Table Cym-24).

Procedure. Depending on the purpose, goals, or needs of a laboratory the callus can be cultured on a medium designed to maintain it (Table Dtps-2) or induce PLBs formation (Table Dtps-3). Once plantlets develop roots they should be moved to modified KC (see Table Cym-24).

TABLE DTPS-2. **P medium for maintenance of an embryogenic callus (Ichihashi and Hiraiwa, 1996)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium phosphate, $NH_4H_2PO_4$[b]	922.4	92.24 g l^{-1}	10	
2	Calcium nitrate, $CaNO_3{\cdot}4H_2O$[b]	236.2	23.62 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	246.5	24.65 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	606.6	60.66 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	272.2	27.22 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2{\cdot}6H_2O$	0.0125	1.25 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.0125	1.25 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	11.2	1.12 g l^{-1}		
(e)	Potassium iodide, KI	0.415	41.5 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
13	Coconut water[f]	125.0 ml	No stock	No stock	Measure
	Sugar				
14	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[f]	To 1000 ml			
	Solidifier				
16	Gelrite[f]	4.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved and the solution turns dark yellow.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4{\cdot}4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4{\cdot}7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[g]Add items 1–13 to 750 ml of distilled water (item 15), adjust pH to 5.6 ± 0.1, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Gelrite or gellan gum [available as such from www.caissonlabs.com or as Phytagel (www.sigmaaldrich.com)] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gelrite, (gellan gum, Phytagel) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave. As a rule amino acids (item 8) and vitamins (items 10–12) are added to the hot solution after autoclaving under sterile conditions with sterilized pipettes, mixed well and the medium is then distributed into preautoclaved culture vessels. In this instance all components are added before autoclaving.

TABLE DTPS-3. **P medium for culture of embyogenic callus (Ichihashi and Hiraiwa, 1996)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium phosphate, $NH_4H_2PO_4$[b]	922.4	92.24 g l^{-1}	10	
2	Calcium nitrate, $CaNO_3 \cdot 4H_2O$[b]	236.2	23.62 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	246.5	24.65 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	606.6	60.66 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	272.2	27.22 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.0125	1.25 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.0125	1.25 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.415	41.5 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyols				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
10	Sorbitol	10.0	No stock	No stock	Weigh
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
14	Coconut water[f]	125.0 ml	No stock	No stock	Measure
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Gelrite[g]	4.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved and the solution turns dark yellow.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[g]Add items 1–14 to 750 ml of distilled water (item 15), adjust pH to 5.6 ± 0.1, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Gelrite or gellan gum [available as such from www.caissonlabs.com and as Phytagel (from www.sigmaaldrich.com)] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gelrite (gellan gum, Phytagel) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave. As a rule amino acids (item 8) and vitamins (items 11–13) are added to the hot solution after autoclaving under sterile conditions with sterilized pipettes, mixed well and the medium is then distributed into preautoclaved culture vessels. In this instance all components are added before the medium is autoclaved.

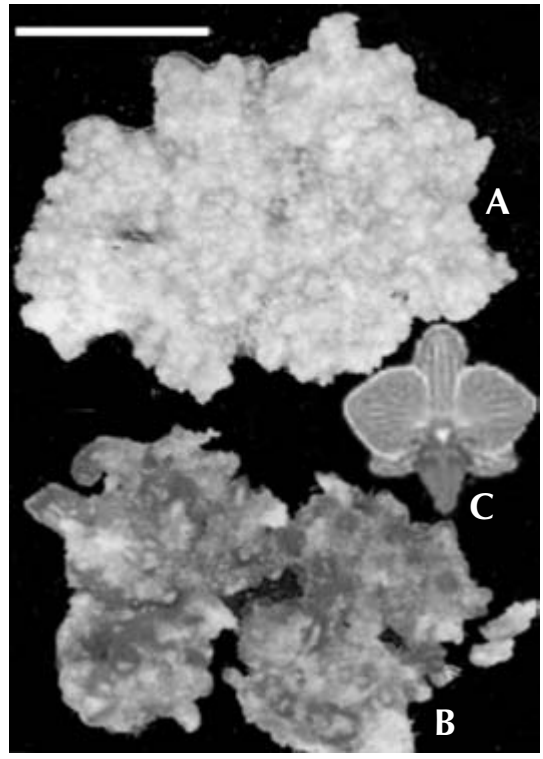


FIG. DTPS-1. **Embryogenic callus of *Doritaenopsis* New Toyohashi. A. Yellow friable callus on sucrose containing P medium. B. Green embryogenic callus on sucrose-free medium. C. *Doritaenopsis* New Toyohashi. Scale bar ≈ 1 cm. (Sources: A, B, Ichihashi and Hiraiwa, 1996; C, www.orchiddigest.com.)**

Developmental Sequence. This callus may remain friable, yellow and very prolific on one medium (Table Dtps-2) or turn green and form PLBs on the other (Table Dtps-3).

General Comments. A friable proliferating callus offers many possibilities (Fig. Dtps-1). Therefore a procedure which can generate and maintain such a callus is of considerable importance.

Micropropagation of *Doritaenopsis* through the Culture of Lateral Buds from Young Flower Stalks

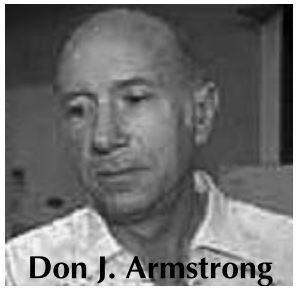
A method for the micropropagation of *Phalaenopsis* through the culture of lateral buds from young flower stalks (Ichihashi, 1992*a*, 1992*b*) can probably also be used for *Doritaenopsis*. See the *Phalaenopsis* section for more details.

Propagation of *Doritaenopsis* through the Culture of Flower-stalk Sections on Thidiazuron-containing Medium

Phalaenopsis flower-stalk sections (Rotor, 1949), not *Cymbidium* or other shoot tips (Morel, 1960), were the first orchid explants to be cultured. For *Doritaenopsis* and *Phalaenopsis* the culture of inflorescence sections is still an important means of micropropagation, but most of the available methods produce only a few plants per explants.

Chance and prepared minds: thidiazuron (TDZ) as a cytokinin

In the mid 1970s Dr. Machteld C. Mok (Departments of Horticulture and Genetics), Dr. David W. S. Mok (Department of Horticulture), and Dr. Don J. Armstrong (Department of Botany and Plant Pathology) at Oregon State University were searching for cytokinin antagonists. After they heard a talk about the cotton defoliant Dropp (TDZ) in 1978 it occurred to them that they "might have found [their] antagonist." When they "tested it in callus bioassays . . . to [their] great amazement, thidiazuron turned out to be a very potent cytokinin" (quotes from an e-mail by Machteld Mok). They reported their findings in 1979 and 1980 and published them in 1982. The discovery generated interest immediately. In 1987 Dr. Paul E. Read and his associates in Nebraska studied TDZ effects on meristem formation and shoot proliferation. Badzian et al. in Poland investigated TDZ effects on organogenesis of *Brassaia actinophylla* shoot explants in 1987. Dr. John E. Preece and his group at the University of Illinois studied the effects of TDZ in tissue culture of woody plants in 1992 and 1993. Dr. Robert Ernst at the University of California, Irvine was the first to use TDZ in orchid micropropagation and published his findings in 1994. Since then TDZ has been used successfully in a number of other orchid micropropagation procedures.



Note: Louis Pasteur is reported to have said that chance favors the prepared mind. This is a classic example of prepared minds seizing a chance.

When the extremely high cytokinin acitivity of TDZ became known, Dr. Robert Ernst at the Universisty of California, Irvine tested its effects on *Doritaenopsis* and *Phalaenopsis* stem sections (Ernst, 1994).

Plant Material. Flower stalks which finished blooming were the sources of explants. After removing the upper small-flower-bud-bearing portions and the lowermost node the stalks were cut into 3-cm sections, each with a bud in the middle.

Surface Sterilization. After sealing the ends of the sections with paraffin wax to prevent entry of sterilant into the tissues, they were wiped with 95% ethanol and then stirred for 20 min in a 1% solution of sodium hypochlorite (20 ml of household bleach containing 5–5.25% sodium hypochlorite) partially neutralized to pH 9.0 ± 0.2 with 2N hydrochloric acid [17.2 ml of concentrated HCl diluted to 100 ml with distilled water; to prevent splattering and possible injury to the operator the acid must be poured into the water (never the water into the acid) slowly with stirring while wearing a face guard and gloves]. Bracts which cover the buds were removed after that and the sections were submerged in the same sterilant for an additional 10 min. The sections were placed in culture after rinsing them three times with sterile distilled water and cutting the paraffin wax seals. The paraffin seals seem to be an unnecessary precaution which overly complicates the procedure without serving much of a useful function because the volume of sterilant which can enter the sections during 30 min is very small and will probably have no effects of any kind. Also, cutting about 0.25–0.5 cm off the ends of sections will probably remove tissue which contains sterilant. Countless *Phalaenopsis* stem sections are cultured successfully in many laboratories without being sealed. This is convincing evidence that paraffin sealing is not necessary. The neutralization of the sterilant (a procedure used with other plants before being adapted to *Doritaenopsis*; the original paper does not cite a source for it) also seems to be an excessive precaution and unnecessary complication because no other successful orchid procedures use it. Still, both the paraffin seal and sterilant neutralization should not be eliminated without a preliminary test.

Culture Vessels. Erlenmeyer flasks, 250 ml capacity containing about 50 ml medium, closed with perforated rubber stoppers plugged with cotton can be used. Other containers and/or different plugs are also suitable.

Culture Conditions. Research cultures were maintained at 23–24°C under 12-h photoperiods of approximately 25 μmol m^{-2} s^{-1} provided by Sylvania F 3OTB 30-W Gro-Lux lamps (Sylvania GTE, Versailles, KY, USA).

Culture Media. Explants should be cultured initially in a medium designated XER by its author (eXperimental Ernst, Robert) containing 0.45 μmol TDZ (Table Dtps-4). PLBs produced on the first medium should be cultured in XER medium containing 30–45% coconut water (CW) from ripe nuts (Table Dtps-5) to induce further proliferation. To enhance vegetative growth PLBs can also be cultured (before or after proliferation) on XER containing 0.2% activated charcoal (www.sigmaaldrich.com) and 5% (w/v) ripe banana pulp homogenate (Table Dtps-6).

TABLE DTPS-4. **XER medium for the culture of *Doritaenopsis* flower-stalk sections (Ernst, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	500.0	50.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	122.0	12.2 g l^{-1}	10	
4	Dibasic potassium phosphate, K_2HPO_4	30.0	3.0 g l^{-1}	10	
5	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	500.0	50.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	2.0	200.0 mg l^{-1}	10	One solution
(b)	Cobaltous chloride, $CoCl_2 \cdot 6H_2O$	0.03	3.0 mg l^{-1}		
(c)	Copper chloride, $CuCl_2 \cdot 2H_2O$	0.015	1.5 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	2.5	250.0 mg l^{-1}		
(e)	Sodium molybdate, $NaMoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Cytokinin				
9	Thidiazuron	1.0	100 mg 100 ml^{-1} 1N NaOH in 95% ethanol[e]	1	
	Sugar				
10	Fructose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[e,f]	To 1000 ml			
	Solidifier				
12	Gelrite[f,g]	5.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]The original recipe does not include EDTA as a chelating agent. It is included here because iron salts tend not to stay in solution. Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. The XER original recipe for this medium calls for 28 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$, l^{-1}. The difference (0.2 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored. Sticklers for accuracy can add 28 mg l^{-1}.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]To prepare the solvent (1N NaOH in ethanol) dissolve 4 g NaOH in 100 ml 95% ethanol. Use this as a sterilizing solvent for thidiazuron if the cytokinin is to be added to the medium after autoclaving. According to several reports thidiazuron can be added to the medium before autoclaving. If so it can be dissolved in aqueous 1N NaOH. In either case the stock solution should be kept frozen between uses. Thidiazuron can also be dissolved in dimethyl sulfoxide (DMSO). Both thidiazuron and DMSO can be obtained from www.sigmaaldrich.com and other sources. For more information about incorporation of thidiazuron in culture media see Mok et al., 1982; Mok and Mok, 1985; Fellman et al., 1987; Badzian et al., 1989; Bates et al., 1992; Huetterman and Preece, 1993 (excellent source of information); Neuman et al., 1993.

[f]Add items 1–9 to 900 ml of distilled water (item 11), adjust pH to 5.5, add sugar (item 10), and bring volume to 1000 ml with distilled water (item 11). Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. When the gellan gum is completely dissolved pour the solution into culture vessels and autoclave.

[g]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels.

TABLE DTPS-5. **Coconut water-containing XER medium for proliferation of protocorm-like bodies (PLBs) produced by flower-stalk explants of *Doritaenopsis* (Ernst, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitratete, NH_4NO_3[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$[b]	500.0	50.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	122.0	12.2 g l^{-1}	10	
4	Dibasic potassium phosphate, K_2HPO_4	30.0	3.0 g l^{-1}	10	
5	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
6	Potassium nitrate, KNO_3	500.0	50.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	10.0	1.0 g l^{-1}	10	One solution
(b)	Cobaltous chloride, $CoCl_2{\cdot}6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}6H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	25.0	2.5 g l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.25	25.0 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	10.0	1.0 g l^{-1}		
	Complex additive				
9	Coconut water from ripe nuts[e]	400.0 ml	No stock	No stock	Measure
	Sugar				
10	Fructose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f,g]	To 1000 ml			
	Solidifier				
12	Gelrite[f,g]	5.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]The original recipe does not include EDTA as a chelating agent. It is included here because iron salts tend not to stay in solution. Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. The XER original recipe for this medium calls for 28 mg ferrous sulfate, $FeSO_4{\cdot}7H_2O$, l^{-1}. The difference (0.2 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored. Sticklers for accuracy can add 28 mg l^{-1}.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endospermor "meat" inside the nut). It is preferable to use water from unripe (green) nuts except when indicated otherwise as in this case. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe nuts are not available, water from ripe (brown) ones can be used even for media which require water from green fruits. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels.

[g]Add items 1–9 to 500 ml of distilled water (item 11), adjust pH to 5.5, add sugar (item 10), and bring volume to 1000 ml with distilled water (item 11). Gelrite (gellan gum, Phytagel) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. When the gellan gum is completely dissolved pour the solution into culture vessels and autoclave.

TABLE DTPS-6. **Banana pulp homogenate and activated charcoal-containing XER medium for enhancement of vegetative growth of plantlets produced by flower-stalk explants of *Doritaenopsis* (Ernst, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitratete, NH_4NO_3[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$[b]	500.0	50.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	122.0	12.2 g l^{-1}	10	
4	Dibasic potassium phosphate, K_2HPO_4	30.0	3.0 g l^{-1}	10	
5	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
6	Potassium nitrate, KNO_3	500.0	50.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	10.0	1.0 g l^{-1}	10	One solution
(b)	Cobaltous chloride, $CoCl_2{\cdot}6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}6H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	25.0	2.5 g l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.25	25.0 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	10.0	1.0 g l^{-1}		
	Complex additive				
9	Ripe banana pulp homogenate[e]	50.0 g	No stock	No stock	Weigh
	Sugar				
10	Fructose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f,g]	To 1000 ml			
	Solidifier				
12	Gelrite[f,g]	5.0 g	No stock	No stock	Weigh
	Darkening agent				
13	Activated charcoal[h]	2.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]The original recipe does not include EDTA as a chelating agent. It is included here because iron salts tend not to stay in solution. Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. The XER original recipe for this medium calls for 28 mg ferrous sulfate, $FeSO_4{\cdot}7H_2O$, l^{-1}. The difference (0.2 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored. Sticklers for accuracy can add 28 mg l^{-1}.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Opinions differ regarding the use of banana in culture media for orchids. The predominant opinion is that only homogenized pulp of ripe banana should be used. Other views are that: (1) unripe (green) bananas are preferable; (2) the peel rather than the pulp should be used; (3) unpeeled banana homogenate should be incorporated in media; and (4) slices, ripe or unripe, peeled or unpeeled, rather than homogenate are most effective. Opinions also differ regarding which is the most effective variety (Gros Michel, Raja, Pisang Susu and others). Since there is no experimental evidence to support any view regarding which is the best approach, follow the recommendations in the original reports and use what is suggested there. If there are no specific suggestions, or if there are but specific bananas are hard to find, the best approach is to use those that are easily available.

[f]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels.

[g]Pour 900 distilled water (item 11) into a blender, add items 1–9, homogenize throughly, adjust pH to 5.5, add sugar (item 10), homogenize again and bring volume to 1000 ml with distilled water (item 11). Gelrite (gellan gum, Phytagel) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. When the gellan gum is completely dissolved pour the solution into culture vessels and autoclave.

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite should not be used except in special circumstances.

Procedure. Surface-sterilized sections are placed in culture and maintained on the first medium (Table Dtps-4) until PLB or plantlet formation. PLBs can be cultured on CW containing XER (Table Dtps-5) for proliferation. Plantlets or PLBs are cultured on medium supplemented with banana pulp (Table Dtps-6) for vegetative growth.

General Comments. This very useful method is the first one for orchids to use TDZ. Its important advantage is that it generates several plantlets per explant node.

Doritaenopsis Micropropagation by the Culture of Thin Sections of Leaves

There are a number of methods for the culture of *Phalaenopsis* and *Doritaenopsis*, but most of them do not produce enough plants fast enough or without somaclonal variations. A method utilizing thin sections of leaves seems to be rapid and efficient (Park et al., 2002).

Plant Material. Sections, five to seven, each 1 mm thick, were cut from leaves of 3-month-old plantlets generated from PLBs derived from flower-stalk sections of *Doritaenopsis* New Candy × (*Doritaenopsis* Mary Ames × *Doritaenopsis* Ever Spring).

Surface Sterilization. There is no need to sterilize explants taken from plants growing in vitro. However, they should be rinsed with sterile distilled water to remove medium residues.

Culture Vessels. Plastic Petri dishes, 10 cm in diameter containing 25 ml of medium, were used to culture the thin sections in the original research. Other containers can also be used. Erlenmeyer flasks, test tubes, jars, or other containers can be used to culture PLBs for plantlet production. Plantlets are grown in pots.

Culture Conditions. In the original research the cultures were maintained initially for a week in the dark at 27°C and then transfereed to 25 ± 1°C under 16-h photoperiods of 10 μmol m^{-2} s^{-2} photosynthetic photon flux provided by cool white fluorescent lamps (Kumho FL 40D). Cultures for plantlet production were maintained at 25°C under 16-h photoperiods of 30 μmol m^{-2} s^{-1}. Standard culture room conditions will probably also be suitable. Plantlets, 5–6 cm in height, were potted and grown in a greenhouse under 60–70% relative humidity (RH) and 16-h photoperiods of 500 μmol m^{-2} s^{-1} and 25°C. The temperature during the 8-h dark period was 15°C.

Culture Media. Thin sections should be cultured on half-strength MS medium (Murashige and Skoog, 1962) containing 2 mg TDZ l^{-1} (Table Dtps-7; the actual amount used was 9 μmol or 1982.7 μg, which can be rounded up to 2 mg). For plantlet production PLBs should be grown on modified Hyponex (KH) medium (Kano, 1965) containing potato homogenate and peptone (Table Dtps-8). Hyponex (www.hyponex.co.jp) may not be easy to find in some parts of the world, therefore banana homogenate and activated charcoal containing XER medium (Table Dtps-6) can be used instead. Plantlets should be potted in sphagnum moss.

TABLE DTPS-7. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of thin sections of *Doritaenopsis* leaves (Park et al., 2002)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Purine				
10	Adenine sulfate, $2C_5H_5N_5 \cdot H_2SO_4$	10.0	100 mg 10 ml^{-1} 0.5N HCl[f]	1	
	Cytokinin				
11	Thidiazuron (TDZ)	2.0	200 mg 100 ml^{-1} 1N NaOHl[g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
15	Coconut water[h]	200.0 ml	No stock	No stock	Measure
	Sugar				
16	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[i]	To 1000 ml			
	Solidifier				
18	Agar[i]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]To prepare 0.5 N HCl pour 4.3 ml of concentrated hydrochloric acid into 95.7 ml distilled water slowly while stirring. To prevent splatter and possible injury acid must always be poured into water. Water must never be poured into acid [mnemonic by Prof Arnold S. Dunn of the University of Southern California: **A**(cid) comes before **W**(ater)]. The person preparing this solution must wear rubber gloves and a face shield. Also it is preferable to use a volumetric flask (www.fishersci.com or www.wvr.com). Adenine sulfate (www.sigmaaldrich.com, www.fishersci.com, www.wvr.com) is dissolved in 0.5N HCl to prepare the stock solution which must be kept frozen between uses.

[g]To prepare 1N NaOH dissolve 4 g of sodium hydroxide (www.sigmaaldrich.com, www.fishersci.com, www.wvr.com) in 100 ml distilled water. To prevent splatter and possible injury the NaOH pellets must always be dropped a few at a time into the water. Water must never be poured on the pellets. The person preparing this solution must wear rubber gloves and a face shield. Also it is preferable to use a volumetric flask (www.fishersci.com or www.wvr.com). Thidiazuron (www.sigmaaldrich.com) is dissolved in 1N NaOH to prepare the stock solution which must be kept frozen between uses.

[h]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not

TABLE DTPS-8. **Kano's Hyponex (KH) medium (Kano, 1965) modified for plantlet production from protocorm-like bodies derived from *Doritaenopsis* leaf sections (Park et al., 2002)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]
	Macroelements	
1	Hyponex[b]	3.0
	Complex additive	
2	Potato homogenate[c]	30.0
	Sugar	
3	Sucrose	20.0
	Solvent	
4	Water, distilled[d]	To 1000 ml
	Solidifier	
5	Agar[d]	5.5
	Darkening agent	
6	Activated charcoal[e]	0.5

[a]Amounts are given in g unless indicated otherwise.

[b]This is a soluble fertilizer which is becoming hard to find outside of Japan. The address in Japan is Hyponex Co., Tokyo, Japan. A website for the Hyponex brand name in the USA is www.scotts.com. If Hyponex cannot be located, modfied XER medium (Table Dtps-6) can be used instead.

[c]To prepare potato homogenate peel the potatoes, cut them into cubes approximately 1 cm^3 in size, and follow directions in footnote *d*.

[d]Add items 1 and 2 to 800 ml distilled water (item 4) in a homogenizer, homogenize thoroughly, let stand for 30–60 s and homogenize again, adjust pH to 5.5, add the sugar (item 3) and raise the volume to 1000 ml with distilled water (item 4). Bring the mixture to a gentle boil and add the agar (item 5) while stirring vigorously. When the agar is completely dissolved add the activated charcoal (AC, item 6) slowly with stirring. Keep stirring until the charcoal is dispersed completely. After that dispense the medium into culture vessels and autoclave.

[e]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources. Charcoal functions not only by darkening the medium, but also (and probably mainly) by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite should not be used except in special circumstances.

Procedure. Thin sections should be placed on the agar cut side down on the first medium (Table Dtps-7), maintained in the dark for a week, moved to 16-h photoperiods and kept there until PLBs are formed (approximately 6 weeks). Plantlets on the second medium (Table Dtps-8) should be subcultured every 8 weeks. When plantlets reach a height of 5–6 cm they should be moved to pots and grown in a greenhouse.

Developmental Sequence. Globular structures develop after 2 weeks of culture (Fig. Dtps-2A). They become distinct on the edges of the thin sections after an additional week (Fig. Dtps-2E). PLBs become evident after that (Fig. Dtps-2B, D). They are always formed near the epidermis of the sections and never from callus. The PLBs form normal plantlets on the Hyponex medium (Fig. Dtps-2C) which develop normally after being potted (Fig. Dtps-2F).

be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH as required, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10, 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels.
Agar is not added to liquid media.

First description of the Petri dish

Petri, R. J. 1887. Eine kleine Modification des Koch'schen Plattenverfahrens. *Centralblatt fur Bacteriologie und Parasitenkunde*, Vol. 1, page 279. [A small modification of Koch's plating method. Translated from the original German.]

In order to perform the gelatin plate technique of Koch, it is necessary to have a special horizontal pouring apparatus. The poured plates are then placed over one another in layers on small glass shelves in a large bell jar. In many cases it would be desirable to carry out the procedure with less equipment, especially without the pouring apparatus. Since the first of the year I have been using flat double dishes of 10–11 cm diameter and 1–1.5 cm height. The upper dish serves as a lid as usual and has a somewhat larger diameter. These dishes are sterilized by dry heat as usual and after cooling the nutrient gelatin containing the inoculum is poured in. The upper lid is lifted only slightly and used as a shield, while the tube containing the gelatin, its edge previously flamed and cooled in the usual manner, is emptied into the bottom of the dish. Under these conditions contamination from airborne germs rarely occurs. The poured layer of gelatin soon hardens into a layer several millimeters thick which can be kept and observed for a long time because of the protecting upper lid. In studies of soil samples, sand, earth, and similar substances, it is advantageous to place the material in the dish and then pour the liquid gelatin over it. The material is well mixed with the gelatin by rotating the dish with short, intermittent movements. With the dimensions given, every spot on the gelatin surface is accessible with the low power microscope. Only when high power lenses are used is the area at the edge of the dish no longer accessible. The gelatin dries in these dishes quite slowly. The gelatin can be kept moist longer if five or six dishes are placed on top of one another on a disc of moist filter paper in a flat dish over which a bell jar is inverted. These dishes can be especially recommended for agar-agar plates, since agar-agar sticks poorly to simple glass plates unless special methods are used. In addition, it is quite simple to count the colonies that have grown on the plates. The upper lid is replaced by a glass plate that has etched on it squares of known area. The colonies are then counted against a black background using a magnifier. The total area of the plate can be calculated from the diameter.

General Comments. This procedure is actually the second part of a two-step method which starts with the culture of flower stem sections (see method by Park et al., 2000 in the *Phalaenopsis* section, p. 1013, Vol. II). Plantlets produced through this method are the explant sources. Use of this two-step procedures makes possible mass rapid clonal propagation without excessive proliferation of PLBs which can bring about undesirable somaclonal variations.

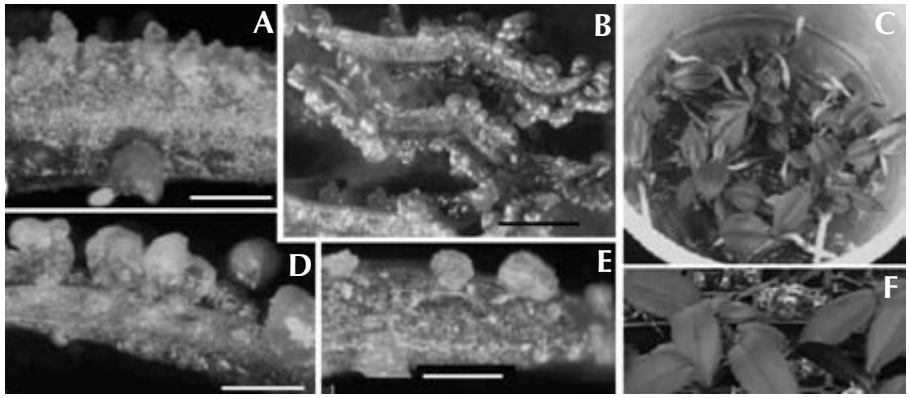


FIG. DTPS-2. **PLB production from thin leaf sections of *Doritaenopsis* and subsequent plantlet development. A. Initiation of PLBs on cut edge (scale bar = 1.5 mm). B, D. Multiple PLBs on cut surfaces after 4 and 6 weeks of culture (scale bars = 3 mm (B), 2 mm (D)). C. Plantlets developing on PLBs after 3 months on Hyponex medium. E. PLB primordia after 3 weeks of culture (scale bar = 2 mm). F. Plants in pots. (Park et al., 2002.)**

Micropropagation of *Doritaenopsis* by Culturing Shoot Tips of Flower-stalk Buds

When the popularity of *Doritaenopsis* as a pot plant increased in recent years so did the need for an efficient rapid micropropagation technique. Such a technique was developed by the Orchid Sanctuary Dogashima and the Faculty of Horticulture, Chiba University (Tokuhara and Mii, 1993).

Plant Material. Flower stalks bearing vegetative buds were taken from plants (Fig. Dtps-3A) and cut into sections leaving 15 mm above each bud and 25 mm below it (Fig. Dtps-3B). Shoot-tip explants 1 mm wide and 0.5 mm high with two or three leaf primordia (Fig. Dtps-3F) were excised following surface sterilization.

Surface Sterilization. Buds should be exposed (Fig. Dtps-3C) by removing the bracts (Fig. Dtps-3D) which cover them and the sections, and then sterilized with the supernatant of a 5% solution of calcium hypochlorite, $Ca(ClO)_2$ (5 g of calcium hypochlorite in 100 ml distilled water, stirred, allowed to stand until a precipitate forms, and stirred again repeating the process three times before decanting the liquid which must be used within 12 h or less; this solution contains about 1% available chlorine), containing a few drops of Tween 20 (www.sigmaaldrich.com, www.fishersci.com, www.vwr.com) or of a mild household detergent by a 15-min immersion with stirring. Following sterilization the buds should be washed with sterile distilled water three times before removing two or three scaly leaves (Fig. Dtps-3E) prior to excision of the explants.

Culture Vessels. Test tubes, 24 × 100 mm containing 15 ml of solid medium and covered with sterilized aluminum foil, were used. Other containers are also suitable.

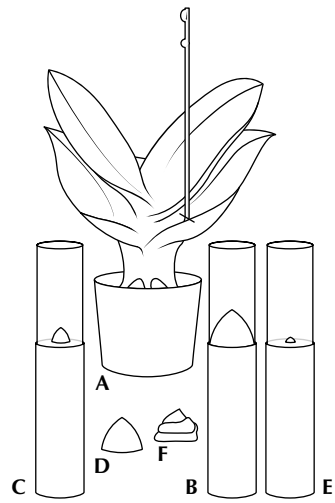


FIG. DTPS-3. **Excision of *Doritaenopsis* buds. A. Plant with bud bearing flower stalk. B. Bud-bearing section. C. Exposed bud following removal of bract which covered it. D. The bract. E. Bud after scales which cover it have been removed. F. Explant to be cultured. (Tokuhara and Mii, 1993.)**

Culture Conditions. The research cultures were maintained at $23 \pm 1°C$ under 14-h photoperiods of 33 μmol m^{-2} s^{-1} provided by Toshiba FLR40S W/M 36 lamps. Standard culture room conditions are probably also suitable.

Culture Media. New Dogashima (ND) medium containing 0.1 mg NAA l^{-1} and 1 mg BA l^{-1} (Table Dtps-9) should be used for PLB induction and subculture. Hormone-free ND medium (Table Dtps-10) is used for plantlet development from PLBs.

Procedure. Flower stalks are cut into sections after being removed from plants. The bracts which cover the buds are removed, the sections are surface sterilized and then rinsed with sterile distilled water. Scale leaves are removed from the buds after the rinse, and shoot tips are excised and placed into culture on hormone-containing medium (Table Dtps-9). When PLBs form they should be subcultured on the same medium (Table Dtps-9). To produce plantlets PLBs should be cultured on hormone-free medium (Table Dtps-10).

Developmental Sequence. Explants form PLBs on the first medium (Table Dtps-9). When subcultured on the same medium (Table Dtps-9) the PLBs proliferate. The PLBs produce plantlets on the second medium (Table Dtps-10).

General Comments. This procedure can be used for both *Doritaenopsis* and *Phalaenopsis*. The protocol is easy, simple, and efficient. With it propagation is rapid. More than 10,000 PLBs were obtained in 1 year from a few buds excised from a single flower stalk. Unfortunately, the efficiency and speed are also the major drawbacks of this protocol because rapid and excessive proliferation can and does lead

TABLE DTPS-9. **Medium for root induction on *Doritaenopsis* plantlets (Park et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	3.0	300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Gelrite[g,h]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin do not dissolve, add a few drops of 0.1N KOH or 0.1N HCl respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.2–5.6, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Gelrite (gellan gum, Phytagel) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. Pour solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Solidifier is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear, and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels.

TABLE DTPS-10. **Medium for elongation of roots on *Doritaenopsis* plants (Park et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins[f]				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[g]	To 1000 ml			
	Solidifier				
15	Gelrite[g,h]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]There is no auxin or cytokinin in this medium.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 14), adjust pH as required, add sugar (item 13), and adjust volume to 1000 ml with distilled water (item 14). Gelrite (gellan gum, Phytagel) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium, it will form clumps and will not gel properly after autoclaving. Add the amino acid (item 8) and vitamins (items 10–12) to the warm and still liquid solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels.

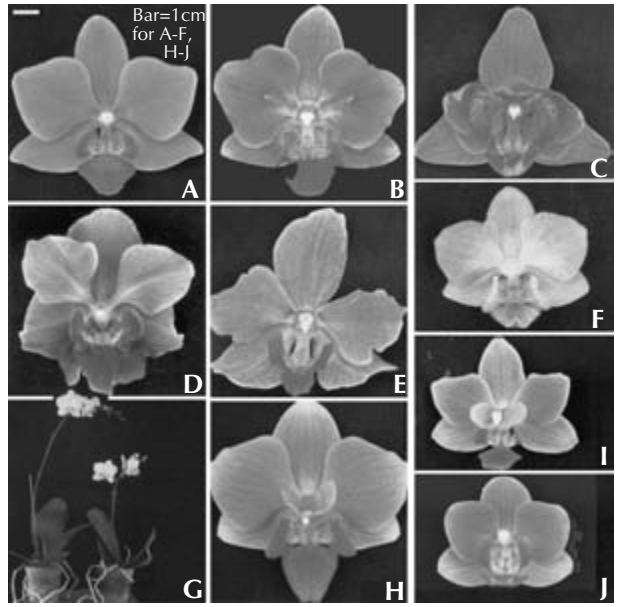


FIG. DTPS-4. **Micropropagation induced somaclonal variations in *Phalaenopsis* flowers (A–F, H–J). G. Different lengths of inflorescences in *Doritaenopsis*. (Tokuhara and Mii, 1998.)**

to mutations, somaclonal variations, and flower shapes which can be undesirable (Fig. Dtps-4; Tokuhara and Mii, 1998).

Micropropagation of *Doritaenopsis* through Root-tip Cultures

Root tips are attractive explants for micropropagation of monopodial orchids because removing them does not threaten the survival of the plants from which they are taken. Therefore a micropropagation method for *Doritaenopsis* using root tips was developed in a cooperative research project involving the Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University in Korea, and the Department of Biological Science, Aichi University of Education in Japan (Park and Paek, 2001; Park et al., 2001).

Plant Material. To obtain enough roots for explants in vitro raised plantlets of *Doritaenopsis* New Candy × (*Doritaenopsis* Mary Ames × *Doritaenopsis* Ever Spring) with two to three leaves were cultured for 2 months on a medium containing 3 mg NAA (Table Dtps-9). Approximately 18 roots per plant were produced on this medium. After that the plantlets were moved to an auxin-free medium (Table Dtps-10) for a month to enhance root growth. Root tips were then excised and cultured. There is no indication in the original paper regarding the size of the explant. The only statement is "root tips." This suggests that the explants consisted of all or part of the green root tip (Fig. Dtps-5).

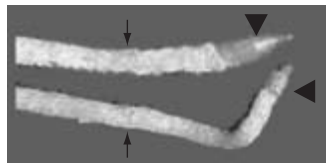


FIG. DTPS-5. **Orchid roots. Arrows indicate velamen covered part of root (which can be white, grey, or silvery). Wedges and root tip are velamen-free and are green. (Source: www.botgard.ucla.edu.)**

Surface Sterilization. It is not necessary to sterilize explants taken from plants which are grown in vitro. However they should be rinsed with sterile distilled water to remove medium residues.

Culture Vessels. Erlenmeyer flasks, plastic cultures vessels, or jars containing medium equal to 20–30% of their volume are suitable.

Culture Conditions. The research cultures were maintained at 26°C and 16-h photoperiods of 30 μmol m^{-2} s^{-1} (the light source is not listed). Standard culture room conditions are also suitable.

Culture Media. Plantlets (both seedlings and ones produced through micropropagation) should first be cultured on modified MS medium (Murashige and Skoog, 1962) containing 3 mg NAA l^{-1} (Table Dtps-9). After 2 months on this medium the plantlets should be moved to an auxin-free substrate (Table Dtps-10) for a month. Root-tip explants taken from plants on the second medium (Table Dtps-10) must be cultured on a third solution (Table Dtps-11) for induction of PLBs. The PLBs produce plantlets when moved to Hyponex medium (Table Dtps-8). Plantlets produced on the Hyponex medium (Table Dtps-8) are potted in sphagnum moss.

Procedure. Plantlets should be cultured on the first medium (Table Dtps-9) for 2 months to induce production of adventitious roots. Once such roots are produced (18 per plant on the average) it is necessary to move them to the second medium (Table Dtps-10) for a month so that the roots will elongate. Explants taken from the elongated roots should be moved to the third medium (Table Dtps-11) for PLB formation which will take approximately 8 weeks. The PLBs must be moved to Hyponex medium (Table Dtps-8) for plantlet production. When plantlets on the Hyponex medium are large enough they should be potted in sphagnum moss and grown in a greenhouse.

Developmental Sequence. The relatively high auxin (NAA) level in the first medium (Table Dtps-9) will induce the formation of multiple roots (an average of 18 in the original research) on the plantlets. However these roots will not elongate due to the high auxin concentration. The roots will elongate on the second medium (Table Dtps-10) because it is free of auxins. The root tips will produce PLBs on the third medium (Table Dtps-11) due to the presence of coconut water (CW) and the cytokinin TDZ. On the relatively simple Hyponex medium (Table Dtps-8) the PLBs

TABLE DTPS-11. **Medium for induction of protocorm-like bodies (PLBs) from *Doritaenopsis* root explants (Park et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration for repeated and medium), mg[a]	Stock solution (concentrate prepared per liter of convenient use)	Volume of stock solution culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Thidiazuron (TDZ)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
14	Coconut water[e,f]	200.00	No stock	No stock	Measure
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Gelrite[g, h]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[g]Add items 1–7, 9 and 14 to 700 ml distilled water (item 16), adjust pH to 5.2–5.6, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 15). Gelrite (gellan gum, Phytagel) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. Pour solution into a 2-l flask and autoclave Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Solidifier is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels.

will produce plantlets. When potted in sphagnum moss these plantlets should become larger and flower after being moved to a standard potting mix.

General Comments. Root tips can be removed safely from a plant and because of that they are very useful as explants. However, if taken from seedlings they cannot be used to propagate desirable forms. When taken from plantlets produced through micropropagation roots tips can be used to propagate superior clones. Root tips from mature plant could be explants which can be cultured. But, roots usually contain mycorrhizal fungi which can contaminate and smother cultures. Therefore only tips from aerial roots which have not come into contact with surfaces or potting mix can be used.

Propagation of Protocorm-like Bodies in Liquid Medium

See procedure under the same name in the *Phalaenopsis* section (p. 1004, Vol. II; Park et al., 1996).

Effects of Complex Organic Additives on the Growth of *Doritaenopsis* Callus

Apple, potato, and taro extracts and coconut water (CW) enhanced the growth of *Doritaenopsis* callus depending on the quantity added. Taro at 50–200 ml per liter of new *Phalaenopsis* medium (Ichihashi, 1992*a*, 1992*b*) was the most effective of these extracts. To prepare the extract, 100 g tissue was diced and boiled for 20 min in 100 ml distilled water, the hot supernatant was filtered through a kitchen sieve, and then adjusted to 100 ml with distilled water (Ichihashi and Islam, 1999). Just in case too much of the water boils away it is wise to have available nearby a container of boiling water which can be added to the taro extract to maintain its volume.

Differences between the Capacities of *Doritaenopsis* Axillary Buds and Shoot Tips to Form Shoots and Protocorm-like Bodies

Axillary buds and shoot tips from flower stalks of eight *Doritaenopsis* hybrids were cultured on new Dogashima medium (Tokuhara and Mii, 1993) supplemented with 0.1 mg NAA l^{-1} and 1 mg or 5 mg BA l^{-1}. Approximately 70% of the explants survived after 5 months of culture. Shoot and PLB formation differed among hybrids (Yamazaki et al., 1997).

Establishment of *Doritaenopsis* Cell Suspension Cultures

There are relatively few methods for the establishment of orchid cell suspension cultures. The method described here was developed as part of a vitrification research project (Tsukazaki et al., 2000).

Plant Material. Flower stalks bearing vegetative buds were taken from plants of *Doritaenopsis* strain 343. Other *Doritaenopsis* hybrids can probably also be used. The stalks should be cut into sections leaving 15 mm above each bud and 25 mm below it (see Fig. Dtps-3B). Shoot-tip explants 1 mm wide and 0.5 mm high with one or two leaf primordia (see Fig. Dtps-3F) should be excised following surface sterilization.

Surface Sterilization. Buds should be exposed (see Fig. Dtps-3C) by removing the bracts (see Fig. Dtps-3D) which cover them and the sections, and then sterilized with the supernatant of a 5% solution of calcium hypochlorite, $Ca(ClO)_2$ (5 g of calcium hypochlorite in 100 ml distilled water, stirred, allowed to stand until a precipitate forms, and stirred again repeating the process three times before decanting the supernatant which must be used within 12 h or less; this solution contains about 1% available chlorine), by a 15-min immersion with stirring. A few drops of Tween-20 (www.sigmaaldrich.com, www.fishersci.com, www.vwr.com) or of a mild household detergent should be added to the sterilizing solution. Following sterilization the buds should be washed with sterile distilled water three times before removing two or three scaly leaves (see Fig. Dtps-3E) prior to excision of the explants.

Culture Vessels. Erlenmeyer flasks, 100-ml capacity, containing 40 ml of medium and covered with sterilized aluminum foil should be used. Other containers are also suitable.

Culture Conditions. Explant cultures were maintained at 23°C under 14-h photoperiods of 33 μmol m^{-2} s^{-1} (light sources not listed). Callus in liquid medium should be placed on rotary shakers at approximately 100 rpm at 25°C under continuous illumination of 33 μmol m^{-2} s^{-1} (white fluorescent lights were used in the original research; other sources may also be suitable). Standard culture room conditions are probably also suitable. Cells on the medium which contains 0.2 mmol sucrose l^{-1} (Table Dtps-13) should be cultured at 25°C in the dark for 2 days. For PLB production on the fourth medium (Table Dtps-14), cells should be cultured in the light. The original paper is not specific regarding the conditions, but 23°C under 14-h photoperiods of 33 μmol m^{-2} s^{-1} or standard culture room conditions will probably be appropriate.

Culture Media. A medium designated as new Dogashima (ND) containing 0.1 mg NAA l^{-1}, 1 mg BA l^{-1}, 0.56 mmol sucrose, and 8 g agar l^{-1} (Table Dtps-12) should be used to culture the explants. Explants should be cultured on the same medium, but without agar for production of cells (Table Dtps-12). After that cells can be cultured on ND containing 0.2 mmol sucrose and 2 g Gelrite l^{-1} (Table Dtps-13) for 1 month before being transferred to a fourth medium (Table Dtps-14) for PLB formation. No medium is suggested for the culture of plantlets to maturation. Plantlets can be cultured on one of the versions of the Knudson C (KC) medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate (BH) or charcoal (see Table Pln-1) or the Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and

TABLE DTPS-12. **New Dogashima (ND) medium (Tokuhara and Masahiro, 1993) modified for the culture of vegetative buds of *Doritaenopsis* (Tsukazaki et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	480.0	48.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	470.0	47.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	550.0	55.0 g l^{-1}	10	
5	Potassium chloride, KCl	150.0	15.0 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	200.0	20.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Sulfuric acid, concentrated	0.5 µl	0.5 ml		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50.0 mg l^{-1}		
	Amino acid				
8	*l*-cysteine	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Purine				
10	Adenine	1.0	100 mg in 100 ml acidified 70% ethanol[e,f]	1	
	Auxin				
11	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,g]	1	
	Cytokinin				
12	6-Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,g]	1	
	Vitamins				
13	Biotin	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Calcium pantothenate	1.0	100 mg 100 ml^{-1} 70% ethanol[e,h]	1	
15	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
16	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
17	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
18	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[i]	To 1000 ml			
	Solidifier				
20	Agar[i,j]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Dissolve the adenine in up to 30 ml 0.1N hydrochloric acid and make up the solution to 100 ml with 95% ethanol.

[g]If the auxin or cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl respectively.

[h]Dissolve the calcium pantothenate in 30 ml distilled water and make up the solution to 100 ml with 95% ethanol.

[i]Add items 1–17 to 900 ml of distilled water (item 19), adjust pH as required, add sugar (item 18), and raise volume to 1000 ml with distilled water (item 19). Bring the mixture to a gentle boil and add the agar with vigorous stirring. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Amino acid (item 8), hormones (items 11, 12), and vitamins (items 13–17) are not heat stable and are often added to autoclaved and still warm and liquid media with sterilized pipettes and mixed well before distribution to preautoclaved culture vessels. In this case all components of the medium are autoclaved.

[j]Do not add agar to the liquid version of this medium.

TABLE DTPS-13. **New Dogashima (ND) medium (Tokuhara and Masahiro, 1993) as used for the culture of callus proliferation from cells of *Doritaenopsis* (Tsukazaki et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	480.0	48.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	470.0	47.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	550.0	55.0 g l^{-1}	10	
5	Potassium chloride, KCl	150.0	15.0 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	200.0	20.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2{\cdot}6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Sulfuric acid, concentrated	0.5 µl	0.5 ml		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	0.5	50.0 mg l^{-1}		
	Amino acid				
8	*l*-cysteine	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Purine				
10	Adenine	1.0	100 mg in 100 ml acidified 70% ethanol[e,f]	1	
	Auxin				
11	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,g]	1	
	Cytokinin				
12	6-Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,g]	1	
	Vitamins				
13	Biotin	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Calcium pantothenate	1.0	100 mg 100 ml^{-1} 70% ethanol[e,h]	1	
15	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
16	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
17	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
18	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[i]	To 1000 ml			
	Solidifier				
20	Gelrite[j]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4{\cdot}4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4{\cdot}7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Dissolve the adenine in up to 30 ml 0.1 N hydrochloric acid and make up the solution to 100 ml with 95% ethanol.

[g]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively.

[h]Dissolve the calcium pantothenate in 30 ml distilled water and make up the solution to 100 ml with 95% ethanol.

[i]Add items 1–17 to 900 ml of distilled water (item 19), adjust pH as required, add sugar (item 18), and adjust volume to 1000 ml with distilled water (item 19). Add the Gelrite (Phytagel, gellan gum) slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. When the gellan gum is completely dissolved pour the solution into culture vessels and autoclave. Amino acid (item 8), hormones (items 11, 12), and vitamins (items 13–17) are not heat stable and are often added to autoclaved and still warm and liquid media with sterilized pipettes and mixed well before distribution to preautoclaved culture vessels. In this case all components of the medium are autoclaved.

[j]Gellan gum [(available as such from www.caissonlabs.com) and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE DTPS-14. **Half-strength new Dogashima (ND) medium (Tokuhara and Mii, 1993) for the production of protocorm-like bodies from cell cultures (Tsukazaki et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	240.0	24.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	235.0	23.5 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	125.0	12.5 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	275.0	27.5 g l^{-1}	10	
5	Potassium chloride, KCl	75.0	7.5 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	100.0	10.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2{\cdot}6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Sulfuric acid, concentrated	0.5 µl	0.5 ml		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	0.5	50.0 mg l^{-1}		
	Amino acid				
8	*l*-cysteine	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Purine				
10	Adenine	1.0	100 mg in 100 ml acidified 70% ethanol[e,f]	1	
	Auxin				
11	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,g]	1	
	Cytokinin				
12	6-Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,g]	1	
	Vitamins				
13	Biotin	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Calcium pantothenate	1.0	100 mg 100 ml^{-1} 70% ethanol[e,h]	1	
15	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
16	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
17	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
18	Maltose	10.0 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[i]	To 1000 ml			
	Solidifier				
20	Gelrite[j]	30.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4{\cdot}4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4{\cdot}7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Dissolve the adenine in up to 30 ml 0.1 N hydrochloric acid and make up the solution to 100 ml with 95% ethanol.

[g]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively.

[h]Dissolve the calcium pantothenate in 30 ml distilled water and make up the solution to 100 ml with 95% ethanol.

[i]Add items 1–17 to 900 ml of distilled water (item 19), adjust pH as required, add sugar (item 18), and adjust volume to 1000 ml with distilled water (item 19). Add the Gelrite (gellan gum, Phytagel) slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. When the gellan gum is completely dissolved pour the solution into culture vessels and autoclave. Amino acid (item 8), hormones (items 11, 12), and vitamins (items 13–17) are not heat stable and are often added to autoclaved and still warm and liquid media with sterilized pipettes and mixed well before distribution to preautoclaved culture vessels. In this case all components of the medium are autoclaved.

V-4). If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal (AC) should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *d*).

Procedure. Flower stalks are cut into sections after being removed from plants. The bracts which cover the buds are removed, the sections are surface-sterilized and then rinsed with sterile distilled water. Scale leaves are removed from the buds after the rinse, and shoot tips are excised and placed into culture on the solid medium (Table Dtps-12). When callus is formed it should be transferred to a liquid medium of the same composition (Table Dtps-12). Cells should be collected by filtering the liquid medium through a 40-μm nylon mesh (www.spectrapor.com) and used as needed. About 3 g of fresh cells should be subcultured every 2 weeks on fresh medium (Table Dtps-12). Cells should be cultured on the third medium (Table Dtps-13 for a month before being transferred to half-strength ND medium supplemented with 1% maltose and solidified with 3 g Gelrite l^{-1} (Table Dtps-14) for PLB formation. For further growth the plantlets can be moved to one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, Cym-1 to Cym-3, Cym-5, Cym-24, C-19, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, and V-4) with or without BH or AC. If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). AC should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *d*).

Developmental Sequence. Explants produce a yellow-green callus after 3–5 months in culture in the first medium (Table Dtps-12). Cells can be harvested every 2 weeks after transferring this callus to liquid medium (Table Dtps-12). They multiply on the third medium (Table Dtps-13) and produce PLBs on the fourth solution (Table Dtps-14).

General Comments. To quote the authors of this procedure, they "have produced a useful method . . . [for] *Doritaenopsis* suspension culture cells that should be applicable to . . . other Orchidaceae." Their aim was to develop a cryopreservation procedure, but the cell culture method is a valuable byproduct.

[i]Gellan gum [(available as such from www.caissonlabs.com) and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

Comparison between Hyperhydric and Normal Protocorm-like Bodies of *Doritaenopsis*

Hyperhydric PLBs (HPLBs) are translucent and turgid due to a lack of air in their intercellular air spaces. They have a lower capacity for shoot formation than PLBs. On the other hand, HPLBs have an increased ability to produce new PLBs compared to normal PLBs (NPLBs). The new HPLBs can be rendered to NPLBs by culturing them on Vacin and Went medium containing 10% (v/v) fresh potato homogenate (peeled potato slices homogenized and added to the medium), adjusted to pH 5.3, and solidified with 0.8% (w/v) agar (Zhou, 1995).

Mass Micropropagation of *Doritaenopsis* Protocorm-like Bodies using an Air-driven Periodic Immersion Bioreactor (ADPIB)

Doritaenopsis PLBs derived from flower stalks were cultured in liquid medium using an ADPIB consisting of a timer, small electronic pump, inner container, humidifier, and two plastic jars connected with silicone tubing which allows the medium to circulate between them. The results show that the ADPIB cultures are superior to conventional ones on solid medium. This suggests an economic potential for ADPIB (Liu et al., 2002).

Doritaenopsis Micropropagation in Different Types of Bioreactors

Doritaenopsis PLBs were cultured in air-driven periodic immersion, rolling bottles, spinner flask and airlift bioreactors. The best results were obtained with the air-driven periodic immersion bioreactor (ADPIB). Proliferation rates in the ADPIB ranged from 4.2- to 6.5-fold by weight. Each ADPIB can generate 100 g of PLBs, which amounts to 3000–5000 plantlets. Biomass increases in a rolling bottle bioreactor were similar. The conclusion by the authors is that these bioreactors can be used for the micropropagation of *Doritaenopsis* and *Phalaenopsis* (Liu and Wu, 2002).

Doritis

A genus consisting of a few species and small to medium-sized plants which do not have pseudobulbs, *Doritis* is found in India, Indonesia, Myanmar (formerly Burma), Nepal, Malaysia, Sri Lanka, and Thailand.

In Vitro Culture of *Doritis* Ovaries and Immature Embryos

A method has been developed for the in vitro culture of *Doritis* ovaries and immature embryos [which are erroneously called ovules in the original paper (Yasugi, 1984)]. Those wishing to culture *Doritis* explants could use this procedure as a starting point. Procedures used for *Doritaenopsis* and *Phalaenopsis* could also be tested. *Doritis* leaves have been cultured in a medium containing 15–50% coconut water (Sagawa and Kunisaki, 1982), but not enough details are presented about this procedure to allow its inclusion here.

Culture of Embryogenic Callus of *Doritis*

The culture method for embryogenic callus of *Doritaenopsis* (Ichihashi and Hiraiwa, 1996) may also be suitable for *Doritis*.

Encyclia

At one time species which now belong to the genus *Encyclia* were part of *Epidendrum*. Currently there are about 150 *Encyclia* species, most of them found in Mexico and the Caribbean with some extending into South America. Due to the close affinities between *Encyclia*, *Epidendrum*, and *Cattleya*, micropropagation methods developed for members of one of these genera can probably be used for species or hybrids in any of the other two especially since over the years a number of taxa were assigned to more than one them.

Epidendrum

A number of species and hybrids of *Epidendrum* are grown widely in greenhouses and outdoors. Some are relatively easy to propagate and others are not, but few are of as much interest as clones of *Cattleya*, *Cymbidium*, and *Dendrobium*. Therefore fewer methods have been developed for *Epidendrum* propagation from tissue culture (Scully, 1967; Churchill et al., 1970, 1971*c*, 1972*a*, 1973; Arditti et al., 1971, 1972; Stewart and Button, 1976*a*; Khaw et al., 1978*a,* 1978*b*; Kushnir and Budak, 1980). *Epidendrum* is one of the few orchids for which there is a micropropagation method using root tips (Stewart and Button, 1978) and a procedure for the culture of root explants (Churchill et al., 1972*b*). *Epidendrum* tissues have also been used in studies of hormone effects on anthocyanin production (Rudolph et al., 1972). It is interesting to note that only reed-type *Epidendrum* hybrids and species have been cultured to date. Methods used for related genera may prove suitable for pseudobulb-producing species and hybrids.

Rapid Vegetative Multiplication of *Epidendrum* O'brienianum in Vitro

Under some circumstances plantlets may develop from dormant buds on the stems and flower stalks of reed-stem *Epidendrum*. Many more dormant buds never develop. These buds can be used as explants for mass rapid clonal propagation (Stewart and Button, 1976*a*).

Plant Material. Node sections (length is not given in the original research, but 1.5–2 cm with the bud in the middle would seem appropriate) and flower-stalk sections (length also not given) with "buds [that] were damaged or removed prior to culture" of *Epidendrum* O'brienianum are used. The original paper does indicate that the stems "were separated into single node sections by cutting across the middle of the internode" and in some "instances the buds were partially or entirely removed prior to culture." It is not entirely clear from this description whether the partially removed bud or the stem with part of a bud remaining on it or both were cultured. However, the statement, ". . . despite the attempt to remove the entire bud, a high percentage of the node sections retained sufficient meristematic tissue to permit the direct development of a plantlet," suggests that perhaps the nodes were cultured. If so, the plantlets could have developed from accessory or newly formed buds. Flower-stalk nodes were presumably prepared in a similar manner.

Surface Sterilization. Leaves and sheaths are removed and the stems are washed with water and household liquid detergent. Stems are cut into node sections after that, and each section should be wiped with cotton soaked in 85% ethanol. The wiped stems are immersed in freshly prepared, filtered calcium hypochlorite (a concentration is not given in the original paper, but 7 mg per 100 ml should be appropriate) containing a few drops of Tween 20 for a period of time that was not listed (15–30 min should be enough). Following removal from the calcium hypochlorite the sections should be washed two to three times with sterile distilled water. Bleached tissue at the ends of sections should be removed at this point. It is reasonable to assume that flower-stalk nodes can be surface-sterilized by the same procedure.

Culture Vessels. Pyrex culture tubes 80×25 cm containing 10 ml medium and covered with Cap-O-Test lids were used in the original research. Other containers would also be suitable.

Culture Conditions. In the original research, cultures were maintained under 16-h photoperiods of approximately 1000 lx provided by Sylvania Gro Lux lamps and $25 \pm 2°C$.

Culture Media. Stem nodes produce plantlets on Knudson C (see Table Aranda-7), Vacin and Went (see Table Cym-5), and MS (Table Epi-1) media. Undamaged flower stalks can presumably be cultured in the same solutions. Flower-stalk node "sections whose buds were damaged or removed prior to culture" produce single plantlets on the MS (see Table Cym-10) and Lindeman–Gunckel–Davidson (see Table C-1) media. Multiple plantlets are produced on a modification of the MS medium (Table Epi-1).

Procedure. Insert node sections in the medium with their morphological bases down and allow the buds to develop. When the plantlets become large enough, pot in a potting mix. Plantlets that were part of a group will develop normally and form roots from their lowest nodes within 20 weeks. These plantlets can be moved to community pots.

Developmental Sequence. Node sections form plantlets quickly and "spontaneously." Photographs in the original paper (Stewart and Button, 1976) indicate that they develop like plantlets that form on mature plants in situ. Damaged buds form a "proliferative body" after 5 weeks in culture. Plantlets develop from this body after an additional 3 weeks. Roots and leaves develop after a further 8 weeks, and then the plantlets can be moved to pots.

General Comments. Although developed with *Epidendrum* O'brienianum, this procedure should prove suitable for other reed-type epidendrums.

Putrescine

Names:	1,4-diaminobutane; 1,4-butanediamine; putrescine; tetramethylenediamine	
Molecular formula:	$C_4H_{12}N_2$	
Percent composition:	C 54.50% H 13.73%	
	N 31.77%	$H_2NCH_2CH_2CH_2CH_2NH_2 \cdot 2HCl$
Molecular weight:	88.15 g/mol	Putresmine hydrochloride
Functional groups:	Amine	1,4-butanediamine dihydrochloride
Appearance:	Colorless oil	1,4-diaminobutane dihydrochloride
Melting point (°C):	23–24	Tetramethylenediamine dihydrochloride
Boiling point (°C):	158–160	M. W. 161.07
Solubility:	Very soluble in water	
Notes:	Putrescine is a poisonous oil present in rotting flesh and produced during decomposition of the amino acid arginine. It is partially responsible for the odor of urine and bad breath	

TABLE EPI-1. **Murashige–Skoog medium (Murashige and Skoog, 1962) for the production of multiple plantlets of *Epidendrum* (Stewart and Button, 1976*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Indoleacetic acid (IAA)	1	100 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
11	Kinetin	0.04	4 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
15	Coconut water	150 ml	No stock	No stock	Weigh
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]This medium can contain various amounts of auxin and/or cytokinin depending on specific needs. The original paper for this method (Stewart and Button, 1976*a*) states that the medium was used "alone and with the addition of 5 mg/litre . . . NAA, . . . 15 percent (v/v) coconut milk [sic correct term is 'coconut water'], . . . or both. . . ." A table shows that proliferation is best in the Murashige–Skoog medium with coconut water but gives no indication of amounts (if any) of auxin and/or cytokinin. Therefore we give the minimal amounts listed in the original recipe, since lack of these hormones can do harm. If auxin or cytokinin does not dissolve, add a few drops of KOH or HCl, respectively, to solubilize them.

[g]Keep refrigerated between uses.

[h]Add items 1–7, 9, and 15 to 700 ml distilled water (item 17). Adjust pH to 5.2–5.5, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid, hormones, and vitamins (items 8 and 10–14) to hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

Clonal Propagation of *Epidendrum O'brienianum* through the Culture of Root Tips

A number of orchids produce buds and shoots from root tips. If this is "an inherent trait of the Orchidaceae which could be 'turned on,'. . . the result . . . of propagating orchids by culturing excised roots might be of considerable importance to the orchid industry" (Stewart and Button, 1978).

Plant Material. In the original research, roots were obtained from plantlets that formed in vitro from stem sections. They were divided into green root tips 1–4 mm long and sections 4–8 mm long excised 1–10 cm above the tip.

Surface Sterilization. There is no need to surface-sterilize roots taken from plantlets in vitro. Should tips be taken from mature plants growing outdoors or in pots, they should be surface-sterilized by immersing them in a 7% (w/v) solution of calcium hypochlorite (7 g calcium hypochlorite dissolved in 100 ml distilled water stirred and allowed to settle three times at 10–15-min intervals, filtered or decanted, and used within 4 h of preparation) for 5–10 min. They should be washed three times with sterile distilled water after the sterilization.

Culture Vessels. Pyrex culture tubes 80×25 cm containing 10 ml medium and covered with Cap-O-Test lids were used in the original research (Stewart and Button, 1978). Other containers would also be suitable.

Culture Conditions. In the original research, cultures were maintained under 16-h photoperiods of approximately 1000 lx provided by Sylvania Gro Lux lamps, 25 ± 2°C, and at a relative humidity of 75 ± 5%.

Culture Media. Callus formation is best on a modified Ojima–Fujiwara medium (Table Epi-2). The callus should be subcultured often on the same medium. "Callus, and callus with roots . . . [was] transferred to the media of Schenk and Hildebrand . . . [see Table Epi-3] and Knudson [C; see Table Aranda-7] without auxin." A plantlet was produced in these experiments, but it is not clear whether this happened on the Schenk–Hildebrand medium (Table Epi-3) or modified Ojima–Fujiwara medium (Table Epi-2).

Procedure. Place the tips on the first medium (Table Epi-2), and allow them to develop callus. This callus grows slowly in either medium (Tables Epi-2 and Epi-3) and should be transferred several times. PLBs and shoots, if they form, can be moved to the Knudson C medium for differentiation (see Table Aranda-7).

Developmental Sequence. Root tips either elongate or form "'disorganized' growths," which (in photographs at least) appear to consist of PLBs. The outer layers of the growth are composed of "dense layers of meristematic cells." These growths proliferate and on rare occasions produce plantlets.

TABLE EPI-2. **Modified Ojima–Fujiwara medium (Ojima and Fujiwara, 1962) modified for the culture of *Epidendrum* root tips (Stewart and Button, 1978)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	273.4	27.34 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	96	9.6 g l^{-1}	10	
3	Potassium nitrate, KNO_3[b]	20.2	4.04 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	8.16	816 mg l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
6	**Microelements**[d]				
(a)	Ammonium molybdate, $(NH_4)_2MoO_4$	0.02	2 mg l^{-1}		
(b)	Boric acid, H_3BO_3	0.02	2 mg l^{-1}		
(c)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	1.0	100 mg l^{-1}	10	One solution
(d)	Potassium iodide, KI	0.02	2 mg l^{-1}		
(e)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.02	2 mg l^{-1}		
	Auxin				
7	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.5	50/100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Vitamins				
8	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
9	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
10	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
11	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[f]	To 1000 ml			
	Solidifier				
13	Agar, Difco Bacto[f]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 5a) and iron salt (item 5b) to the same 1 l distilled water, and stir and/or heat until both are dissolved. The original formulation does not use chelated iron, but this form is preferable.

[d]add all microelements to the same 1 l distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep refrigerated or frozen between uses.

[f]Add items 1–6 in 900 ml distilled water (item 12), adjust pH to 5.2–5.5, add sugar (item 11), and adjust volume to 1000 ml with distilled water (item 12). Bring solution to a gentle boil, and add agar (item 13) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add hormone and vitamins (items 7–10) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

General Comments. This method was developed with root tips from plantlets grown in vitro; it is not clear whether it would also be suitable for root explants from mature plants. If root tips from mature plants were to be used it would be necessary to select roots free of mycorrhiza. The method is slow and not very effective since only one plantlet was obtained from among many cultures. On the other hand, this is a simple method that does not endanger the plants, and it could serve as a starting point for further research.

TABLE EPI-3. **Modified Schenk–Hildebrand medium (Schenk and Hildebrand, 1972) used for the culture of *Epidendrum* root-tip explants (Stewart and Button, 1978)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium phosphate, $NH_4H_2PO_4$[c]	300	30 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot H_2O$	200	20 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	400	40 g l^{-1}	10	
4	Potassium nitrate, KNO_3[c]	2500	250 g l^{-1}	10	
5	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.9	2.79 g l^{-1}		
6	**Microelements**[e]				
(a)	Boric acid, H_3BO_3	10	1 g l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	25	2.5 g l^{-1}		
(d)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(e)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	10	1 g l^{-1}		
	Auxin				
7	2,4-Dichlorophenoxyacetic acid (2,4-D)	1	100 mg 100 ml^{-1} 95% ethanol[f]	1	
	Sugar				
8	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[g]	To 1000 ml			
	Solidifier				
10	Agar, Difco Bacto[g]	10 g	No stock	No stock	Weigh

[a]The exact modification(s) used in this case are not clear. The authors state that the medium was used with three modifications: "(i) with . . . 2,4-D at a higher concentration, and (ii) and (iii) with some of the growth substances omitted" (Stewart and Button, 1978). Table 1 in their paper shows that the highest percentage of callus formation occurred in the presence of 1 mg l^{-1} 2,4-D but does not indicate which if any other substances were omitted.

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium.

[f]This auxin can be solubilized with a few drops of dilute KOH. Refrigerate between uses.

[g]Add items 1–6 to 900 ml distilled water (item 9), adjust pH to 5.2–5.5, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l Erlenmeyer flask and autoclave. Add auxin (item 7) to the warm or hot, still liquid solution, swirl vigorously to ensure complete mixing, and dispense into preautoclaved culture vessels.

Production of *Epidendrum* Plants from Leaf Tips

Use of leaf tips as a source of explants has the advantage of not endangering or even seriously damaging a plant. The donor plant is only disfigured slightly by the removal of the explants (Churchill et al., 1970, 1971*a*, 1971*b*, 1971*c*, 1972*a*, 1973; Arditti et al., 1971*a*, 1971*b*, 1972, Arditti, 1977*a*, 1978). At the same time the use of leaves and leaf tips was a great innovation. Everyone wanted a version of the article and we obliged.

Plant Material. Leaf tips from 1-year-old (but possibly older) seedlings growing in flasks under normal conditions are used (Arditti, 1967; Harrison, 1970; Harrison and Arditti, 1970).

Surface Sterilization. This is not necessary since the seedlings are already axenic.

Culture Vessels. Use 125-ml Erlenmeyer flasks containing 50 ml medium.

Culture Conditions. Liquid cultures are placed on a reciprocating shaker moving at the rate of 60 oscillations per minute, 50 cm below banks of Sylvania Gro Lux tubes and incandescent bulbs producing 150 ft-c during 18-h photoperiods. The temperature is maintained at 22–25°C. Solid cultures are kept under the same conditions on a stationary surface.

Culture Media. A modified MS medium (Table Epi-4) is used for initial culture. Callus cultures are maintained on solid modified Linsmaier–Skoog medium (see Table C-18) or MS medium (see Table C-17). Differentiation of plantlets occurs on modified Knudson C medium (see Table C-19).

Procedure. Remove seedlings from flasks and place on sterile Petri dishes, microscope slides, or aluminum foil. Remove tips no larger than 4–5 mm (the best size being 2 mm, about the dimension of a pinhead) with sterile scalpels, razor blades, or tips of syringe needles, and suspend in culture medium. Then place the flasks on the shaker. When tissue masses form, they should be divided and placed on solid Murashige–Skoog (Table Epi-4) or Linsmaier–Skoog (see Table C-18) media. After a new tissue mass forms, it should also be subdivided. For plantlet development, portions of the mass (which are in fact PLBs) are transferred to Knudson C medium (see Table C-19).

Developmental Sequence. Leaf tips remain green without any apparent changes or growth for nearly 60 days. After that, approximately 7% form calli. When divided and transferred to solid MS or Linsmaier–Skoog media, these form large callus masses. After a while abnormal plantlets form on these media. Following 3 weeks on Knudson C medium, callus sections form normal plantlets.

General Comments. Since the explants are obtained from unflowered seedlings, the procedure cannot be used for the propagation of desirable clones. Perhaps with further research a way can be found for the culture of leaf tips from adult plants. In the meanwhile, this method can be useful for the production of *Epidendrum* callus cultures for research purposes. These cultures can apparently be subdivided indefinitely (some of our cultures are still growing well after 3 years).

TABLE EPI-4. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for the culture of *Epidendrum* leaf tips (Churchill et al., 1970, 1972*a*, 1973; Arditti et al., 1971*a*, 1971*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
3	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	95 g l^{-1}	20	Or weigh
5	Ammonium nitrate, NH_4NO_3[b]	1650	82.5 g l^{-1}	20	Or weigh
6	**Iron chelate**[c]				
(a)	Disodium EDTA, NA_2EDTA	74.6	7.46 g l^{-1}	10	One solution
(b)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[c]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(c)	Zinc chloride, $ZnCl_2$	3.93	393 mg l^{-1}		
(d)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(f)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(g)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
	Amino acid				
8	Glycine	2	2 g l^{-1} 95% ethanol[d]	1	
	Auxin				
9	2,4-Dichlorophenoxyacetic acid (2,4-D)	1	50 mg 25 ml^{-1} 95% ethanol[e,f]	0.5	
	Cytokinin				
10	6-Benzylaminopurine (benzyladenine)	0.5	50 mg 50 ml^{-1} 95% ethanol[e,f]	0.5	
	Vitamin				
11	Thiamine (vitamin B_1)	0.4	80 mg 100 ml^{-1} 95% ethanol[f]	0.5	
	Sugar				
12	Sucrose[g]	30 g	No stock	No stock	Weigh
	Solvent				
13	Water, distilled	To 1000 ml			
	Solidifier				
14	Agar[g]	10–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelated iron (items 6a, 6b) and all microelements (items 7a–7g) to the same 1 l of distilled water, stir and/or heat until dissolved. Add 10 ml per liter of culture medium.

[d]Keep frozen between uses.

[e]If auxin or cytokinin does not dissolve, add a few drops of dilute KOH or HCl, respectively.

[f]Keep refrigerated.

[g]Add items 1–10 to 700 ml distilled water (item 13) and dissolve, adjust pH to 5.5, add sugar (item 12), and adjust volume to 1000 ml with distilled water (item 13). Sterilize by filtration and distribute into preautoclaved culture vessels. Alternately, dissolve items 1–7 and 12 in 1000 ml distilled water (item 13) and autoclave, add items 8–11 with sterile pipettes under sterile conditions to the autoclaved salt solution, and distribute into preautoclaved culture vessels. When solid medium is required, dissolve sugar (item 12) and agar (item 14) in 750 ml distilled water (item 13) and autoclave. Add items 1–11 to 250 ml distilled water (item 13), and adjust pH to 5.5. Sterilize by filtration, mix with the autoclaved solution while it is still hot and liquid, swirl to mix well, and dispense into sterile culture vessels. Alternately, add items 1–7 to 800 ml distilled water (item 13), adjust pH to 5.5, add the sugar (item 12), and bring volume to 1000 ml with more distilled water (item 13). Dissolve agar (item 14) and autoclave the solution. After autoclaving and while it is still hot and liquid, add items 8–11 with sterile pipettes under sterile conditions, swirl to mix well, and distribute into preautoclaved culture vessels.

Culture of *Epidendrum* Root Tips

It seems reasonable to assume that, since orchid root tips contain meristematic zones, they can be made to grow in vitro. If so, another logical assumption is that they can be made to form callus cultures and eventually plantlets. As of this writing, the first assumption has been proven correct. Proof for the second, at least insofar as orchids are concerned, is still lacking. *Epidendrum* root tips, then, can be cultured, but all they produce is roots, albeit longer roots (Churchill et al., 1972*b*).

Plant Material. Free-hanging, aerial, mycorrhiza-free roots must be used. Roots that have reached the potting medium, touched the pot, or crawled over benches become infected by mycorrhizal fungus, which grows out of them and contaminates the culture medium.

Surface Sterilization. Roots are soaked in saturated calcium hypochlorite solution (10 g per 140 ml water) for about 5 min.

Culture Vessels. Use 100-ml prescription bottles containing 10–20 ml solid medium that has been allowed to solidify on the under (flat) surface; 125- or 250-ml Erlenmeyer flasks containing 25 or 50 ml medium, respectively, can also be used. For the subculture of larger roots, 1000-ml prescription bottles containing 200 ml medium should be used.

Culture Conditions. The conditions employed for *Epidendrum* leaf tips (Churchill et al., 1970, 1972*b*, 1973; Arditti et al., 1971*a*, 1971*b*, 1977) should be used, except that the root explants are grown on solid medium.

Culture Medium. Modified Ojima–Fujiwara medium (Table Epi-5) is most suitable for root tips.

Procedure. Place root tips on the medium and allow to grow. After 2 months they elongate and can be subcultured by removing and transferring the tips to new medium. The procedure can be repeated 3 months later. Thereafter, transfers can be made every 6 months, or the tips can be placed in a large bottle from the outset and not transferred.

Developmental Sequence. Roots simply grow in length, but their diameters and growth rate decrease with time. After 1 year some roots may be 5–6 cm long while others reach 15 cm, but all become thinner than roots of equal length growing on plants. After 2 years in culture the roots can still grow, but they lose their chlorophyll. Detipped roots do not grow.

General Comments. The culture medium apparently does not provide roots with sufficient amounts of some or all requirements. Neither is it capable of inducing callus formation. Whether the medium is MS, Heller's, or a special "meristem" medium (Ball et al., 1971), liquid or solid, shaken or not, roots die. Some, like those of *Phalaenopsis*, swell tantalizingly, as if to form a callus, but die just the same.

TABLE EPI-5. **Modified Ojima–Fujiwara medium (Ojima and Fujiwara, 1962) for the culture of *Epidendrum* root tips (Churchill et al., 1972*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Potassium nitrate, KNO_3[b]	20.2	2.02 g l^{-1}	10	Or weigh
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	136.7	13.67 g l^{-1}	10	Or weigh
3	Monopotassium phosphate, KH_2PO_4	4.08	408 mg l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	48	4.80 g l^{-1}	10	
5	Ferric sulfate, $Fe_2(SO_4)_3$	1	1 g l^{-1}	1	
6	**Microelements**[c]				
(a)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	1	1 g l^{-1}		
(b)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.02	20 mg l^{-1}		
(c)	Potassium iodide, KI	0.02	20 mg l^{-1}	1	One solution
(d)	Boric acid, H_3BO_3	0.02	20 mg l^{-1}		
(e)	Ammonium molybdate, $(NH_4)_2MoO_4$	0.02	20 mg l^{-1}		
	Vitamins[d]				
7	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol	1	
8	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol	1	Or combine
9	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol	1	
	Complex additive				
10	Neopeptone	300	No stock	No stock	Weigh
	Sugar				
11	Sucrose[e]	20 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[e]	To 1000 ml			
	Solidifier				
13	Agar[e]	10–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all dissolved. Add 1 ml per liter of culture medium.

[d]The vitamins may be combined into one stock solution. Add 50 mg each of niacin and pyridoxine and 10 mg thiamine to the same 100 ml of 95% ethanol, and use 1 ml of this per liter of culture medium. Refrigerate between uses.

[e]Add items 1–10 to 800 ml distilled water (item 12) and dissolve, adjust pH to 5.0 (it will change to 5.1, which is the desired pH), add the sugar (item 11) and bring volume up to 1000 ml with more distilled water (item 12). For solid medium, dissolve agar (item 13) by adding it to gently boiling solution while stirring. When fully dissolved, distribute into culture vessels and autoclave. According to the original report, Ojima and Fujiwara (1962), all components of this medium were sterilized by autoclaving.

In commenting about this procedure some years ago I wrote:

> Perhaps someday a method for the production of callus cultures and plants from orchid root tips may be developed. Several orchids naturally produce buds and plants on their roots. For the time being, however, root-tip culture of orchids may be a subject for wishful thinking and a means for the production of roots to be used as experimental organs (Arditti, 1977*a*, 1977*b*).

The prediction and wishful thinking came to pass: Methods for plantlet production from root tips were developed for several orchids, including *Phalaenopsis* (by Professor K. Zimmer and his associates).

Propagation of *Epidendrum* by the Culture of Explants from Vegetative Shoots

The method was developed as part of the work with the *Cattleya* alliance (Scully, 1967). In cases described as "excepted instances," PLBs arose from *Epidendrum conopseum* explants. Subculture of the bodies was unsuccessful. This simply means that additional work is needed to perfect the method.

Proliferation, Propagation, and Organogenesis of *Epidendrum* in Vitro

Shoot-tip explants of *Epidendrum radicans* (*Epidendrum ibaguense*) cultured on modified MS medium formed PLBs and plantlets (Kusumoto, 1981*b*). The original paper is in Japanese.

Transformation of Isoprenoids by *Epidendrum* in Tissue Culture

Tissue cultures of *Epidendrum ochraceum* maintained in vitro on media used for other orchids (Kukułczanka, 1985; Kukułczanka and Wojciechowska, 1983) transformed some isoprenoids (Mironowicz et al., 1987).

Isolation of Mesophyll Protoplasts of *Epidendrum*

The procedure for the isolation of mesophyll chloroplasts of *Calanthe discolor* as used for *Dendrobium* can be employed for *Epidendrum*. Isolation was 40% after 30 min and 74.6% following 2 h (Yasugi et al., 1986).

Protoplast Isolation from Leaves and Roots of *Epidendrum radicans*

The method developed for *Acampe praemorsa* has been used to isolate 3.2×10^4 and 1.2×10^4 protoplasts from leaves and roots, respectively, of *Epidendrum radicans* (Seeni and Abraham, 1986).

Transformation of Menthyl Acetate by *Epidendrum ochraceum* Tissues in Vitro

Tissues of *Epidendrum ochraceum* cultured in the Reinert–Mohr liquid medium (constant shaking 20–24°C, 18-h photoperiods, 2000 lx) transformed a racemic mixture of menthyl acetate to menthol (Mironowicz et al., 1987).

Protocorm-like Body and Plantlet Production from Stalk Explants of *Epidendrum radicans*

There are several micropropagation procedures for *Epidendrum*, including one from our laboratory (Churchill et al., 1972*a*) which could be more efficient than it is (but it was developed during the early days of orchid micropropagation), one which is probably unreliable (Stewart and Button, 1976*a*), and another mostly intended to generate publicity if it works at all (Kuhn, 1981*a*). The procedure for *Epidendrum radicans* described here is efficient and very commendable because it utilizes several completely defined media (Chen et al., 2002*a*).

Plant Material. Sections, 10–15 cm in length, should be taken from inflorescence stalks of 2–3-year-old greenhouse grown plants when flower buds are fully formed, but before color is showing.

Surface Sterilization. Sodium hypochlorite ($NaOCl_3$, domestic bleach), 1% (17 or 19 ml of 6 or 5.25% Clorox, respectively, diluted to 100 ml with distilled water), should be used to surface-sterilize the explants. They should be immersed in this solution for 12 min and then rinsed three times with sterile distilled water.

Culture Vessels. Culture tubes, 20 × 150 mm, were used in the original research. Other containers are also suitable.

Culture Conditions. Cultures should be maintained at 26 ± 1°C under 16-h photoperiods of 28–36 μmol m^{-2} s^{-1} provided by 40W China Electric Co. (Taiwan) daylight fluorescent tubes FL-30D/29.

Culture Media. Explants should be cultured on half-strength MS medium (Murashige and Skoog, 1962) supplemented with 0.45 μmol TDZ l^{-1} (Table Epi-6) for 40 days to bring about the formation of PLBs. The PLBs should be cultured on a medium containing 40 g sucrose l^{-1} (Table Epi-7) for proliferation. For shoot formation, PLBs should be cultured on a substrate with 10 g sucrose l^{-1} (Table Epi-8). Homogenized PLBs should be placed on a medium containing 4.44 μmol BA l^{-1} (Table Epi-9) to enhance the production of new PLBs. The homogenate should be cultured on a solution supplemented with 1.39 μmol kinetin (Table Epi-10) for maximal shoot production on. To produce plants PLBs with shoots should be cultured on the first medium (Table Epi-6). Plants should be potted in sphagnum.

Procedure. Internode sections, 5–7 mm in length should be excised from the surface-sterilized inflorescence segments and placed on the first medium (Table Epi-6). PLBs which form on this medium should be cultured on a substrate with 40 g sucrose l^{-1} (Table Epi-7) for proliferation. Shoot formation will take place on 10 g sucrose l^{-1} (Table Epi-8) or 0.44 μmol BA l^{-1} (Table Epi-9). The sucrose medium (Table Epi-8) is preferable because of the possibility (even if small) that BA (Table Epi-9) can be mutagenic. Shoots should be moved to the first medium (Table Epi-6) for plant formation. Plants are potted in sphagnum and grown in a greenhouse.

TABLE EPI-6. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the production of protocorm-like bodies (PLBs) from inflorescence explants of *Epidendrum radicans* (Chen et al., 2002*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
11	Thidiazuron	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
15	Peptone	1.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Gelrite[h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.7, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Add the Gelrite (item 18) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com, and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require higher concentrations of gellan gum or additional calcium or magnesium salts. Gellan gum must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE EPI-7. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the proliferation of protocorm-like bodies (PLBs) produced by inflorescence explants of *Epidendrum radicans* (Chen et al., 2002*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins[e,f]				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	40.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[g]	To 1000 ml			
	Solidifier				
15	Gelrite[h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]There are no auxins or cytokinins in this medium.

[g]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH to 5.7, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Add the Gelrite (item 15) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media.

[h]Gellan gum [(available as such from www.caissonlabs.com), and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require higher concentrations of gellan gum or additional calcium or magnesium salts. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE EPI-8. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for shoot induction on protocorm-like bodies (PLBs) produced by inflorescence explants of *Epidendrum radicans* (Chen et al., 2002*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins[e,f]				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[g]	To 1000 ml			
	Solidifier				
15	Gelrite[h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]There are no auxins or cytokinins in this medium.

[g]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH to 5.7, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Add the Gelrite (item 15) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media.

[h]Gellan gum [(available as such from www.caissonlabs.com), and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require higher concentrations of gellan gum or additional calcium or magnesium salts. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE EPI-9. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the production of protocorm-like bodies (PLBs) from homogenized PLBs produced by inflorescence explants of *Epidendrum radicans* (Chen et al., 2002*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
11	Benzyladenine (BA)	1.0	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Gelrite[h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murasiqe and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.7, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Add the Gelrite (item 17) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media.

[h]Gellan gum [(available as such from www.caissonlabs.com), and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require higher concentrations of gellan gum or additional calcium or magnesium salts. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE EPI-10. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for shoot induction on protocorm-like bodies (PLBs) produced from homogenized PLBs produced by inflorescence explants of *Epidendrum radicans* (Chen et al., 2002*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
11	Kinetin	300.0	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Gelrite[h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1N KOH or HCl.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.7, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Add the Gelrite (item 17) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com, and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require higher concentrations of gellan gum or additional calcium or magnesium salts. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

Developmental Sequence. Explants form PLBs on the first medium (Table Epi-6). PLBs proliferate on the second solution (Table Epi-7). The PLBs produce shoots on either the third (Table Epi-8) or fourth (Table Epi-9) substrate. Shoots develop into plants on the first medium (Table Epi-6).

General Comments. Like other procedures from Professor Wei-Chin Chang's laboratory, this method is well conceived and based on impressive research. The paper is clear and well written.

Isolation, Culture, and Fusion of Protoplasts of *Epidendrum radicans*

The method used for *Dendrobium* (Yasugi, 1990) is also suitable for *Epidendrum radicans*.

In Vitro Morphogenesis of *Epidendrum radicans*

When cultured on half-strength MS medium with or without TDZ for 1–2 weeks, root, stem, leaf, and flower-stalk explants formed small masses of transparent tissue on the cut surfaces. Under illumination this tissue became larger and "turned into green organized masses," which formed PLBs and proliferated on hormone-free basal half-strength MS. TDZ enhanced the proliferation. The PLBs produced plantlets on TDZ-containing half-strength MS after 1 month of culture (Chang et al., 2001).

Epiphronitis

The first hybrid was *Epiphronitis* Veitchii [*Epidendrum ibaguense* (*Epidendrum radicans*) × *Sophronitis grandiflora*] registered in 1890 by the well-known orchid establishment Messrs J. Veitch & Sons, which at that time was located at Chelsea and Slough, Bucks., England (the Slough nursery eventually became the Black and Flory Orchid Co.). Since 1890 more *Epiphronitis* hybrids have been produced by crossing additional *Epidendrum* and *Sophronitis* species.

A method for tissue culture propagation of *Epiphronitis* Veitchii was developed in Japan (Kusumoto, 1981*b*). Information that can be gleaned from illustrations and tables (the paper is in Japanese) suggests that shoot tips, and axillary buds from stems and flower stalks proliferate well and form PLBs on the MS medium (see Table Cym-11, Epi-1 and Epi-4). Best results were obtained "when axillary buds of peduncles [flower stalks] were taken. . . ." The best method for "propagating PLBs of *Epiphronitis* [is] the same as that of *Cattleya*" (Kusumoto, 1981*b*).

It is reasonable to assume that the procedures developed for *Epiphronitis* Veitchii, *Epidendrum*, *Sophronitis*, *Cattleya*, *Laelia*, and related genera would be suited for *Epiphronitis* hybrids in general.

Eulophia

Eulophia is a pantropic, chiefly Asiatic terrestrial genus consisting of approximately 200 genera. A method for clonal propagation of *Eulophia hormusjii* was developed in India (Vij et al., 1989), where the genus is represented by 26 species.

Plant Material. In the original research "rhizome segments (3–4 mm) with or without differentiated nodes" were excised from 24-week-old seedlings in vitro.

Surface Sterilization. There is no need to sterilize the explants or their sources because they are already axenic.

Culture Vessels. Test tubes, Erlenmeyer flasks, jars, and other containers are suitable.

Culture Conditions. Cultures should be maintained under 12-h photoperiods of 3500 lx at $25 \pm 2°C$. The sources of illumination are not described in the original research, but it is reasonable to assume that fluorescent and/or incandescent lamps would be suitable.

Culture Media. The explants formed PLBs in modified Mitra–Prasad–Roychowdhury (MPR) medium (Mitra et al., 1976) containing peptone (MPRP; Table Eul-1). Shoot buds developed on MPR containing yeast extract, NAA, and kinetin (MPRY; Table Eul-2). Plantlets regenerated on MPR with peptone, NAA, and kinetin (MPRN; Table Eul-3).

Procedure. Recommendations for a practical procedure are not included in the original paper. A reasonable approach would be to place the explants on MPRP until PLBs form. These should be moved to MPRY to stimulate the formation of shoot buds and to MPRN for plantlet regeneration.

Developmental Sequence. On MPRP the explants form chlorophyll-containing PLBs that elongate and become rhizomatous before organogenesis starts. Shoot buds appear on MPRY. Plantlets 3–4 cm tall develop 6 weeks after transfer to MPRN.

General Comments. Cytological studies of plantlets raised by this method suggest that the chromosome number ($2n = 54$) remains stable. This is an indication that plantlets produced by this procedure would be true to type. The method should be useful in cases where only a few seedlings of a rare form or hybrid are available.

TABLE EUL-1. **Mitra–Prasad–Roychowdhury medium (Mitra et al., 1976) as modified for the induction of protocorm-like bodies from rhizome segments from seedlings of *Eulophia hormusjii* (Vij et al., 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100	10 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200	20 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180	18 g l^{-1}	10	
5	Monosodium phosphate, NaH_2PO_4	150	15 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Complex additive				
14	Peptone	2 g/l	No stock	No stock	Weigh
	Sugar				
15	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[f]	7 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original recipe used 3 ml $Na_2FeEDTA$, a preparation that may not be readily available. The substitute listed here is used very commonly in orchid tissue culture media. Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium.

[e]Keep refrigerated or frozen between uses.

[f]Add items 1–14 to 900 ml distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense solution into culture vessels and autoclave. Omit agar if preparing liquid medium. Media that contain hormones and vitamins should not be autoclaved without first determining that this method of sterilization will have no deleterious effects. This is such a case (Mitra, 1971; Mitra, et al., 1976), so the medium may be autoclaved.

TABLE EUL-2. **Mitra–Prasad–Roychowdhury medium (Mitra et al., 1976) as modified for the induction of shoot buds on rhizome segment explants from seedlings of *Eulophia hormusjii* (Vij et al., 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100	10 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200	20 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180	18 g l^{-1}	10	
5	Monosodium phosphate, NaH_2PO_4	150	15 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5 mg l^{-1}		
	Auxin				
8	Napthaleneacetic acid (NAA)	1	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	Kinetin	1	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
10	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
14	Riboflavin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
15	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Complex additive				
16	Yeast extract	2 g l^{-1}	No stock	No stock	Weigh
	Sugar				
17	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Agar, Difco Bacto[g]	7 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original recipe used 3 ml $Na_2FeEDTA$, a preparation that may not be readily available. The substitute listed here is used very commonly in orchid tissue culture media. Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium.

[e]Keep refrigerated or frozen between uses.

[f]If the auxin for cytokinin fails to dissolve, add a few drops of dilute KOH or HCl, respectively.

[g]Add items 1–16 to 900 ml distilled water (item 18), adjust pH to 5.6, add sugar (item 17), and adjust volume to 1000 ml with distilled water (item 18). Bring solution to a gentle boil, and add agar (item 19) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense solution into culture vessels and autoclave. Omit agar for liquid medium. Media that contain hormones and vitamins should not be autoclaved unless prior experiments have determined that this method of sterilization has no deleterious effects. This is such a case (Mitra, 1971; Mitra, et al., 1976), and the medium may be autoclaved.

TABLE EUL-3. **Mitra–Prasad–Roychowdhury medium (Mitra et al., 1976) as modified for plantlet regeneration from rhizome-segment explants from seedlings of *Eulophia hormusjii* (Vij et al., 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100	10 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200	20 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180	18 g l^{-1}	10	
5	Monosodium phosphate, NaH_2PO_4	150	15 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5 mg l^{-1}		
	Auxin				
8	Napthaleneacetic acid (NAA)	1	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	Kinetin	1	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
10	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
14	Riboflavin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
15	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Complex additive				
16	Peptone	2 g l^{-1}	No stock	No stock	Weigh
	Sugar				
17	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Agar, Difco Bacto[g]	7 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original recipe used 3 ml $Na_2FeEDTA$, a preparation that may not be readily available. The substitute listed here is used very commonly in orchid tissue culture media. Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium.

[e]Keep refrigerated or frozen between uses.

[f]If auxin or cytokinin fails to dissolve, add a few drops of dilute KOH or HCL, respectively.

[g]Add items 1–16 to 900 ml distilled water (item 18), adjust pH to 5.6, add sugar (item 17), and adjust volume to 1000 ml with distilled water (item 18). Bring solution to a gentle boil, and add agar (item 19) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense solution into culture vessels and autoclave. Omit agar for liquid medium. Media that contain hormones and vitamins should not be autoclaved unless prior experiments have determined that this method of sterilization will have no deleterious effects. This is such a case (Mitra, 1971; Mitra, et al., 1976), so the medium may be autoclaved.

Gastrochilus

See *Saccolabium calceolare* (pp. 1097–1099, Vol. II).

Geodorum

Geodorum, a small terrestrial genus, is distributed from India, Sri Lanka and China through tropical Asia, Australia, Malaysia, Papua New Guinea and a number of Pacific islands (Seidenfaden and Wood, 1992; O'Byrne, 1994).

Isolation of *Geodorum densiflorum* Protoplasts

The method developed for *Acampe praemorsa* (Seeni and Abraham, 1986) was used to isolate 6.1×10^4 protoplasts from leaves of *Geodorum densiflorum*.

In Vitro Propagation of *Geodorum densiflorum* through the Culture of Rhizome Sections

Asymbiotic seed germination of *Geodorum densiflorum* resulted in the development of protocorms which produced rhizomes that "continue to grow and ramify without the formation of other organs for a long time and occasionally shoots are formed from rhizome apices." Sections of these rhizomes were cultured in an effort to induce organogenesis (Sheelavantmath et al., 2000).

Plant Material. Four-week-old rhizomes produced by protocorms which developed from seeds germinated on Knudson C medium (Knudson, 1946) ". . . were initially cut at nodal regions in small pieces and then internodal regions were longitudinally cut into 5–10 mm length" (Sheelavantmath et al., 2000).

Surface Sterilization. Explants taken from plants growing in vitro do not require surface sterilization.

Culture Vessels. Photographs in the original paper show that cultures were maintained in test tubes. Other containers can also be used.

Culture Conditions. The research cultures were maintained at $22 \pm 2°C$ under 12-h photoperiods of 40 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps.

Culture Media. The seeds which produced the protocorms that gave rise to the 4-week-old rhizomes were germinated on Knudson C (KC) medium (Knudson, 1946) supplemented with 10% (v/v) coconut water (CW). Rhizome sections produced multiple shoots on modified MS medium (Murashige and Skoog, 1962) containing 5 μmol (1.13 mg) BA l^{-1} and 0.1% charcoal (Table Gdrm-1). The shoots rooted on MS containing 1 μmol (0.2 mg; actually 1 μmol is 0.186 mg, but this can be rounded

TABLE GDRM-1. **Modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of rhizome sections of *Geodorum densiflorum* (Sheelavantmath et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzylaminopurine (BAP)	1.13	113 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	8.0 g	No stock	No stock	Weigh
	Darkening agent				
17	Activated vegetable charcoal (AC)[h]	1.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.7, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a homogenizer, add the charcoal and mix thoroughly. After that pour medium into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the warm and still liquid solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite should not be used except in special circumstances.

off to 0.2 mg which is easier to weigh; the difference of 0.014 mg is insignificant, but sticklers for accuracy can use the exact amount) NAA l^{-1} and 0.1% charcoal (Table Gdrm-2). Well-developed plants were potted in vermiculite in plastic pots and watered with half-strength MS (presumably minerals only, Table Gdrm-3).

Procedure. Seeds (harvested 30 days after pollination in the original research) should be sown on KC medium and cultured until rhizomes are formed. When the rhizomes are 4 weeks old they should be sectioned. The sections should be cultured on the BAP containing medium (Table Gdrm-1). Shoots which form on the explants after 4–6 weeks must be subcultured onto fresh medium at 4-week intervals. Well-developed shoots should be moved to the second medium (Table Gdrm-2) for rooting. Rooted plants that are large enough should be potted in vermiculite. Once well established potted plants should be moved to a greenhouse.

Developmental Sequence. Small green protuberances form on the explants after 2–4 weeks on the first medium (Table Gdrm-1). These protuberances develop into multiple shoots in 4–6 weeks (Fig. Gdrm-1). During this time the explants release into the medium phenolic compounds which can inhibit growth. Therefore the addition of charcoal which absorbs the phenolics is of great importance. The shoots are well developed after 14 weeks, but lack roots. Root development takes place after 4 weeks on the second medium (Table Grdm-2). Large plants develop after several weeks on this medium. Seedlings grow to maturity after being potted and moved to a greenhouse.

General Comments. *Geodorum densiflorum* is an endangered orchid. This micropropagation method is intended as a means of ex situ conservation. It can also be used for large scale horticultural propagation of seedlings. Other research has also shown that BA and NAA can affect PLB and rhizome development in *Geodorum densiflorum* (Roy and Banerjee, 2002).

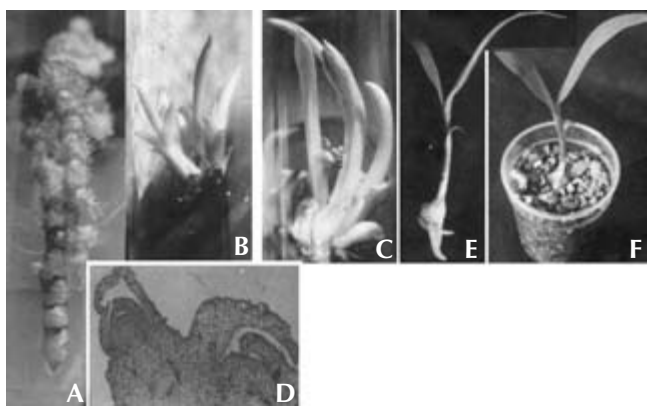


FIG. GDRM-1. **In vitro propagation of *Geodorum densiflorum*. A. Rhizome proliferating on MS with 2 µmol NAA. B, C. Multiple shoots on MS with 2 and 5 µmol BA respectively. D. Section through rhizome showing development of root primordia. E. Rooting on MS with 1 µmol of NAA. F. Plantlet in pot. (Sheelavantmath et al., 2000.)**

TABLE GDRM-2. **Modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for root induction on shoots of *Geodorum densiflorum* (Sheelavantmath et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.2	120 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	8.0 g	No stock	No stock	Weigh
	Darkening agent				
17	Activated vegetable charcoal (AC)[h]	1.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH or NaOH respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.7, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a homogenizer, add the charcoal, and mix thoroughly. After that pour medium into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10) and vitamins (items 11–13) to the warm and still liquid solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite should not be used except in special circumstances.

TABLE GDRM-3. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for irrigating *Geodorum densiflorum* plants (Sheelavantmath, 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Solvent				
8	Water, distilled[e]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add items 7a–g to 1 l of distilled water, heat for a while if some of the salts do not dissolve, mix and use as indicated. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Add items 1–7 to 900 ml of water (item 8), adjust pH to 5.4–5.7, bring volume to 1 l, mix well and use.

Rapid Micropropagation of *Geodorum densiflorum* in Liquid Culture

At one time *Geodorum densiflorum* was very common in the forests of the north-eastern Himalayas. However its existence is being threatened by commercial exploitation (i.e., overcollection) and human encroachment (expansion of cultivated areas). A mass rapid propagation method, useful as a means of conservation and production of plants for the horticulture trade, was developed in India (Kanjilal and Datta, 2000).

Plant Material. Leaves and roots of seedlings were removed to expose the stems which were sectioned into thin transverse sections, 1–1.5 mm thick. These sections were placed in culture.

Surface Sterilization. Explants excised from plants growing in vitro do not require surface sterilization.

Culture Vessels. Flasks described as "conical [probably meaning Erlenmeyer] . . . (100 ml; Borosil)" were used to culture explants on both liquid and solid media.

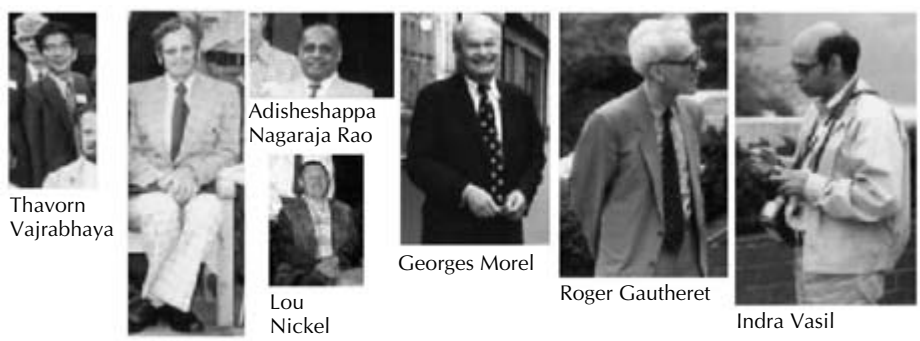
Culture Conditions. The research cultures were maintained at 25 ± 2°C under 16-h photoperiods of 25 μE m^{-2} s^{-1} provided by cool white fluorescent lamps. Standard culture room conditions are also suitable. Liquid cultures should be placed on a rotary shaker at 80–120 rpm.

Culture Media. Explants should be cultured first in liquid Knudson C (KC) medium (Knudson, 1946) supplemented with NAA, BA, and peptone (Table Gdrm-4). PLBs produced in the liquid medium should be moved to solid substrate of the same composition. Well-developed plantlets should be taken out of culture and potted in a mixture of brick pieces, coal chips, sand, and soil (in a ratio of 1 : 1 : 1 : 1, v/v/v/v).

Procedure. The explants should be placed in the liquid medium (Table Gdrm-4). When clusters of PLB form they should be separated and subcultured in liquid medium (Table Gdrm-4) for further proliferation or on solid substrate (Table Gdrm-4) for plantlet production. Subculturing may be necessary every 2 weeks. Well-developed plantlets should be washed carefully with water at body temperature, treated with Bavastin (or another suitable fungicide), and potted.

Developmental Sequence. The explants produce PLBs in the liquid version of the medium (Table Gdrm-4). Well-developed PLBs cultured on solid substrate (Table Gdrm-4) develop plantlets. Large and well-developed plantlets can be obtained in 10–12 weeks and should be moved to pots in a potting mix.

General Comments. This is a very rapid, simple, and efficient micropropagation method because shoots can be cut into several sections and every section produces many PLBs. It cannot be used to select specific cultivars because the explants are taken from seedlings.



Tissue culture pioneers. Vajrabhaya, Nickel, Rao and Morel worked with orchids. Gamborg formulated a waidely used tissue culture medium. Gautheret was a pioneer in France and for a while Morel's superior. Vasil is responsible for numerous advances and is a well known editor of journals and books.

TABLE GDRM-4. **Modified liquid and solid Knudson C (KC) medium (Knudson, 1946) for protocorm-like body (PLB) and plantlet production from shoot sections of *Geodorum densiflorum* (Kanjilal and Datta, 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement[d]				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
7	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
8	Benzylaminopurine (BAP)	3.0	300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
9	Peptone[g]	2 g	No stock	No stock	Weigh
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[h]	To 1000 ml			
	Solidifier				
12	Agar[h]	8–10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. Kundson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$ l^{-1}, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored.

[d]There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If incorporation of additional microelements becomes necessary those used in the Murashige–Skoog (MS) medium are suitable.

[e]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH or HCl respectively.

[f]Keep frozen between uses.

[g]Available as powder (catalog number SPPE117) from www.caissonlabs.com. There are other sources, but Caisson Laboratories is suggested here because it specializes in plant tissue culture media and chemicals.

[h]Add items 1–9 to 900 ml of distilled water (item 11), adjust pH to 5–5.2, add sugar (item 10), and bring volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. As a rule vitamins, hormones and other heat labile items are added after autoclaving, but in this case they can be autoclaved. No agar should be added to the medium for culturing explants because it must be liquid. Agar should be added to the medium for culturing PLBs.

The first solution for asymbiotic orchid seed germination published by Professor Lewis Knudson in 1921 and 1922 is his medium B. In 1946 he published his medium C. There is no medium A. Knudson did not name any of his media after himself. He merely designated them solutions B and C. Others started calling them "Knudson B medium" and "Knudson C medium."

Goodyera

Goodyera, a terrestrial north temperate genus, is widely distributed and found in North and Central America, Africa, Asia, Australia, Europe, Japan, Madagascar, and New Guinea. It is named after John Goodyer (1592–1664) who was considered the best British botanist of his time (Schultes and Pease, 1963; Bechtel et al., 1992; www.goodyer.com/family2002.html).

Propagation of *Goodyera schlechtendaliana* through Callus Derived from Leaf Segments

This species is found in Bhutan, China, India, Indonesia (Sumatra), Japan, Korea, Nepal, Sikkim, Taiwan, Thailand, and Vietnam (Seidenfaden, 1978). In Japan *Goodyera schlechtendaliana* is popular due to its attractive foliage variations. Like many other orchids it has a colorful nomenclatural history (for a review see Seidenfaden, 1978). Propagation through division is very slow. Therefore a method of mass rapid propagation was developed at Hirosaki University, Aomori, Japan (Tomita, 2002).*

Plant Material. Mature seeds were sown on Knudson C (KC) medium (Knudson, 1946) in 100-ml Erlenmeyer flasks containing 30 ml medium. The cultures were maintained at 22 ± 2°C under continuing illumination of 25 μmol m^{-2} s^{-1} provided by fluorescent lamps (type not described). Seedlings were transplanted onto fresh medium every 8 weeks. Leaves, up to 2 cm in length, were taken from 144-week-old seedlings and sectioned into segments measuring 5 mm × 5 mm. These segments were cultured.

Surface Sterilization. Explants taken from plants grown in vitro do not require sterilization. However, they should be rinsed with sterile distilled water to remove medium residues.

Culture Vessels. Polystyrene Petri dishes (9 cm diameter, Iwaki, Co., Ltd.) were used to culture explants. Callus produced by the segments should be cultured in Erlenmeyer flasks (100-ml capacity containing 30 ml medium). Other containers can also be used.

Culture Conditions. Leaf explant and callus (up to 40 weeks) cultures were kept in the dark at 20°C. Sections taken from 40-week-old calli were maintained at 22 ± 2°C under continuing illumination of 25 μmol m^{-2} s^{-1} provided by fluorescent lamps (type not described). Standard culture room conditions are probably also suitable.

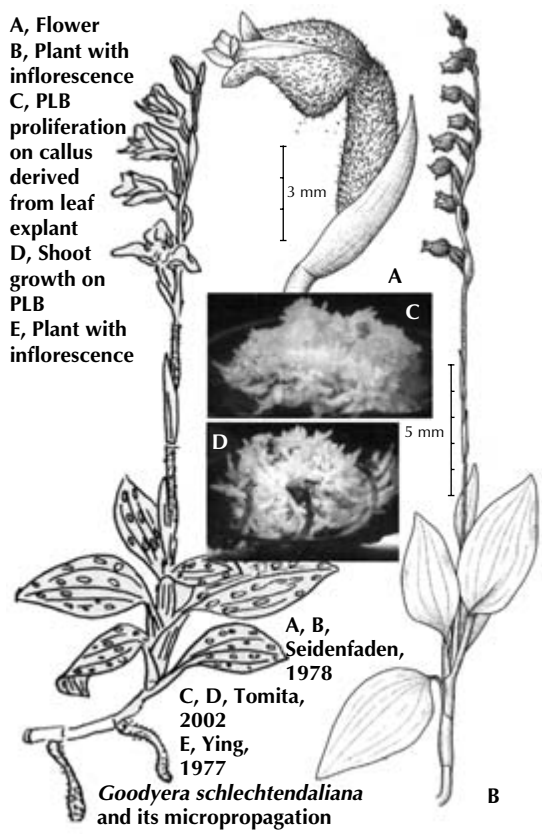
* Thanks are due to Professor Masnaori Tomita, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki 036-8561 Aomori, Japan for providing a reprint and much additional information via e-mail (tomita@cc.hirosaki-u.ac.jp).

Culture Media. Leaf explants should be cultured on modified half-strength MS medium (Murashige and Skoog, 1962) containing 2.21 mg Dicamba (3,6-dichloro-*o*-anisic acid) l^{-1} and 0.225 mg BA l^{-1} (Table Gdya-1). Callus sections are cultured on modified Norstog medium (Table Gdya-2). Shoots which form on this medium produce green plantlets on oat medium (Table Gdya-3).

Procedure. Young leaves (up to 2 cm in length) are taken from 5-month-old seedlings and cut into 5 × 5 mm sections which should be cultured, ten per dish, in Petri dishes, 9 mm in diameter. The abaxial side (that is the side away from the axis) of each segment is placed on the first medium (Table Gdya-1). Callus should be excised from the sections after 24 weeks of culture and subcultured onto the same solution (Table Gya-1) in 100-ml flasks covered with aluminum foil and maintained in the dark at 20°C. This should be done every 4 weeks. Most of them will die. The surviving callus masses should be cultured for 40 weeks (64 weeks from the start of culture) before small sections, 10–12 mm^3 in size, are taken from each mass and moved to the second medium (Table Gdya-2). Shoots will form on the second medium after 20 weeks of culture (84 weeks from the start of culture).

Developmental Sequence. The leaf segments should start to form white callus at the cut surface after 8 weeks of culture on the first medium (Table Gdya-1). When this callus is moved to the second medium (Table Gdya-2) PLBs will form on the surface of some callus masses after 12–16 weeks of culture. Green shoots should form on these PLBs after 20 weeks on the second medium (Table Gdya-2). To produce plants the shoots and PLBs should be moved to the oat medium (Table Gdya-3) with a suitable *Rhizoctonia* strain. When these plants become large enough they can be grown in a non-heated greenhouse.

General Comments. This procedure is ingenious and of considerable scientific interest. However its slow pace may be a practical drawback.



***Goodyera schlechtendaliana* and its micropropagation**

TABLE GDYA-1. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Goodyera schlechtendaliana* leaf explants (Tomita, 2002)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Herbicide				
10	Dicamba (3,6-dichloro-*o*-anisic acid)	2.2	220 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylamionopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g,h]	To 1000 ml			
	Solidifier				
17	Gellan gum[g,h]	3.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. Dicamba is available from http://search.wako-chem.com.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses. Dicamba is available from http://search.wako-chem.com/

[f]If the herbicide or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), raise volume to 1000 ml with distilled water (item 16), add the gellan gum (item 17) in accordance with the instructions in footnote *h* below, distribute the medium into vessels and autoclave.

[h]Gellan gum (available as such from www.caissonlabs.com and as Phytagel from www.sigmaaldrich.com) or Gelrite is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE GDYA-2. **Half-strength Nortstog medium (Norstog, 1973; Tomita and Tomita, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium chloride, $CaCl_2 \cdot 2H_2O$[b]	370.0	37.0 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
3	Potassium chloride	375.0	37.5 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	455.0	45.5 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
6	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50.0 mg l^{-1}		
7	**Amino acids**[e,f]				
(a)	Alanine	50.0	500.0 mg	2	Dissolve all amino acids in the 20 ml of 70% ethanol[f]
(b)	Arginine	10.0	100.0 mg		
(c)	Cysteine	20.0	200.0 mg		
(d)	Glutamine	400.0	4.0 g		
(e)	Leucine	10.0	100.0 mg		
(f)	Phenylalanine	10.0	100.0 mg		
(g)	Tyrosine	10.0	100.0 mg		
	Polyol[f]				
8	*myo*-inositol	50.0	5 g 100 ml^{-1} 70% ethanol[f]	1	
	Organic acid				
9	Malic acid	1.0 g	No stock	No stock	Weigh
10	**Vitamins**[e]				
(a)	Calcium pantothenate	0.25	25.0 mg	1	One solution. Dissolve all vitamins in the same 100 ml of 95% ethanol[e]
(b)	Pyridoxine·HCl	0.25	25.0 mg		
(c)	Thiamine	0.25	25.0 mg		
	Sugar				
11	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[g]	To 1000 ml			
	Solidifier				
13	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If a precipitate forms shake vigorously before dispensing into medium.

[g]Add items 1–6, 8 and 9 to 900 ml of distilled water (item 12), adjust pH to 5.5, add sugar (item 11), and raise volume to 1000 ml with distilled water (item 12). Bring the solution to a gentle boil and add the agar (item 13) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acids (item 7) and vitamins (item 10) to the warm solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE GDYA-3. **Oat medium for production of *Goodyera schlechtendaliana* plants (M. Tomita, personal communication)**[a]

Oats (rolled) powdered in a blender	10 g
Agar	10 g
Distilled water	1000 ml

[a]Add oats to water, adjust pH to 5.5, bring mixture to a gentle boil, add agar, stir until the mixture is homogenous, pour 50 ml of medium per 250-ml Erlenmeyer flask (or 100 ml per 500-ml flask) and autoclave. Inoculate some of the flasks with an appropriate *Rhizoctronia* strain and transfer PLB or shoots to these flasks.

Grammatophyllum

Grammatophyllum is one of the earliest Asian orchids to be described in the Western literature. In his *Herbarium Amboinense* (published in Holland between 1741 and 1750, but completed in Ambon in 1690) Georgius Everhardus Rumphius, the blind seer of Ambon (de Wit, 1977; Wehner et al., 2002; Beekman, 2003), listed among what he called "the Aristocracy" of plants a plant he called *Angraecum scriptum*, *Bonga loki* (probably an Ambonese name), *Anggrek krinsing* (an Indonesian name) and *het geschreven Anggrek* (The Inscribed Anggrek), which is now known as *Grammatophyllum scriptum*.

Isolation of Protoplasts of *Grammatophyllum elegans*

The method developed for *Acampe praemorsa* was used to isolate 11.6×10^4 and 9.4×10^4 protoplasts from leaves and roots (the latter taken from seedlings growing in vitro), respectively, of *Grammatophyllum elegans* (Seeni and Abraham, 1986).

Callus Induction and Plant Regeneration of *Grammatophyllum scriptum*

This, Rumphius's orchid aristocrat, is found in Indonesia, the Malay archipelago, the Philippines, and the Solomon Islands. Its name is derived from the maroon blotches on its petals and sepals which Rumphius likened to Hebrew letters. A method for the micropropagation of this species was developed at the Rizal Technological University in the Philippines (Quilang et al., 2002).

Plant Material. In the original research leaf and node explants were obtained from seedlings in vitro. However the report describing the method deals only with leaf explants. Presumably, node explants should be handled in a similar manner.

Surface Sterilization. Explants taken from plants growing in vitro do not require surface sterilization. However, they must be washed to remove all medium residues.

Culture Vessels. Culture tubes, Erlenmeyer flasks, and other containers can be used.

Culture Conditions. For callus formation explants should be maintained at 25 ± 1°C in the dark. Callus section for plant formation should be maintained at the same temperature under 14-h photoperiods. The light intensity used in the original research was not described. Standard culture room light intensity should be suitable.

Culture Media. Explants should be cultured in a modified Vacin and Went (VW) medium (Vacin and Went, 1949) supplemented with coconut water and 4 μmol 2,4-D (Table Gram-1). Sections of the callus which formed on this medium should be cultured on a medium free of 2,4-D (Table Gram-2). No medium is suggested for the culture of plantlets to maturation. The second medium (Table Gram-2) may be

TABLE GRAM-1. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of *Grammatophyllum speciosum* leaf explants (Quilang et al., 2002)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	1	
	Auxin				
8	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.88	88 mg 100 ml^{-1} 95% ethanol[e]	1	0.4 µmol
	Complex additive				
9	Coconut water (CW)[f]	150.0 ml	No stock	No stock	Measure
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 11) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]If the 2,4-dichlorophenoxyacetic acid (2,4-D) will fail to dissolve add a few drops 0.1 N HCl. Keep frozed between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water (CW), not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Filtered CW must be stored in a freezer until it is used. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[g]Add items 1, 3–9 to the 500–700 ml of distilled water (item 11) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 11, adjust pH to 4.8–5.0 add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 10) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

TABLE GRAM-2. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of callus obtained from *Grammatophyllum speciosum* leaf explants and for plantlet regeneration (Quilang et al., 2002)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	1	
	Complex additive				
8	Coconut water (CW)[e]	150.0 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]The correct term for the liquid endosperm of coconuts is coconut water (CW), not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Filtered CW must be stored in a freezer until it is used. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Add items 1, 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 2), raise the volume to 900 ml with distilled water (item 10), adjust pH to 4.8–5.0, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

suitable, or plantlets can be cultured on one of the versions of the Knudson C (KC) medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate (BH) or charcoal (see Table Pln-1) or the Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal (AC) should be added to the medium selected for use as suggested for KC that does contain this darkening agent (Table Pln-1, footnote *f*).

Procedure. Explants should be cultured on the first medium (Table Gram-1) until a sufficient amount of callus for subculturing becomes available. This may take 2–4 months. Sections of this callus should be subcultured on the second medium (Table Gram-2) until plantlets are formed. These plantlets can be subcultured onto the same medium (Table Gram-2) or moved to one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, Cym-1 to Cym-3, Cym-5, Cym-24, C-19, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, and V-4) with or without BH or AC. If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). AC should be added to the medium selected for use as suggested for KC that does contain this darkening agent (see Table Pln-1, footnote *f*).

Developmental Sequence. On being placed on the first medium (Table Gram-1) the explants will swell. This will be followed by the "formation of globular structures and protocorm-like bodies and then progress to formation and proliferation of the callus." Plantlet formation on the second medium (Table Gram-2) proceeds through somatic embryogenesis.

General Comments. The report starts by referring to leaf and node explants but dwells only on the former. However one can assume that the procedure will work for both. This procedure should also be tested with leaves from mature plants.

***Grammatophyllum scriptum* flower (courtesy J. Arditti)**

Habenaria

A mostly terrestrial genus consisting of 500–600 species, *Habenaria* is essentially circumglobal and found in Africa, the Americas, Asia, Australia, and Europe.

Micropropagation and in Vitro Flowering of *Habenaria crinifera*

In the state of Bihar, India *Habenaria crinifera* is part of an antiheadache drug formulation. It may also be suitable for commercialization in the horticultural trade, and as a more widely used drug formulation. *H. crinifera* is also one of the more important and rarer species in the Western Ghats in India. These seem to be the reasons why a micropropagation method was developed at the Tropical Botanic Garden and Research Institute, Palode, Thrivanathapuran, Kerala, India (Latha, 1999a).

Plant Material. Young plantlets collected from the ground in the field following the northeast monsoon showers in June and July were potted in 13-cm (6-inch) community pots in a potting mixture consisting of river sand, charcoal, and dried cattle manure (in a ratio of 1 : 2 : 1, v/v/v) and watered daily. Following 2 months of growth in the pots, shoots, approximately 5 cm tall, were excised below their point of attachment to the tubers from which they were growing. All leaves were removed from the shoots after that and the stems were cut into 1-cm-long segments.

Surface Sterilization. Shoot-tip segments should be washed with running water for 15 min and then soaked in 1% (v/v) solution of the detergent Teepol (www.sigmaaldrich.com) for the same length of time. After that the shoot tips should be dipped in 70% ethanol (74 ml 95% ethanol diluted to 100 ml with distilled water) for 30 s, submerged in 0.01% (w/v) mercuric chloride ($HgCl_2$; www.sigmaaldrich.com, www.fishersci.com, www.vwr.com, www.thomassci.com) for 3 min (this is a dangerous and toxic substance which should be handled with great care), and washed with sterile distilled water three times. Shoot tips excised from the stems should be immersed in 0.01% $HgCl_2$ for 2 min and washed three times (10 s per wash) with sterile distilled water.

Culture Vessels. Erlenmeyer flasks, 250-ml capacity, containing 100 ml medium were used in the original research. Other containers are also suitable.

Culture Conditions. The original cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx (light sources not indicated). Standard culture room conditions are also suitable.

Culture Medium. Explants should be cultured on woody plant (WPM) medium (Lloyd and McCown, 1981) containing 0.5 mg BA l^{-1} (Table Hab-1) for vegetative bud and shoot formation. The shoots should be moved to WPM supplemented with 0.05 mg NAA l^{-1} (Table Hab-2) for rooting. Flower-bud formation can be induced in vitro on WPM with 0.5 mg BA l^{-1} and 0.1 mg NAA l^{-1} (Table Hab-3).

TABLE HAB-1. **Woody plant (WPM) medium (Lloyd and McCown, 1981) modified for the culture of *Habenaria crinifera* explants (Latha, 1999a)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400.0	40.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	96.0	9.6 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)$	556.0	55.6 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	Potassium sulfate, K_2SO_4	990.0	99.0 g l^{-1}		
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.25	25.0 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(d)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(e)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
11	6-Benzyladenine (BA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	6.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. This medium was originally formulated for the culture of a woody plant, mountain laurel, *Kalmia latifolia*.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe lists $MnSO_4 \cdot H_2O$ which seems to be a typographical error because the formulae and concentrations of all other microelements are the same as in the Murashige–Skoog (MS) medium (Murashige and Skoog, 1962).

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.2, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media. As a rule it is preferable to add heat-labile substances after autoclaving. However in this case the cytokinin and vitamins can be added before sterilization.

Habenaria from the Latin *habenia* (reins) alluding to the long, strap-like divisions of the two lateral petals and the lebellum (Schultes and Pease, 1963).

TABLE HAB-2. **Woody plant (WPM) medium (Lloyd and McCown, 1981) modified for rooting of *Habenaria crinifera* shoots (Latha, 1999a)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400.0	40.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	96.0	9.6 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)$	556.0	55.6 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	Potassium sulfate, K_2SO_4	990.0	99.0 g l^{-1}		
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.25	25.0 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(d)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(e)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	α-Naphthaleneacetic acid (NAA)	0.05	5 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	6.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. This medium was originally formulated for the culture of a woody plant, mountain laurel, *Kalmia latifolia*.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe lists $MnSO_4 \cdot H_2O$ which seems to be a typographical error because the formulae and concentrations of all other microelements are the same as in the Murashige–Skoog (MS) medium (Murashige and Skoog, 1962).

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.2, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media. As a rule it is preferable to add heat-labile substances after autoclaving. However in this case the cytokinin and vitamins can be added before sterilization.

TABLE HAB-3. **Woody plant (WPM) medium (Lloyd and McCown, 1981) modified for in vitro flowering of *Habenaria crinifera* explants (Latha, 1999a)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400.0	40.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	96.0	9.6 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)$	556.0	55.6 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	Potassium sulfate, K_2SO_4	990.0	99.0 g l^{-1}		
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.25	25.0 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(d)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(e)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	α-Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
12	6-Benzyladenine (BA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	6.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. This medium was originally formulated for the culture of a woody plant, mountain laurel, *Kalmia latifolia*.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe lists $MnSO_4 \cdot H_2O$ which seems to be a typographical error because the formulae and concentrations of all other microelements are the same as in the Murashige–Skoog (MS) medium (Murashige and Skoog, 1962).

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.2, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media. As a rule it is preferable to add heat-labile substances after autoclaving. However in this case the cytokinin and vitamins can be added before sterilization.

Procedure. Shoots, 5 cm tall, are removed from tubers, defoliated, surface-sterilized, sectioned, and shoot tips are placed in culture on BA containing medium (Table Hab-1). When shoots form they must be moved to a NAA containing substrate (Table Hab-2) to induce rooting. If the intent is to induce flowering in vitro explants should be cultured on a medium which contains both BA and NAA (Table Hab-3).

Developmental Sequence. Explants cultured on BA containing medium (Table Hab-1) produce vegetative buds which give rise to shoots (Fig. Hab-1). These shoots form roots following transfer to a medium which contains NAA (Table Hab-2). The explants flower in vitro when cultured on a substrate which includes both NAA and BA (Table Hab-3).

General Comments. This procedure is unique for two reasons. One is the source of explants. The second is the clever use of the same type of explant for both propagation and flowering in vitro.

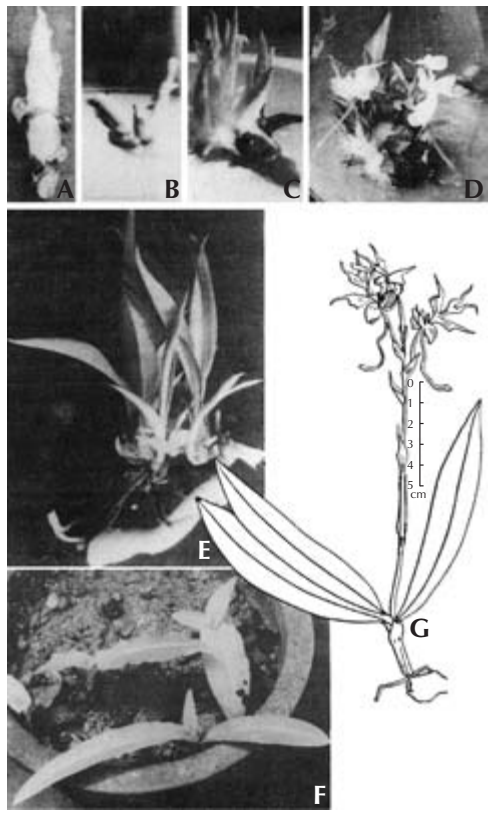


FIG. HAB-1. **Tissue culture of *Habenaria crinifera.* A. Shoot-tip explant. B. Young shoots. C. Shoots. D. Flowers in vitro. E. Rooted plantlet. F. Plants in pot. G. Plant.**

Hetaeria

Plantlets can be produced from stem (creeping rhizome) sections by the method developed for *Bletilla striata* (Yam, 1989).

Holttumara

Richard Eric Holttum (1895–1990) was director (one could say "the legendary director") of the Singapore Botanic Gardens from 1925 to 1949, where among his other achievements he produced the first artificial (i.e., human-made) orchid hybrid in that former British colony, *Spathoglottis* Primrose. After retiring he remained active at Kew Gardens until his death at the age of 95. He was interned during the Japanese occupation but used the time and benevolent attitude of General Kwan Koriba (a botanist, former university dean, and after the war a university president; for a biography see Arditti, 1989), the Japanese wartime director of the Gardens, to write a book on the orchids of Malaya – easily one of the very best orchid books in existence. After the war Holttum became the first professor of botany at the University of Singapore, where one of his successors was the eminent A. N. Rao. The trigeneric hybrid *Holttumara* (*Arachnis* × *Renanthera* × *Vanda*) was named in his honor. The first cross, *Holttumara* Cochineal [*Aranda* Hilda Galistan (*Arachnis hookeriana* × *Vanda tricolor*) × *Renanthera storiei*], was raised at the Singapore Botanic Gardens (Anonymous, 1958). Since then several additional *Holttumara* crosses have been produced with a number of *Aranda*, *Renantanda*, *Aranthera*, *Vanda*, and *Arachnis* parents.

A method for the clonal propagation of *Holttumara* Cochineal from apical and axillary buds was developed at the Singapore Botanic Gardens (Lim-Ho, 1981). The procedure is the same as the method used for apical and axillary buds of *Aeridachnis*, *Arachnis*, *Arachnostylis*, *Aranda* Hilda Galistan, and *Aranthera* (Lim-Ho, 1981). This procedure should be suitable for other *Holttumara* crosses and clones.

Ipsea

A small terrestrial genus of approximately three species, *Ipsea* is related to *Calanthe* and *Phaius* (Bechtel et al., 1992). It is found in India and Sri Lanka. As is so often the case with orchids, at one time or another taxonomists have assigned members of this genus to *Ipsea*, *Pachystoma*, *Spathoglottis*, and *Tainia* (Pradhan, 1976–1979; Bechtel et al., 1992).

Micropropagation of the Malabar Daffodil Orchid, *Ipsea malabarica*

The Malabar daffodil orchid, *Ipsea malabarica*, is a horticulturally desirable terrestrial species found at 1000-m elevation in the Western Ghats, Southern India. It is a protected species and is not being overcollected, but its populations are too small for survival. A micropropagation method was developed "to strengthen the resource base through reintroduction and restoration" (Gangaprasad et al., 1999).

Plant Material. Seeds from immature capsules, 40–45 days after self-pollination, were germinated on liquid modified Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) containing 2% sucrose and 0.05% (w/v) casein acid hydrolysate (CH), peptone, or yeast extract (YE) and coconut water (CW; all available from www.alphabiosciences.com or www.plantmedia.com). Protocorms which formed on this medium were subcultured on a solid modification of the MPR medium containing 0.6% agar, 0.05% CH, 0.05% peptone, 0.05% YE, 20% CW, 0.05% activated charcoal (AC), and 3–7% banana pulp (BP). The protocorms produced seedlings with one or two small leaves. After two subcultures at 30-day intervals (i.e., a total of 60 days) the seedlings had two to four leaves and stem tubers 0.5–1.0 cm in diameter. For tissue culture (i.e., micropropagation) stem tubers of 60-day-old seedlings were dissected and cultured.

Surface Sterilization. Explants taken from seedlings growing in vitro do not require surface sterilization. However, they must be washed with sterile distilled water to remove medium residues.

Culture Vessels. Test tubes were used in the original research (Fig. Ipsea-1). Other containers can also be used.

Culture Conditions. The original cultures were maintained at 25 ± 2°C under 12-h photoperiods provided by cool white fluorescent tubes (no information was provided about the number and wattage of the tubes or light intensity). Standard culture room conditions are also suitable.

Culture Media. Explants should be cultured first in MPR medium containing 0.5 mg BA l^{-1} (Table Ipsea-1) to bring about shoot formation. After 90 days (and four transfers to fresh medium, each following 25 days of culture) shoots with two to four leaves should be moved to MPR medium supplemented with 1 mg IAA l^{-1} (Table Ipsea-2) for rooting.

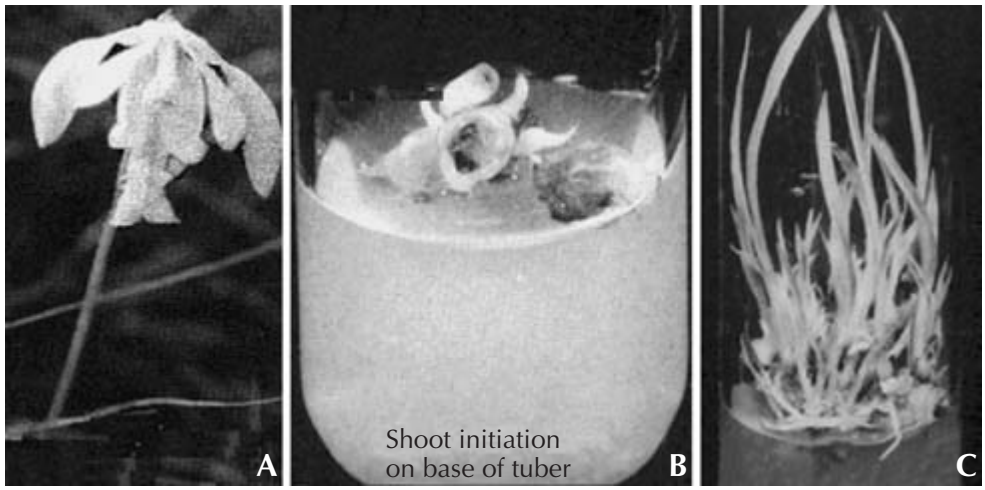


FIG. IPSEA-1. ***Ipsea malabarica*. A. Flower in tissue culture. B. Shoot initiation on base of tuber. C. Multiple shoots. (Gangaprasad et al., 1999.)**

Procedure. Explants are placed on the first medium (Table Ipsea-1). After 20–30 days the medium will turn brown due to exudations. Therefore the explants and the shoots which develop from them must be transferred to fresh medium every 30 days. Following 90 days of culture shoots should be transferred to the second medium (Table Ipsea-2) to induce root formation. If the medium turns brown the shoots/plantlets must be transferred to fresh medium at 20–30-day intervals.

Developmental Sequence. Shoots develop on the first medium (Table Ipsea-1) and roots form on the second substrate (Table Ipsea-2).

General Comments. Both the seed germination and micropropagation parts of this procedure are simple, efficient, and effective. The micropropagation protocol can be used to increase the number of plants which are obtained from one batch of seeds. However, since the explants are taken from seedlings the method cannot be used to propagate outstanding forms unless it proves to be suitable for explants from mature plants. This method could possibly also be suitable for *Ipsea speciosa*. Only experiments can determine if this method, or at least the media, can also be used with explants from mature plants.

TABLE IPSEA-1. **Mitra, Prassad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Ipsea malabarica* stem tubers (Gangaprasad et al., 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4{\cdot}H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2{\cdot}6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2{\cdot}4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	0.05	5.0 mg l^{-1}		
	Cytokinin				
8	Benzylaminopurine (BAP)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Vitamins				
9	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
14	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Agar[f]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses. If the cytokinin does not dissolve add a few drops of 0.1 N HCl.

[f]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976; Gangaprasad et al., 1999).

TABLE IPSEA-2. **Mitra, Prassad, and Roychowdhury medium (Mitra et al., 1976) modified for rooting of *Ipsea malabarica* stem shoots (Gangaprasad et al., 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Auxin				
8	Indoleacetic acid	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Vitamins				
9	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
14	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Agar[f]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses. If the auxin does not dissolve add a few drops of 0.1 N KOH.

[f]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and bring volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976; Gangaprasad et al., 1999).

Kagawara

The first *Kagawara* (*Ascocentrum* × *Renanthera* × *Vanda*) hybrid, *Kagawara* Firebird [*Renanthera storiei* × *Ascocenda* Red Gem (*Vanda merrillii* × *Ascocentrum curvifolium*)], was registered in 1968 by R. K. Mizuta in Hawaii. Other parents have been used since then to produce additional *Kagawara* crosses.

A method for the clonal propagation of *Kagawara* Teoline Fair [*Ascocenda* Meda Arnold (*Ascocentrum curvifolium* × *Vanda* Rothschildiana) × *Renanthera coccinea*] from apical and axillary buds developed at the Singapore Botanic Gardens is the same as the procedures used for similar explants of *Aeridachnis*, *Arachnis*, *Arachnostylis*, *Aranda* Hilda Galistan, and *Aranthera* (Lim-Ho, 1981). This procedure should be suitable for other *Kagawara* crosses and clones.

Laelia

The procedures used for *Brassocattleya* (Kako, 1973) can also be employed with *Laelia*. Methods used for *Brassolaelia*, *Cattleya*, *Laeliocattleya*, and related species and hybrids should also be suitable.

Laeliocattleya

One of the earliest intergeneric orchid hybrids, *Laeliocattleya* is very popular among growers. Its name was first published in the *Botanial Journal of the Linnean Society* 24:168 (1887). The first taxon in the genus is *Laeliocattleya elegans* which is considered to be a natural hybrid, *Cattleya leopoldii* × *Laelia purpurata* (or *Laeliocattleya* Schilleriana if the hybrid is considered to be horticultural). Few methods were developed specifically for *Laeliocattleya* because procedures used for *Cattleya* are also suitable for this hybrid genus (for one example see Ishii, 1974).

Laeliocattleya Propagation by Tissue Culture

The method used for *Cattleya* is also suitable for *Laeliocattleya* (Huang, 1984).

Clonal Propagation through the Culture of *Laeliocattleya* Leaf Tips

The method developed for *Cattleya* can also be used for *Laeliocattleya* (Arditti et al., 1971; Ball et al., 1971; Churchill et al., 1971*a*, 1971*c*, 1972*a*, 1973). As with *Cattleya* it is important to keep in mind that as the leaf ages, cells and tissues at its tips may lose their regenerative capability. Therefore explants must be taken before the characteristic notch develops at the leaf tip (Fig. Lc-1).

Plant Production from Young Leaves of *Laeliocattleya*

Rather than writing something new or paraphrasing (with attribution, of course) a published statement, it is sometimes better to present a direct quote. The introduction

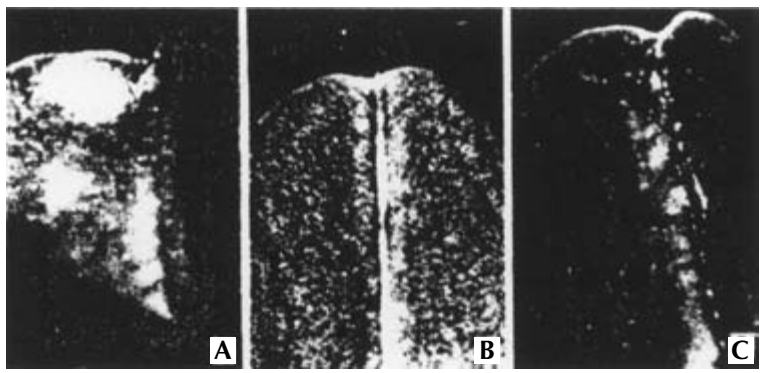


FIG. LC-1. **Notch formations on *Laeliocattleya* leaves. A. Young leaf from a mature plant. The tip is still pointed and, if cultured, is capable of callus and/or PLB formation (× 4). B. A notch starting to form (× 4). C. The notch is fully formed, and excised tips cannot be cultured (× 4.3).**

of the paper which describes this procedure states that "the propagation of orchids through tissue culture has been commercially successful due to the production of quality plants in large quantities by clonal multiplication. These facts have motivated us to study the clonal propagation of different species of orchids, testing several types of explants: apical meristems and lateral buds from *Cymbidium* and Venezuelan species of *Cattleya*; microcuttings from *Dendrobium*; complete and sectioned leaves from *Laeliocattleya*. Researchers in orchid tissue culture have been looking for an explant other than the meristem, to minimize the disturbance caused to the plant during the isolation of the explant. Leaves have been pointed out as a suitable tissue . . ." (Matos and De Garcia, 1991). The use of leaves as explants from axenic seedlings is an excellent idea. However if the purpose of micropropagation is "the production of quality plants in large quantities" it is hard to see how this can be achieved if explants are taken from seedlings because the quality of unflowered plants is not known. Use of explants is a good starting point for the development of a new protocol since it eliminates the need for surface sterilization (a process which can damage the tissues and bring about failure. But, to be useful as a means of "production of quality plants in large quantities" a procedure must be applicable to explants taken from plants of known quality.

Plant Material. In the original research "leaves 1–1.5 cm long . . . bearing their basal buds were" taken from "*Laeliocattleya* John Cunningham plantlets grown for one or two years from seed germinated in solid Knudson C medium." Since there is no mention of surface sterilization it is reasonable to assume that the "plantlets" were seedlings still growing on Knudson C medium in vitro 1–2 years after the seeds were germinated. The paper does not make clear whether the seeds were produced by *Laeliocattleya* John Cunningham through selfing or crossing with another plant of the same cultivar or by repeating the cross that gave rise to this hybrid (*Laeliocattleya* Schilleriana × *Laeliocattleya* Elegans). If it is the former, the seedlings were not *Laeliocattleya* John Cunningham as such but the F_2 generation of *Laeliocattleya* Schilleriana × *Laeliocattleya* Elegans, the cross which produced *Laeliocattleya* John Cunningham.

Surface Sterilization. There is no mention of surface sterilization in the original paper. This suggests that the explants were taken from seedlings growing in vitro. Explants from seedlings growing in vitro do not require sterilization. However they should be washed with sterile distilled water to remove medium residues.

Culture Vessels. Tubes containing 10 ml of medium were used in the original research. Other containers filled with medium to 20–30% of their volume are also suitable.

Culture Conditions. Cultures should be maintained at 27°C under continuous illumination of 7000 lx (the source of illumination was not listed) or standard culture room conditions.

Culture Media. Explants were cultured on half-strength MS medium (Murashige and Skoog, 1962) supplemented with citric acid (this organic acid is added to media to solubilize ferrous or ferric salts; the rationale of adding it to a medium which

contains chelated iron is not obvious), ascorbic acid (this is the heat labile vitamin C; how much of it will remain in a medium after autoclaving is not certain), auxin, and cytokinin (Table Lc-1). It is hard to determine which and how much hormone(s) was/were added. The "Composition" section in "Materials and methods" states that the medium contains 1 mg 2,4-D l^{-1}, but table II in the paper shows that the best results were obtained on media which did not contain this auxin. Also, the abstract states that the best yield of shoots was on BA containing medium and makes no reference to 2,4-D. In relation to BA the abstract asserts that "with 0.5–1 mg/l BA the best yield of 67 shoots per explant, was achieved after 23 weeks," but table II in the paper shows that after 23 weeks the number of shoots (presumably per explant) was 30 on 0.5 mg BA l^{-1}, 63 on 1 mg BA l^{-1}, and 67 on 2 mg BA l^{-1}. Shoot vigor was highest on the 2 mg BA l^{-1} level. The amount recommended here (Table Lc-1) is 2 mg BA l^{-1}, but those using this procedure may wish to experiment with 0.5 and 1 mg BA l^{-1}. Shoots obtained on the first medium "were transferred to [an undescribed] suitable medium to induce roots." The recommendation here is to use half-strength MS with 1 mg NAA l^{-1} (Table Lc-1). If roots are not produced on this medium the NAA concentration can be doubled, tripled, or even quintupled. In the event 1 mg NAA l^{-1} has deleterious effects the concentration can be cut in half or reduced to 0.25 mg l^{-1}.

Procedure. "One leaf was placed in each tube with the lower side in contact with the medium." This "lower side" can be the underside or the cut surfaces. Also, since "leaves bearing their basal buds" were cultured it stands to reason that the bases of the buds were on the medium. It seems that shoots formed on the first medium (Table Lc-1) were transferred to the second "suitable" medium (Table Lc-2, which is suggested here on the assumption that it may work and not because it may have been used originally).

Developmental Sequence. Shoots form on the first medium (Table Lc-1). They develop roots on the second substrate (Table Lc-2).

General Comments. As published this procedure was only used to culture leaves taken from seedlings. There is no evidence that it may be suitable for leaves from mature plants. If it is only suitable for seedling leaves this method is of limited practical value. The title of the paper suggests that plants are produced from leaves, but two statements in the paper cast doubts on whether this is the case. In "Materials and methods" the authors state that "leaves bearing their basal buds were isolated" and in "Results and discussion" they assert that "the complete leaf bearing its basal bud was required to establish a competent culture." Since buds were present it is possible that they, not the leaves, produced shoots. If this is the case this method could perhaps be used to culture axillary buds of mature plants. The paper which describes this method is: (1) unclear; (2) not detailed enough; (3) not well written; and (4) weak in the interpretation and discussion of the results it presents.

TABLE LC-1. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of young leaves of *Laeliocattleya* (Matos and de García, 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Organic acid				
10	Citric acid[g]	100.0	No stock	No stock	Weigh
	Cytokinin				
11	Benzylaminopurine (BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Ascorbic acid (vitamin C)[h]	150.0	Suspend in 5 ml 70% ethanol	No stock	Weigh
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
16	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[i]	To 1000 ml			
	Solidifier				
18	Gelrite[i,j]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]The reason for the addition of citric acid is not clear. In some cases it is added to solubilize iron, but a chelating agent is used in this medium. There is no evidence that *Laeliocattleya* explants or those of any other orchid require citric acid.

[h]Ascorbic acid (vitamin C) is usually not added to culture media. The reasons for its addition to this medium are not clear.

[i]Add items 1–7, 9, and 10 to 900 ml of distilled water (item 17), adjust pH to 5.3, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous sitting to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. When the gellan gum is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 11), and vitamins (items 12–15) to the warm solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels.

[j]Gellan gum (available as such from www.caissonlabs.com, and as Phytagel www.sigmaaldrich.com or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels.

TABLE LC-2. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the rooting of *Laeliocattleya* shoots (Matos and de García, 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Organic acid				
10	Citric acid[g]	100.0	No stock	No stock	Weigh
	Auxin				
11	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Ascorbic acid (vitamin C)[h]	150.0	Suspend in 5 ml 70% ethanol	No stock	Weigh
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
16	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[i]	To 1000 ml			
	Solidifier				
18	Gelrite[i,j]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]The reason for the addition of citric acid is not clear. In some cases it is added to solubilize iron, but a chelating agent is used in this medium. There is no evidence that *Laeliocattleya* explants or those of any other orchid require citric acid.

[h]Ascorbic acid (vitamin C) is usually not added to culture media. The reasons for its addition to this medium are not clear.

[i]Add items 1–7, 9 and 10 to 900 ml of distilled water (item 17), adjust pH to 5.3, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous sitting to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. When the gellan gum is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 11), and vitamins (items 12–15) to the warm solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels.

[j]Gellan gum (available as such from www.caissonlabs.com and as Phytagel, www.sigmaaldrich.com) or Gelrite is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels.

Liparis

Plantlets can be produced from stem sections of *Liparis nervosa* by the method developed for *Bletilla striata* (Yam, 1989). Leaf tips of *Liparis viridiflora* can be cultured by the method used for *Acampe rigida* (Yam, 1989).

Tobacco and Old DNA: The Discovery of Cytokinins

Enhancement of bud formation in tobacco callus by KH_2PO_4 led Professor Folke Skoog at the Botany Department, University of Wisconsin to an exploration of the effects of adenosine and adenine. On finding that adenine can stimulate production of buds, Skoog concluded that nucleic acids were "critically involved in plant development" (Professor Carlos Miller in an e-mail message dated April 30, 1995). By 1951 when Professor Miller joined Professor Skoog's laboratory "many chemicals . . . part of the nucleic acid area" were being tested. One recollection of events is that a new technician prepared DNA containing medium and autoclaved it. He did not know that DNA must not be sterilized by autoclaving. Tissues grew well on the autoclaved medium but failed to do so on a medium which contained filter-sterilized DNA. The tissues grew well again when DNA containing medium was autoclaved. Another recollection is that the technician prepared the medium that supported growth using DNA from an old and dusty bottle of herring sperm DNA. When all of the old herring sperm DNA was used up a fresh supply was purchased and found to be inactive. The fresh herring sperm DNA became active on standing or after being autoclaved in a weakly acid solution. A crystalline active compound was isolated from the autoclaved solution and named kinetin. Its structure was established as 6-furfurylaminopurine by Professors Miller and Skoog with the help of Professor Frank Strong and his associates in the Department of Biochemistry. Kinetin was synthesized in 1955.

Professors Robert Cleland, Carl Leopold, Carlos Miller, and Machteld Mok provided information for this box.

Ludisia (*Haemaria*)

Most orchids are grown for their flowers. Only a few are cultivated for their beautiful leaves. *Ludisia* (*Haemaria*) *discolor* is such an orchid. It is found in southern China, Burma, Indonesia, and the Malay archipelago. This species was abundant in Malaysia at one time, but its population has been reduced drastically. A tissue culture procedure was formulated at the University Sains in Penang, Malaysia, "with a view to conservation of the species" (Teo, 1978).

Plant Material. Single-node sections are taken from succulent shoots 3–4 cm long.

Surface Sterilization. Shoots are washed with detergent and water before being immersed in 10% Clorox solution (10 ml Clorox diluted to 100 ml with distilled water). A time period for this immersion is not given in the original paper, but 8–15 min should be appropriate. Following this sterilization the leaves (especially the petioles) are removed from around each node and the shoots are again immersed in 10% Clorox, this time for 5–6 min. On being removed from the Clorox solution the shoots are washed with sterile distilled water two to three times and cut into sections.

Culture Vessels. Erlenmeyer flasks were used in the original research, but other containers can also be employed.

Culture Conditions. Cultures should be maintained under continuous illumination (intensity and sources were not given, nor was temperature) and, if liquid, agitation "as is done with other orchids (Kunisaki et al., 1972; Intuwong and Sagawa, 1973; Teo et al., 1973)."

Culture Media. Liquid Knudson C medium (see Tables Aranda-7 and Cym-3 with the agar omitted) containing "either auxin or cytokinin or their combinations at varying concentrations did not" induce proliferation. Stem sections placed on solid Knudson C medium (see Tables Aranda-7 and Cym-3) produced shoots.

Procedure. Stem sections placed in liquid medium do not proliferate. After the sections are moved to solid medium they turn yellowish-green, swell, and produce leaves and shoots. Excision of the shoot tip induces branching. When the plantlets are large enough, they can be moved to community pots. It is not clear from the original paper (Teo, 1978) whether a period in liquid medium is required before the sections can grow on solid Knudson C.

Developmental Sequence. Sections in liquid medium do not proliferate. Shoots are produced only on solid medium.

General Comments. This appears to be a slow procedure since each node seems to produce only a single plant.

Luisia

Named after the explorer Don Luis de Torres by Charles Gaudichaud in 1826, the genus *Luisia* consists of approximately 40 species which are found in tropical Asia, Australia, Japan, Malaysia, Papua New Guinea, and Polynesia. The type species of the genus is *Luisia teretifolia* which was found by Gaudichaud in Guam. This species also occurs in India, Indonesia, New Caledonia, South East Asia, and Sri Lanka. Both *Luisia teretifolia* and *Luisia trichorhiza* have terete leaves.

Micropropagation of *Luisia teretifolia* through Leaf Segments

According to the noted expert on Indonesian orchids, James B. Comber, "differences between [*Luisia javanica*] and *L. teretifolia* . . . are very small if indeed they do exist." He was unable to see any difference between them. In India the leaves of *Luisia teretifolia* taper toward the apex and are 15 cm long. Methods for the culture of apical and basal explants from leaves of *L. teretifolia* leaves were developed at the Botany Department, Panjab University (Vij and Pathak, 1990).

Plant Material. Apical and basal explants, 5–10 mm long, were taken from young leaves, up to 2 cm in length, of mature plants and 16- to 40-week-old axenic seedlings.

Surface Sterilization. Explants taken from axenic seedlings do not require sterilization. However they should be washed with sterile distilled water to remove medium residues. No surface sterilization procedure is described for explants taken from mature plants. The method used to surface-sterilize *Cattleya* leaf tips (Arditti et al., 1971; Ball et al., 1971; Churchill et al., 1971*a*, 1971*c*, 1972*a*, 1973) will probably be suitable or can serve as a starting point for the development of a suitable procedure (see *Cattleya* section for details).

Culture Vessels. Test tubes, Erlenmeyer flasks, or other containers can be used.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx or at standard laboratory conditions.

Culture Media. Basal explants cultured on MS medium (Murashige and Skoog, 1962) containing 1 mg kinetin l^{-1} (Table Luisia-1) produce callus and PLBs. Apical explants produce PLBs when cultured on MS containing 1 mg BA l^{-1} and 1 mg NAA l^{-1} (Table Luisia-2). The PLBs can be subcultured on Knudson C medium (Knudson, 1946) supplemented with banana homogenate (BH; see Table Cym-24) for plantlet production.

Procedure. Explants should be placed on the appropriate medium and cultured until PLBs are formed. The PLBs can be proliferated on the initial medium or moved to Knudson C with BH for plantlet production.

TABLE LUISIA-1. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of basal explants from leaves of *Luisia teretifolia* (Vij and Pathak, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Kinetin	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	9.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.5, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media. Amino acids (item 8), hormone (item 10), and vitamins (item 11–14) are heat labile and are often added after autoclaving to the still hot and liquid solution under sterile conditions with sterilized pipettes, mixed well and the medium is distributed to preautoclaved culture vessels. In this case all components are added before sterilization.

TABLE LUISIA-2. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of apical explants from leaves of *Luisia teretifolia* (Vij and Pathak, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	9.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) are heat labile and are often added after autoclaving to the still hot and liquid solution under sterile conditions with sterilized pipettes, mixed well, and the medium is distributed to preautoclaved culture vessels. In this case all components are added before sterilization. Agar is not added to liquid media.

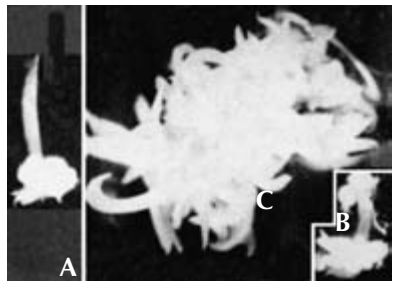


FIG. LUISIA-1. **Micropropagation of *Luisia teretifolia*. A. Protocorm-like body with leaf. B. Callus formation. C. Multiple shoots. (Vij and Pathak, 1990.)**

Developmental Sequence. Calli and/or PLBs form on the initial medium. PLBs proliferate on the same substrate and form plantlets on KC medium + BH (Fig. Luisia-1).

General Comments. This procedure is ideal because both tips and bases of leaves serve as explants which can be cultured. Also, removal of leaves does not damage the plants from which they are taken. If a plant does not have enough leaves only tips can be excised and cultured.

Leaf-segment Culture of *Luisia trichorhiza* in Vitro

Leaf segments of *Luisia trichorhiza* obtained from 16-week-old axenically grown seedlings "responded favorably" when cultured on MS medium and "its various modifications" with 1 mg l^{-1} each of auxins/cytokinins/gibberellin." PLBs were initiated from epidermal cells "at the basal, apical and/or cut ends of the explants depending on media composition."

Rapid multiplication of PLBs occurred on media containing IAA regardless of whether BA or kinetin were present. Charcoal (0.2%) in the medium enhanced further PLB formation and differentiation. Plantlets were regenerated within 18 weeks on MS medium containing IAA, BA, and charcoal. All plantlets contained $2n = 38$ chromosomes (Vij and Pathak, 1988*a*).

Micropropagation of *Luisia trichorhiza* through Leaf Segments

The procedure used for *Luisia teretifolia* can also be employed for *Luisia trichorhiza* except that both apical and basal explants should be cultured on MS containing 1 mg BA l^{-1} and 1 mg IAA l^{-1} (Fig. Luisia-2; Table Luisia-3). PLBs produced on this medium can be subcultured on it or moved to banana homogenate containing Knudson C medium (see Table Cym-24) for plantlet production (Vij and Pathak, 1990).

Protoplast Isolation from *Luisia zeylanica*

The method developed for *Acampe praemorsa* can be used to isolate 0.6×10^4 protoplasts from leaves of *Luisia zeylanica* (Seeni and Abraham, 1986).

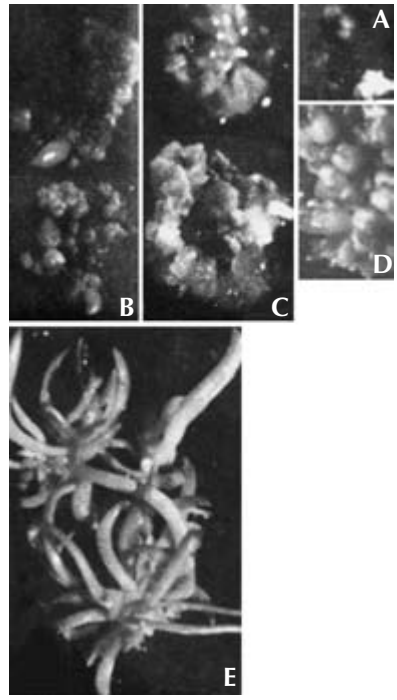


FIG. LUISIA-2. **Tissue culture propagation of *Luisia trichorhiza*. A. Callus formation by apical explant. B. PLBs on apical and basal explants. C. Callus formation by explants. D. Differentiating PLBs. E. Plantlets. (Vij and Pathak, 1990.)**

Luis de Torres (1562–1655), a converted Jew, was an interpreter for Columbus who sailed out on the Santa Maria and returned on the Niña. While exploring his new surroundings de Torres saw a large bird he called Tukki for peacock, thus naming the turkey.

TABLE LUISIA-3. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of apical and basal explants from leaves of *Luisia trichorhiza* (Vij and Pathak, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indoleacetic acid (IAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	9.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively.

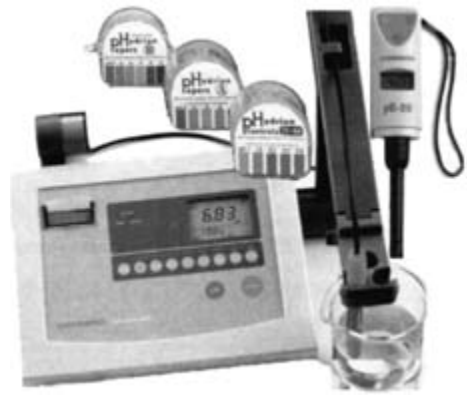
[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media. Amino acid (item 8), hormones (items 10, 11), and vitamins (items 12–14) are heat labile and are often added after autoclaving to the still hot and liquid solution under sterile conditions with sterilized pipettes, mixed well and the medium is distributed to preautoclaved culture vessels. In this case all components are added before sterilization.

Lycaste

The methods used for *Cymbidium* (Morel, 1960, 1963, 1964*a*, 1964*b*) are also suitable for *Lycaste*.

Measuring and Adjusting pH

A measure of the alkalinity or acidity of a medium, pH is an important factor. It must be measured and adjusted to within proper levels of tolerance. Desktop or hand held and portable meters or paper strips can be used to measure pH. If the pH is too high 0.1 N hydrochloric acid (HCl) should be used to reduce it. Low pH can be raised with 0.1 N potassium (KOH) or sodium (NaOH) hydroxide.



Malaxis

First described in 1788 by the Swedish botanist Olof Swartz (1760–1818), the genus *Malaxis* consists of approximately 300 species which can be found throughout most of the world in both tropical and temperate climates. Flowers which do not resupinate are an interesting characteristic of the genus.

Micropropagation of *Malaxis acuminata* through the Culture of Stem Sections

Plantlets can be produced from stem sections of *Malaxis acuminata* by the method developed for *Bletilla striata* (Yam, 1989).

Micropropagation of *Malaxis acuminata* through the Culture of Pseudobulb Segments

In India dried pseudobulbs of *Malaxis acuminata* are an important ingredient of the Ashtavarga drugs which are used in the preparation of an Ayurvedic tonic called Chyavanprash. The species is found in the Himalayas in tropical to subtropical climates and elevations ranging from 1800 to 2300 m. Collection is extensive and exceeds the natural increase in numbers. This jeopardizes species survival and has led to the development of a micropropagation method (Vij and Kaur, 1998).

Plant Material. Sections, 5–10 mm long, from young pseudobulbs shorter than 3 cm, should be cultured.

Surface Sterilization. Pseudobulbs should be washed thoroughly with running tap water and Teepol (www.sigmaaldrich.com) before sterilization with: (1) mercuric chloride (0.1% $HgCl_2$ for 7 min); (2) streptomycin (www.sigmaaldrich.com, www.fishersci.com, www.vwr.com) solution (0.1% for 10 min; 100 mg 100 ml^{-1} distilled water); and (3) 70% ethanol (73–74 ml 95% ethanol made up to 100 ml with distilled water for 10 s) followed by several rinses with sterile distilled water.

Culture Vessels. Test tubes, Erlenmeyer flasks, and other containers are suitable.

Culture Conditions. The cultures should be maintained at 25 ± 2°C and 12-h photoperiods of 3500 lx or standard culture room conditions.

Culture Media. Several media can be used depending on the desired end results. One possibility is to culture the explants on MS medium (Murashige and Skoog, 1962) containing 1 mg BA l^{-1} (Table Mlx-1). On this medium "a single meristematic locus differentiated into a shoot bud which developed into [one] plantlet". Another possibility is to culture the explants on MS medium containing 10 mg NAA l^{-1} (Table Mlx-2). On this medium the explants form multiple shoots and plantlets. If the intent

TABLE MLX-1. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for production of single plantlets from pseudobulb explants of *Malaxis acuminata* (Vij and Kaur, 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzyladenine (BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	9.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH to 5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. It is also possible to prepare this medium by adding items 1–13 prior to autoclaviing as described in Tables Luisia-1 to Luisia-3.

TABLE MLX-2. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for production of multiple plantlets from pseudobulb explants of *Malaxis acuminata* (Vij and Kaur, 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	9.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust to 5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormone (item 10), and vitamins (items 11–13) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. It is also possible to prepare this medium by adding items 1–13 prior to autoclaviing as described in Tables Luisia-1 to Luisia-3.

is to produce PLBs the explants should be cultured on solid MS supplemented with 10 mg BA l^{-1}, 0.1 mg NAA l^{-1}, and 1 g casein hydrolysate (CH) l^{-1} (Table Mlx-3, agar containing). These PLBs can be proliferated on liquid medium of the same composition (Table Mlx-3, agar free). For further growth and development plantlets and PLBs can be moved to Knudson C (KC) medium containing banana homogenate (BH; see Table Cym-24).

Procedure. Young pseudobulbs are washed, surface-sterilized, rinsed with sterile distilled water, and cut into sections. These sections are placed on one of the three initial media (Tables Mlx-1, Mlx-2, or Mlx-3 with agar). If PLBs are produced they can be proliferated in liquid medium (Table Mlx-3 without agar). Plantlets and PLBs should be moved to BH-containing KC (see Table Cym-24) for growth and development.

Developmental Sequence. Only single plantlets develop on BA-containing medium (Table Mlx-1). Multiple plantlets form on the NAA-containing substrate (Table Mlx-2). The explants produce PLBs on solid BA-, NAA-, and CH-containing medium (Table Mlx-3, with agar). These PLBs proliferate if cultured in liquid solution (Table Mlx-3, without agar).

General Comments. As a result of a well thought out experimental approach explants of *M. acuminata* can be cultured on three separate media each designed to produce a different result.

TABLE MLX-3. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the production or proliferation of protocorm-like bodies (PLBs) from pseudobulb explants of *Malaxis* a*cuminata* (Vij and Kaur, 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
15	Casein hydrolysate	1.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	9.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. Solid medium is for PLB production. Liquid medium is for proliferation of PLBs.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively.

[g]Add items 1–7, 9 and 15 to 900 ml of distilled water (item 17), adjust pH to 5.5, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10, 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. Solid (agar containing) medium is for PLB production. Liquid (agar-free) medium is for proliferation of PLBs. It is also possible to prepare this medium by adding items 1–13 prior to autoclaving as described in Tables Luisia-1 to Luisia-3.

Maxillaria

Protoplasts of *Maxillaria tenuifolia* can be released from tissue of 10-cm-long leaves by the procedure used for *Agraecum giryamae* and *Dendrobium* (Price and Earle, 1984; note that this source misspells the species *A. gyrimae*). Incubation time should be 2 days, during which time 0.3×10^5 protoplasts per gram of tissue are released.

Miltonia

The methods used for *Cymbidium* (Morel, 1960, 1963, 1964*a*, 1964*b*) and for *Epidendrum* and *Epiphronitis* (Kusumoto, 1981*b*) are also suitable for *Miltonia*.

Orchid Micropropagation: The First Literature Review

The first (Rotor, 1949), second, and third (Thomale, 1956, 1957) reports on orchid micropropagation generated only limited interest. The same was true for the fourth (Wimber, 1963). Despite being little more than a publicity announcement, the fifth paper (Morel, 1960) drew the most attention, generated interest in orchid tissue culture, and elevated its author to celebrity status. Many papers on the subject were published by the mid 1970s, but they were scattered. Professor Adisheshappa Nagaraja Rao, the long time chair of the Botany Department at the University of Singapore (later the National University of Singapore), wrote the first literature review: *Tissue Culture in the Orchid Industry* (Rao, 1977). This excellent contribution provided impetus for further research, became a classic, and played an important role in establishing orchid micropropagation as a science. Another major contribution to orchid science by Professor Rao was the department he headed for about 20 years. He built it into a powerhouse of research on orchids with prominent orchid scientists like Professors P. N. "Danny" Avadhani, C. S. Hew, Y. H. Lee, S. M. Wong, and others. I spent summers and sabbatical leaves in Singapore working with Rao, Danny, and Choy Sin Hew. All of us retired by 2001, but still remain in contact and maintain our friendship.

Mokara

This trigeneric hybrid (*Arachnis* × *Ascocentrum* × *Vanda*) was produced in Singapore by C. Y. Mok. The first hybrid *Mokara* Wai Liang (*Arachnis* Ishbel × *Ascocenda* Red Gem) was registered in 1969. One of the better-known hybrids that is used extensively in the cut-flower industry in Singapore and Indonesia is *Mokara* Mak Chin On (*Aranda* Christine × *Ascocenda* Blue Boy). It was registered in 1978. This hybrid and a number of others with considerable cut-flower industry potential generated a need for mass rapid propagation procedures.

Clonal Propagation of *Mokara* from Flower Buds

Clonal propagation of orchids through the culture of shoot-tip and axillary buds "requires the sacrifice of a new growth, or even a whole plant in the case of monopodial orchids. . . ." The use of floral buds for clonal propagation causes "no harm or setback to the parent plant"; therefore a method for the clonal propagation of *Mokara* through the culture of flower buds was developed at the Singapore Botanic Gardens (Lim-Ho et al., 1984).

Plant Material. Complete flower buds, 0.5–1.0 cm, are cultured.

Surface Sterilization. Buds are first washed with distilled water (detergent is not mentioned, but the use of a mild formulation is advisable) and immersed for 45 min in 20% Clorox (20 ml Clorox diluted to 100 ml with distilled water plus 1 drop Tween 80). Pedicels are removed after that, and the buds are placed in 5% Clorox (5 ml Clorox diluted to 100 ml with distilled water) for an additional 15 min. After that the buds are rinsed three times with sterile distilled water.

Culture Vessels. Erlenmeyer flasks were used in the original research, but other containers are also suitable.

Culture Conditions. Liquid cultures should be placed on a gyrorotatory shaker at 60 to 80 rpm for 8–12 h day^{-1}. Both liquid and solid cultures are maintained under 12-h photoperiods of 1000 lx provided by Tru-Lite tubes and 21–25°C. Sylvania Gro-Lux and other plant growth fluorescent lights, or a combination of cool white tubes and incandescent bulbs are probably also suitable.

Culture Media. Buds are first cultured on a modified liquid MS medium (Table Mkra-1) and transferred to a modification of the Knudson C solution (Table Mkra-2). Differentiation of PLBs occurs on modified Vacin and Went medium (Table Mkra-3).

Procedure. Buds are cultured in the first medium (Table Mkra-1). When PLBs form "at the base of the flower buds, the cultures are transferred to a basic Knudson C salt with hormone [Table Mkra-2], but . . . without sugar . . . for proliferation." To enhance differentiation the PLBs are moved to the modified Vacin and Went medium (Table Mkra-3). After 9–14 months the plantlets can be potted in the nursery.

TABLE MKRA-1. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for the culture of *Mokara* flower buds (Lim-Ho et al., 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Potassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
9	Indoleacetic acid (IAA)	2	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin and related substance				
10	Kinetin	2	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
11	Adenine sulfate	40	No stock	No stock	Weigh
	Vitamins				
12	Niacin (nicotinic acid)	5	500 mg 100 ml^{-1} 95% ethanol[f]	1	
13	Thiamine (vitamin B_1)	1	100 mg 100 ml^{-1} 95% ethanol[f]	1	
	Sugar				
14	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[g]	8 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference will probably have little effect.

[e]If auxin or cytokinins do not dissolve, use a few drops of KOH or HCl, respectively, to solubilize them.

[f]Keep refrigerated between uses.

[g]Add items 1–13 to 900 ml distilled water (item 15), adjust pH to 5.2, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense solution into culture vessels and autoclave for 20 min. Omit agar if preparing liquid medium. As a rule additives like hormones and vitamins should not be autoclaved, but in this case the medium was autoclaved during the original research, an indication that any changes that do occur are not deleterious.

TABLE MKRA-2. **Modified Knudson C medium (Knudson, 1946) for the culture of *Mokara* flower buds (Lim-Ho et al., 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	400	40 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	400 g	40 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	100	10 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	100	10 g l^{-1}	10	
5	**Chelated Iron**[c]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.84	2.78 g l^{-1}	10	One solution
(b)	Chelating agent, Na_2EDTA	37.24	3.72 g l^{-1}		
6	**Microelements**[d]				
(a)	Aluminum chloride	0.03	3 mg l^{-1}	10	One solution
(b)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(c)	Cupric chloride, $CuCl_2$	0.03	3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}		
(e)	Potassium iodide, KI	1.0	100 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4$	8.6	860 mg l^{-1}		
	Cytokinin				
7	Benzyl adenine (BA)	2	200 mg l^{-1} 95% ethanol[e]	1	
	Complex additive				
8	Coconut water	150 ml	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[f]	To 1000 ml			
	Solidifier				
10	Agar[f]	8 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original recipe does not include chelated iron because it was not available at the time.

[d]Add all microelements to the same 1 l of water, and heat and/or stir until they are dissolved. Water(s) of hydration, if any, are not listed for $ZnSO_4$.

[e]Keep refrigerated or frozen between uses.

[f]Add items 1–8 to 650 ml distilled water (item 9). Adjust pH to 5.2, and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense solution into culture vessels and autoclave for 20 min. As a rule additives like hormones and vitamins should not be autoclaved, but in this case the medium was autoclaved during the original research, an indication that any changes that do occur are not deleterious. Omit agar if preparing liquid medium. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary, adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower the pH if necessary.

Developmental Sequence. PLBs are first formed on the bases of the flower buds. They develop from torus tissues situated between the sepals and petals. These PLBs proliferate on the second medium. Growth is slow initially but accelerates after 6 months of subculturing. Plantlets averaging 5 cm in height are produced after 9–14 months on the second medium. Leaves generally start to appear after 6 months in culture. Roots form 3 months after that.

General Comments. Buds smaller than 0.5 cm and larger than 10 mm did not produce PLBs, while those measuring 0.5–1.0 cm did. This procedure appears to be

TABLE MKRA-3. **Modified Vacin–Went medium (Vacin and Went, 1949) for differentiation of plantlets obtained from *Mokara* flower buds (Lim-Ho et al., 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
6	**Chelated Iron**[d]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.84	2.78 g l^{-1}	10	One solution
(b)	Chelating agent, Na_2EDTA	37.24	3.72 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additives				
8	Coconut water	150	No stock	No stock	Measure
9	Banana homogenate[e]	75	No stock	No stock	Prepare
	Sugar				
10	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Agar[f]	8 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]The original recipe employs 28 mg l^{-1} ferric citrate, $FeC_6H_5O_7 \cdot 5H_2O$, which is difficult to dissolve.

[e]Place 75 g of ripe but still firm banana in a homogenizer, add 150 ml water, and homogenize for 2 min, let stand for 50 min, and homogenize for another minute. Pour the homogenate into the calcium phosphate solution, wash the homogenizer with two separate portions of 50 ml of water, and add that to the mixture.

[f]Add items 1–9 to the 500 ml distilled water (item 11) that contains item 2, adjust pH to 5.2, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring solution to a gentle boil, and add agar (item 12) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar for liquid medium.

ideal because: (1) the parent plant is not damaged by the removal of the explants; (2) flower buds are usually available in large numbers; and (3) it does not require elaborate excision procedures.

Micropropagation of *Mokara* through the Culture of Inflorescence Tips

Abdul Karim B. Abdul Ghani earned his Ph.D. in the laboratory of Folke Skoog, coformulator of the MS medium. Therefore it is not surprising that on returning to Malaysia to accept a position in the Botany Department at the University Kebangsaan, Malaysia, Abdul Ghani initiated an orchid tissue culture program. His

group is currently working on several orchids of interest to Malaysian horticulture and conservation including *Mokara* and *Renantanda* (Abdul Ghani, pers. comm.).*

Plant Material. In the original research, tips 2 mm in length were taken from inflorescences of *Mokara* Chark Kuan. Similar explants from other *Mokara* clones can also be cultured. The apex, which is pin sized, is exposed through the removal of bracts following surface sterilization.

Surface Sterilization. Inflorescences should be washed with water and a mild detergent (Teepol from Shell Chemical, Kuala Lumpur, Malaysia, was used in the original research, but other mild detergents are also suitable) and then rinsed with running water for 2 h. After that and before the uppermost node is excised, remove any bracts that cover the apex. The node should be submerged in 10% household (e.g., Clorox) bleach (10 ml bleach diluted to 100 ml with distilled water) for 15 min. An additional layer of bracts is removed after that, and the tips are immersed in 1% household bleach (1 ml liquid bleach diluted to 100 ml with water) for 10 min. This must be followed by a 5-min rinse with sterile distilled water before all remaining bracts are removed to expose the apex.

Culture Vessels. Erlenmeyer flasks, of 100-ml capacity and containing 50 ml medium, were used in the original research.

Culture Conditions. Cultures were maintained on a gyrorotatory shaker at 100 rpm under 8-h photoperiods of 2000 lx (provided by NEC daylight fluorescent tubes FL 20) at 25 ± 2°C.

Culture Media. PLBs are initiated on liquid modified Vacin and Went medium (Table Mkra-4). The resulting PLBs proliferate on sugar-free Vacin and Went medium (Table Mkra-4), while plantlets form on solid Vacin and Went medium with sugar (Table Mkra-4).

Procedure. Place the shoot tips in the sugar containing medium (Table Mkra-4) to form PLBs. Then move these PLBs to the same medium without sugar (Table Mkra-4) for proliferation. Finally, transfer the new PLBs to solid medium (Table Mkra-4) for plantlet induction.

Developmental Sequence. PLBs form on the explants in liquid modified Vacin and Went medium. These PLBs proliferate on sugar-free liquid Vacin and Went and form plantlets on solid Vacin and Went media.

General Comments. This is an efficient method for mass rapid clonal propagation of *Mokara* that does not endanger the donor plant.

* We thank Dr. Abdul Karim B. Abdul Ghani for allowing us to use unpublished information.

TABLE MKRA-4. **Vacin–Went medium for the germination of orchid seeds (Vacin and Went, 1949) as modified for the initiation and proliferation of protocorm-like bodies, and plantlet induction of *Mokara* (Dr. Abd. Karim Abd. Gahani, University Kebangsaan Malaysia, personal communication)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric citrate, $FeC_6H_5O_7 \cdot 5H_2O$	28	No stock		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water	200 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose[e]	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g of chelating agent (Na_2EDTA) and 2.78 g of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Omit sugar from the PLB proliferation medium.

[f]Add items 1 and 3–8 to the 500 ml of distilled water (item 10) that contains item 2, adjust pH to 5.2. Add sugar (item 9), but not to the PLB proliferation medium. Adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar if preparing liquid medium.

Clonal Propagation of *Mokara* through the Culture of Young Leaves

When only a few plants of a clone are available, the use of axillary or flower buds as well as inflorescence tips can be restricted by the unavailability of explants. To overcome this problem a method utilizing young leaves was developed at the Botany Department, University Kebagsaan, Malaysia (Abdul Ghani and Haris, 1992).*

Plant Material. In the original research whole leaves, 0.5–1.5 cm long and 0.3–0.5 cm wide, were excised from 3-month-old plantlets of *Mokara* Chark Kuan that were produced from inflorescence tips and maintained in vitro.

* Dr. Abdul Karim B. Abdul Ghani is now retired.

Surface Sterilization. Since the explants are taken from aseptic cultures, surface sterilization is not needed.

Culture Vessels. Erlenmeyer flasks, of 100-ml capacity and containing 50 ml medium, were used in the original research. Other containers can also be used.

Culture Conditions. Cultures were maintained on a gyrorotatory shaker at 100 rpm under 8-h photoperiods of 2000 lx (provided by NEC daylight fluorescent tubes FL 20) at 25 ± 2°C.

Culture Media. Liquid Vacin and Went medium containing 10% (v/v) water from green coconuts and 1.0 mg l^{-1} kinetin (Table Mkra-5) is suitable for the induction

TABLE MKRA-5. **Vacin–Went medium for the germination of orchid seeds (Vacin and Went, 1949) as modified for the clonal propagation of *Mokara* from young leaves (Dr. Abd. Karim Abd. Gahani, University Kebangsaan Malaysia, personal communication)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric citrate, $FeC_6H_5O_7 \cdot 5H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Cytokinin				
8	Kinetin	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
9	Coconut water	100 ml	No stock	No stock	Measure
	Sugar				
10	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Agar[f]	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g of chelating agent (Na_2EDTA) and 2.78 g of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]If the cytokinin fails to dissolve, add a small amount of dilute HCl. The stock solution must be refrigerated or frozen.

[f]Add items 1, 3–7, and 9 to the 500 ml of distilled water (item 11) that contains item 2. Adjust pH to 5.2, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring solution to a gentle boil, and add agar (item 12) slowly while stirring until it is dissolved. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour the medium into a 2-l Erlenmeyer flask and autoclave. Add cytokinin (item 8) to the hot, still liquid solution, mix well, and distribute medium into preautoclaved culture vessels. Omit agar if preparing liquid medium.

of PLBs and their proliferation. PLBs cultured on solid modified Vacin and Went medium (Table Mkra-5) formed shoots.

Procedure. Place explants on the liquid modified Vacin and Went medium; when PLBs develop, move them to fresh medium. When enough PLBs become available, move some to solid medium for shoot formation.

Developmental Sequence. Explants swell after 1 week of culture. PLBs can develop on the abaxial and adaxial as well as the proximal and distal sides of explants after approximately 4 weeks of culture. Proliferation is rapid in the presence of 0.5–2 mg l^{-1} kinetin. To reduce the incidence of hormone induced mutation, it is best to use 0.5 mg l^{-1} kinetin. PLBs are produced in clumps. When cultured separately each PLB produces a new clump. Shoots differentiate after 2 months of culture.

General Comments. This procedure can be used to accelerate the clonal propagation of *Mokara* in cases where explant sources are limited.

Mass Rapid Clonal Propagation of *Mokara* through the Culture of Axillary Buds

A method for the culture of axillary buds of *Mokara* Sally Lim (*Arachnis hookeriana* × *Ascocenda* Meda Arnold) developed at the Singapore Botanic Gardens is the same as the procedure used for *Holttumara* axillary buds of *Aeridachnis*, *Arachnis*, *Arachnostylis*, *Aranda* Hilda Galistan, and *Aranthera* (Lim-Ho, 1981). This procedure should be suitable for other *Mokara* crosses and clones.

Clonal Propagation of *Mokara* through Single-cell Culture

Single cells of *Mokara* were obtained from PLBs derived through the culture of axillary buds. These cells divided in culture and formed a cell cluster. As of 1982 "no fully differentiated plantlets [were] . . . obtained from this cultures [but] there is ample indication that plantlets are forthcoming" (Teo, 1985). Approximately 40,000 cells are present on a 2 × 2 cm surface area of PLBs. This is an indication that "single cell culture should have tremendous practical implication. . . . Selected clones could be cloned and large numbers [of plantlets could be] obtained much more rapidly and efficiently than by the normal well-known method of 'meristemming' " (Teo, 1985). Such a propagation method would have the added advantage of not requiring excessive proliferation, which can result in undesirable mutations. However, it is necessary to keep in mind that some plant tissues can be mosaics of different cells, some of which can be genetically undesirable. On the other hand, some cells from a genetic mosaic can produce greatly improved clones.

Culture of Micropropagated *Mokara* Plantlets

Plantlets of *Mokara* White produced through micropropagation were used in experiments on in vitro carbon dioxide enrichments of CAM orchid plants (Hew et al., 1995). The plantlets were obtained from Multico Orchids Pte. Co. in Singapore and maintained in culture at the laboratory of Professor Choy sin Hew, Botany Department, National University of Singapore.

> Modified Vacin and Went medium (1949) supplemented with 2 g l^{-1} tryptone (Bacto, Difco Laboratories, USA), 1 ml l^{-1} Nitsch microelements (Nitsch and Nitsch, 1969) [these are the microelements in Tables C-11, Disa-2, and Disa-3], 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} kinetin was used . . . 2% sucrose (BDH Limited, Poole, UK). . . . Each GA7 [culture vessel] (Magenta™, Sigma Chemical Co., USA) contains 100 ml of culture medium . . . with 1% agar (Bacteriological agar, Oxoid, UK). The pH . . . [was] adjusted to 5.3 before autoclaving . . . 26 ± 4°C, 14 h [photoperiods]. Light intensity (PPFD) 80 µmol m^{-2} s^{-1} (HPLB 700 W lamp, Philips, UK)." (Hew et al., 1995)

Elimination of Cymbidium Mosaic Virus and Odontoglossum Ringspot Virus from *Mokara*

Infections by cymbidium mosaic virus (CyMV) and odontoglossum ringspot virus (ORSV) are a worldwide problem which can have a devastating effect on producers of orchid cut flowers. Planting virus free plants is an effective control measure. A method to free *Mokara* plants of cymbidium mosaic virus and odontoglossum ringspot virus was developed at the Department of Botany, National University of Singapore by Professor Sek Man Wong and S. T. Lim who was a student at the time. Professor Wong, a plant virologist, is currently the leading international authority on orchid viruses whose compendium on the subject (Wong, 2002) is extensive, well written, and most impressive. The report which describes the method also includes Professor Chong Jin Goh (not a plant virologist who was chairman of the department at the time) as a co-author (Lim et al., 1993*b*). There are no further reports.

Plant Material. Plants of *Mokara* Chark Kuan infected with both CyMV and ORSV served as sources of explants by Wong and Lim. For general cultures meristems were excised from young axillary shoots, ca. 9 cm in length, with Aesculap No. 11 surgical blades (www.aesculap.com, other very sharp blades will probably also be suitable) and sectioned into four sizes: 0.01–0.25, 0.26–0.50, 0.51–0.75, and 0.76–1 mm. Survival after 3 months in culture was 0% for sections in the 0.01–0.5-mm range (Fig. Mkra-1). Axillary buds from young shoots were cultured to produce plantlets for thin section virus eradication cultures. Plantlets, about 2 cm in size, obtained from these explants were freed of all leaves except the uppermost two and PLBs, 5 mm in diameter, were cut transversely into 0.5-mm-thick sections for culture.

Surface Sterilization. The young axillary shoots should be washed with copious amounts of tap water, stripped of leaves, immersed in 20% Clorox (20 ml Clorox

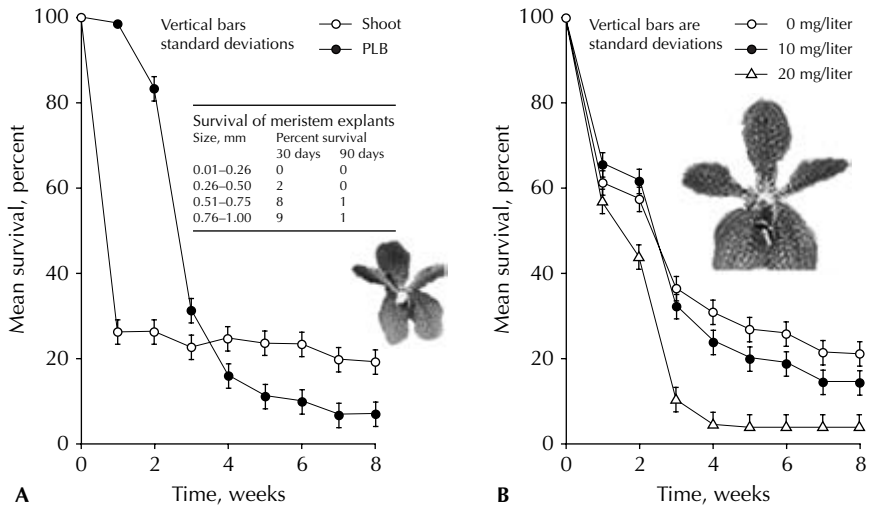


FIG. MKRA-1. **Culture and survival of *Mokara* Chark Kuan 'Pink' explants. A. Survival and culture of thin sections and PLBs. B. Effects of ribavirin on survival of thin section culture. (Modified from Lim et al., 1993*b*.)**

or another household bleach containing 5.25% sodium hypochlorite or 17.5 ml of 6% Clorox diluted to 100 ml with distilled water) with 40 drops Tween 20 (www.sigmaaldrich.com, www.vwr.com, www.fishersci.com, www.duchefa.com) per liter for 20 min and rinsed twice with sterile distilled water.

Culture Vessels. Thin sections for virus eradication should be cultured in Petri dishes, 5 cm diameter. Other explants can be cultured in Petri dishes, Erlenmeyer flasks, test tubes, or other containers.

Culture Conditions. Wong and Lim maintained their cultures at 26 ± 1°C under 14-h photoperiods of 24.5 μE m^{-2} s^{-1} provided by daylight fluorescent tubes. Standard culture room conditions are also suitable.

Culture Media. Explants should be cultured in Vacin and Went (VW) medium (Vacin and Went, 1949) containing 15% coconut water (CW), 0.1 mg NAA l^{-1}, and 0 or 10 mg ribavirin l^{-1} (Table Mkra-6).

Procedure. To free explants of virus, thin sections of both shoots and PLBs should be cultured on medium which contains 10 mg ribavirin l^{-1} (Table Mkra-6). However, virus-free plantlets can also be obtained by culturing thin sections on medium without ribavirin (Table Mkra-7).

Virus Assays. These assays may be beyond the capabilities of most orchid laboratories. Equipment and supplies are available from Agdia Inc. (www.agdia.com) which also performs assays.

TABLE MKRA-6. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for elimination of cymbidium mosaic virus (CyMV) and odontoglossum ringspot virus (ORSV) from *Mokara* Chark Kuan (Lim et al., 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	0.1	100–300 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
9	Coconut water[f]	150.0	No stock	No stock	Measure
	Antiviral agent				
10	Ribavirin (Virazole)	10.0 or none	10 mg in 2.5 ml water/95% ethanol mixture[g]		
	Sugar				
11	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[h]	To 1000 ml			
	Solidifier				
13	Agar[h]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 12) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]If the auxin fails to dissolve add a few drops of 0.1 N KOH. Keep frozen between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[g]Ribavirin (Virazole), 1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide, CAS Number 36791-04S, melting point 165–176°C is an antiviral agent manufactured by ICN Pharmaceuticals, Inc. (3300 Hyland Avenue, Costa Mesa, CA 92626, 714–545-0100, www.icnpharm.com). According to ICN its solubility in water is approximately 0.35 g ml^{-1}. To prepare a sterile ribavirin solution for 1 l of medium dissolve 10 mg ribavirin in 0.5 ml distilled water and bring volume to 2.5 ml with 95% ethanol. Add this solution to the autoclaved, warm and still liquid medium. This medium can be made with or without ribavirin.

[h]Add items 1, 3–7, and 9 to the 500–700 ml of distilled water (item 12) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 12), adjust pH to 5–5.4, add sugar (item 11), and adjust volume to 1000 ml with distilled water (item 12). Bring the solution to a gentle boil and add the agar (item 13) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l Erlenmeyer flask and autoclave. Add the auxin (item 8) and the antiviral agent (item 10) to the warm and still liquid medium with sterile pipettes or syringes, mix well, and dispense into preautoclaved culture vessels. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

TABLE MKRA-7. **Table 2 in the original paper showing survival of explants and production of virus-free plantlets (Lim et al., 1993): *Effect of ribavirin treatment in the production of virus-free regenerants from TS cultures of* Mokara *Char Kuan 'Pink'***

Ribavirin treatment		Virus-free plants in ribavirin-free media (%)			
		After 1 month		After 2 months	
Incubation period	conc. (mg liter^{-1})	CyMV	ORSV	CyMV	ORSV
TS of shoots					
1 month	0	25 (5)	60 (12)	100 (30)	90 (27)
	10	98 (53)**	69 (37)	100 (24)	90 (24)
	20	82 (9)**	82 (9)	100 (16)	81 (16)
2 months	0	10 (2)	15 (3)	86 (25)	90 (26)
	10	93 (37)**	50 (20)**	97 (28)	93 (27)
	20	89 (8)**	56 (5)**	100 (10)	50 (5)**
TS of PLBs					
1 month	0	5 (1)	10 (2)	60 (3)	60 (3)
	10	90 (8)**	22 (2)	100 (10)	60 (6)
	20	100 (6)**	0 (0)	100 (5)	100 (5)
2 months	0	38 (3)	13 (1)	0 (0)	33 (4)
	10	86 (6)	37 (4)	77 (23)**	47 (14)
	20	50 (3)	0 (0)	100 (12)**	42 (5)

Abbreviations: CyMV, cymbidium mosaic virus; ORSV, odontoglossum ringspot virus; PLBs, protocorm-like bodies; TS, thin section cultures; **: $P = 0.01$, chi-square analysis of 2×2 contingency test between treatment and control (untreated) cultures. Figures in parentheses indicate the actual number of virus-free plants.

Developmental Sequence. Explant survival is relatively low, but virus-free plants can be produced from thin sections on media with and without ribavirin (Fig. Mkra-1; Table Mkra-7).

General Comments. The method developed by Wong and Lim can eliminate CyMV and ORSV from *Mokara* and other orchids. The culture procedures are relatively simple and within reach for most orchid laboratories. However the virus assay is complex and requires advanced equipment and skills. This method can also be used to propagate healthy plants.

Mormodes

An interesting orchid genus, *Mormodes* is cultivated by many growers.

Clonal Propagation of *Mormodes histrio* through the Culture of Pseudobulb Sections

Pseudobulbs of *Mormodes histrio* have many nodes depending on age. Some nodes have visible dormant buds. Hölters and Zimmer (1990*a*) induced these to produce shoots.

Plant Material. Leaves were removed since it was shown that they inhibit shoot growth. The pseudobulbs were cut into slices (no thickness given in the original paper), each having one dormant bud. Best results were obtained with basal segments. The least successful segments were the third and fourth nodes from the base.

Surface Sterilization. The plant material was decontaminated with sodium hypochlorite (NaOCl) solution. Best results were obtained with 25–35 g l^{-1} active chlorine (25 g l^{-1} is equivalent to concentrated household bleach) and an exposure time of 5 min. Although not stated, one or more distilled water rinses following sterilization are advisable.

Culture Vessels. Standard vessels (e.g., square plastic culture containers) can be used.

Culture Condition. No specific culture conditions are given, but reference is made to those in Zimmer (1978): a light intensity of 1000–3000 lx (about 100–300 ft-c), 16-h photoperiods, and 25°C.

Culture Medium. Knudson C medium was used (Table Morm-1). Addition of 0.5 mg BA l^{-1} increased the number of shoot segments per node after 5 weeks of growth (Table Morm-1).

Procedure. Cut pseudobulbs following sterilization and lay the slices flat on the nutrient. Buds form shoots that grow up into the air or down into the medium.

Developmental Sequence. The buds start to swell and form leaflets about 7 days later. After an additional 2 weeks of growth the shoots reach a length of about 20 mm and develop one to three short roots. Shoots develop only on the dormant buds. When more than one shoot develops they are either adventitious or grow from a PLB.

General Comments. This aseptic procedure was employed to obtain sufficient root material to carry on the tissue culture of root explants from *M. histrio*.

TABLE MORM-1. **Knudson C medium (Knudson, 1946) as used for the culture of *Mormodes histrio* pseudobulb explants (Hölters and Zimmer, 1990*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1000	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
	Iron[c]				
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Cytokinin				
7	Benzylaminopurine (BAP)	0.5	50 mg/100 ml 95% ethanol[d]	1	
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[d]Keep refrigerated between uses.

[e]Add items 1–6 to 900 ml of distilled water (item 9), adjust pH to 5.0, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary, adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower the pH if necessary. Using a sterilized pipette under sterile conditions, add BAP solution (item 7) to the autoclaved medium while it is warm and still liquid, swirl to mix well, and dispense into sterile containers. Omit agar if preparing liquid medium.

Propagation of *Mormodes histrio* from Root Explants

Mormodes histrio shoots obtained as described in the previous section (Hölters and Zimmer, 1990*a*) were grown in vivo on a grating to promote root development for tissue culture (Hölters and Zimmer, 1990*b*).

Plant Material. Root systems attached to a small amount of shoot were excised below the basal node of the shoot.

Surface Sterilization. No specific decontamination procedure is given, but it can be assumed that the method employed for pseudobulb sections (Hölters and Zimmer, 1990*a*; see above) would be suitable.

Culture Vessels. No reference is made to culture containers in the original paper, but standard culture vessels can be used.

TABLE MORM-2. **Knudson C medium (Knudson, 1946) modified for the culture of *Mormodes histrio* roots (Hölters and Zimmer, 1990*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1000	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
	Iron[c]				
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxin				
7	Potassium naphthaleneacetate (KNA)	0.2	20 mg 100 ml^{-1} 50% ethanol[d]	1	
	Cytokinin				
8	Benzylaminopurine (BAP)	2.0	200 mg 100 ml^{-1} 95% ethanol[d]	1	
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier				
11	Agar[e]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g of chelating agent (Na_2EDTA) and 2.78 g of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[d]Keep refrigerated between uses.

[e]Add items 1–7 to 900 ml of distilled water (item 10), adjust pH to 5.0, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary, adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower the pH if necessary. Using a sterilized pipette under sterile conditions, add BAP solution (item 8) to the autoclaved medium while it is warm and still liquid, swirl to mix well, and dispense into sterile containers. Omit agar for liquid medium.

Culture Medium. Knudson C medium containing 0.2 mg potassium naphthaleneacetate (KNA) l^{-1} and 2.0 mg BA l^{-1} as employed (Table Morm-2).

Procedure. A specific procedure is not given, but it may be assumed that the explants were deposited on the culture medium following sterilization and rinsing with distilled water.

Developmental Sequence. The roots, attached to a small remnant of the shoot, developed PLBs at their tips. A substantial number of these PLBs gave rise to shoots.

General Comments. Root systems attached to shoots whose leaves were removed formed PLBs occasionally, but the latter did not give rise to shoots. Only root systems that were attached to a small shoot segment produced shoot-bearing PLBs.

Neofinetia

A monotypic epiphytic genus *Neofinetia* is found in China, Japan, Korea, and the Ryukyu Islands. It was first described in 1925 by the Chinese botanist H. H. Hu. The only species in the genus, *Neofinetia falcata*, has "traveled" widely through several orchid genera and was known over the years as (in alphabetical order): *Aerides thunbergii*, *Angraecopsis falcata*, *Angraecum falcatum*, *Finetia falcata*, *Holcoglossum falcatum*, *Nipponorchis falcata*, *Oeceoclades falcata*, and *Orchis falcata* (Bechtel et al., 1992).

Culture and Growth of Embryogenic Callus of *Neofinetia falcata*

The method used for *Darwinara*, *Doritaenopsis*, and *Phalaenopsis* is also suitable for *Neofinetia falcata* (Ichihashi and Hirawa, 1996).

Effects of Complex Additives on Callus Growth of *Neofinetia*

The method used for *Doritaenopsis* and *Phalaenopsis* is also suitable for *Neofinetia falcata* (Ichihashi and Islam, 1999). "Effects of T[aro]E[extract], P[otato]E[extract], and C[oconut]W[ater] on calli of . . . and N[*Neofinetia falcata*] revealed that growth was significantly higher on media supplemented with 50 ml l^{-1} of TE, PE, and CW (Fig. 3)" (Ichihashi and Islam, 1999; Fig. Neof-1).

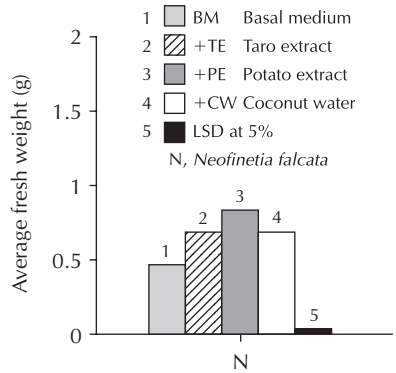


FIG. NEOF-1. **Part of figure 3 in Ichihashi and Islam, 1999; 50 ml l^{-1} of organic extracts were added to the medium. Callus (0.1 g) was transplanted on 10 ml of medium and weighed after 6 weeks.**

Micropropagation of *Neofinetia falcata* through Leaf Segments

Professor S. P. Vij and his associates have directed "efforts . . . to identify an alternate [to shoot meristems] but equally effective explant for . . ." micropropagation. They developed procedures for the culture of leaf segments of a number of orchids including *Neofinetia falcata* (Vij and Pathak, 1990).

Plant Material. Basal explants, 5–10 mm long, from leaves of 16–40-week-old seedlings growing in vitro should be used. Apical segments do not produce PLBs. Explants from leaves of mature plants died in the original experiments.

Surface Sterilization. Explants taken from axenic seedlings growing in vitro do not require surface sterilization.

Culture Vessels. Erlenmeyer flasks, test tubes, and other containers filled with medium to 20–30% of their capacity.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx or standard culture room conditions.

Culture Media. Mitra, Prassad, and Roychowdhury (MPR) medium (Mitra et al., 1976) supplemented with kinetin and IAA (Table Neof-1) must be used for the culture of leaf segments. No medium is suggested for the culture of plantlets to maturation. Plantlets can be cultured on one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate (BH) or charcoal (see Table Pln-1) or the Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal (AC) should be added to media as suggested for Knudson C that does contain this darkening agent (Table Pln-1, footnote *f*).

Procedure. Leaves are taken from plantlets, sectioned, and placed in culture on the initiation medium (Table Neof-1). Plantlets should be cultured on one of the Knudson C or Vacin and Went formulations suggested above, preferably a medium with BH and AC.

Developmental Sequence. PLBs form on the initiation medium (Table Neof-1) and give rise to plantlets (Fig. Neof-2). When transferred to Knudson C or Vacin and Went formulations with BH and AC, the plantlets develop further.

General Comments. Propagation of selected forms is not possible with this method because the explants are taken from seedlings. However it may be possible to adapt the procedure to mature plants. If only kinetin is present in the medium 75% of the explants produce an average of four PLBs (for a total of 300 PLBs per 100 explants). Plantlets produced by these PLBs formed their first leaves after 5 weeks. The first

TABLE NEOF-1. **Mitra, Prassad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Neofinetia falcata* leaf segments (Vij and Pathak, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $Ca(NO_3)_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Auxin				
8	Indoleacetic acid (IAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]		
	Cytokinin				
9	Kinetin	1.0	100 mg 100 ml^{-1} 95% ethanol[e]		
	Vitamins				
10	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[f]	0.25	
11	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[f]	0.25	
12	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[f]	0.25	
13	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[f]	0.25	
14	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[f]	0.25	
15	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[f]	0.25	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	9.0 g	No stock	No stock	Weigh
	Darkening agent				
19	Activated charcoal[h]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH or HCl respectively.

[f]Keep frozen between uses.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.5, add sugar (item 16), and bring volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved add the charcoal with vigorous stirring to ensure complete distribution through the medium, pour the solution into culture vessels and autoclave. After autoclaving when the medium is cooling swirl the flasks to disperse the charcoal. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976; Vij and Pathak, 1990).

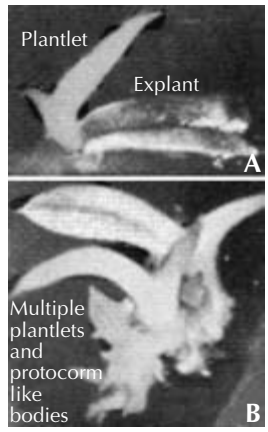


FIG. NEOF-2. **Development of *Neofinetia* plantlets from leaf segments (Vij and Pathak, 1990).**

root appeared following 14 weeks. In the presence of both kinetin and IAA only 25% of the explants produced PLBs. However every explant produced a dozen PLBs (also a total of 300 PLB per 100 explants) and roots appeared after only 10 weeks. That is why the recommendation here is for a medium which contains both hormones. *Neofinetia falcata* is apparently similar enough to *Aerides*, *Angraecopsis*, *Angraecum*, *Finetia*, *Holcoglossum*, *Nipponorchis*, *Oeceoclades*, and *Orchis* to have been assigned to these genera at one time or another. Therefore, this method could perhaps be used for at least some species in these genera.

Neofinetia means new *Finetia* because it is a second genus named in honor of the French botanist M. Achille Finet (1862–1913) who worked on the orchids of China and Japan and added much to the knowledge of angraecoid taxa (Schultes and Pease, 1963). The first genus named for him is *Finetia*.

[b]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite which are very different in nature should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

Neostylis

The methods used for *Ascofinetia* (Intuwong and Sagawa, 1973) can also be used for *Neostylis* (Sagawa and Kunisaki, 1982). A modified Vacin and Went medium containing 15% (v/v) coconut water is used for the initial culture (it is not clear whether the medium should be liquid or solidified with 8–9 g l^{-1} agar). Multiplication (i.e., proliferation) takes place in the same medium without glucose. For differentiation, tissues are cultured in a medium that contains 15% coconut water (v/v), 50 g l^{-1} homogenized green banana, and potato extract (100 g potatoes boiled for 5 min in 200 ml water). Cultures should be maintained under continuous illumination (sources not described) of 2100 lx and 26 ± 3°C.

Neottia

From the English summary (Champagnat 1971; the paper is in French) we learn that:

> . . . at the extremity of a *Neottia nidus-avis* isolated root, kept at 12 or 15°C, a renewal of apical growth is obtained, at will, which results in the formation of a protocorme [sic] quickly crowned by a bud, bearing adventitious roots. Isolation is the principal cause of the evolution.

Nervilia

Unlike most other orchids *Nervilia* has cordate leaves (Figs Nerv-1A, H and Nerv-2A, H). The genus consists of approximately 80 species which can be found in tropical Africa and Asia, northern Australia, Madagascar, the Malay archipelago, and the Pacific Islands (Bechtel et al., 1992).

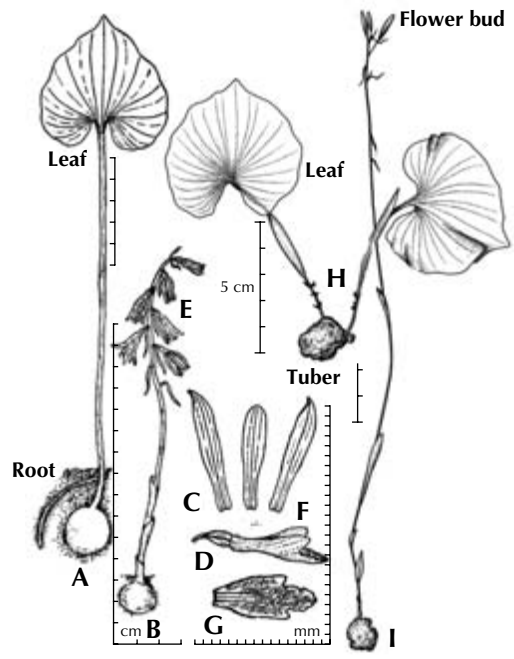


FIG. NERV-1. ***Nervilia aragoana*. A. Local tuber and root. B. Tuber and inflorescence. C. Sepals snd petals. D. Ovary. E. Flower. F. Side view of labellum. G. Flattened labellum. H. Leaves and tuber. (Sources: A–G, Joseph, 1982; H, I, Seidenfaden and Wood, 1992.)**

Nervilia is from the Latin *nervus* (vein). It is descriptive of the veins on the palmate leaves which stand out (Schultes and Pease, 1963; Bechtel et al., 1992).

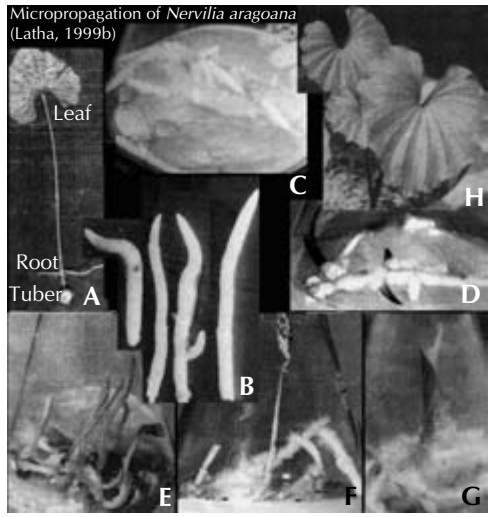


FIG. NERV-2. **Tissue culture of *Nervilia aragoana*. A. Plant with leaf, root, stolon, and tuber. B. Stolon explants. C. New stolons produced in culture. D. Leafy shoot produced in vitro. E. Leafy shoot in vitro. F. Inflorescence in vitro. G. Rooting in vitro. H. Plantlet in community pot. (Latha, 1999*b*.)**

In Vitro Culture of *Nervilia aragoana*

Nervilia aragoana is popularly known in South India as *Orilathamara* which means "single lotus plant" and alludes to its appearance and growth habit (Figs Nerv-1 and Nerv-2A). It is used widely for the preparation of indigenous medicines in India and collected extensively to meet the demand. Therefore it is an endangered species. An in vitro propagation method was developed "to save it from extinction, and to provide a means of obtaining large numbers of plants for the ayurvedic drug industry" (Latha, 1999*b*).

Plant Material. "Tender stolon segments with the growing tips, 2 cm long" (Fig. Nerv-2B) should be cultured after removal of 0.25 cm from the cut ends following sterilization.

Surface Sterilization. Explants should be soaked for 10 min in a 1% (v/v) solution of Teepol (www.sigmaaldrich.com), dipped in 70 ethanol (74 ml 95% ethanol diluted to 100 ml with distilled water) for 30 s, immersed in 5% household bleach like Clorox (Sterilig, Combii Chem, New Delhi was used in the original research) for 5 min, placed in 0.1% $HgCl_2$ (www.fishersci.com) for 3 min (this is a toxic substance and must be handled with care), and rinsed three times with sterile distilled water.

Culture Vessels. A photograph in the original paper shows that 250-ml Erlenmeyer flasks were used. Other containers are also suitable.

Culture Conditions. Cultures can be maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx or standard culture room conditions.

Culture Media. Explants should be cultured on woody plant medium (WPM; Lloyd and McCown, 1980) containing 0.5 mg BA l^{-1} and solidified with 0.7% agar (Table Nerv-1) for the production of new stolons. To induce leafy shoots, new stolons should be moved to the same medium solidified only with 0.4% agar (Table Nerv-2). Roots are produced on WPM with 0.5 mg NAA l^{-1} solidified with 0.7% agar (Table Nerv-3). Plantlets can be cultured on WPM or one of the versions of the Knudson C (KC) medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate (BH) or charcoal (see Table Pln-1) or the Vacin and Went (VW) solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal (AC) should be added to the medium selected for use as suggested for KC that does contain this darkening agent (Table Pln-1, footnote *f*).

Procedure. The explants should be surface-sterilized and placed on the first medium (Table Nerv-1) after removing 0.25 cm from the cut ends. Leafy shoots, 2 cm long, which form within 6–8 weeks should be moved to the second medium (Table Nerv-2) for rooting. The rooted plantlets can be moved directly to community pots or cultured for a period on BH- and AC-containing versions of WPM, KC, or VW.

Developmental Sequence. Each explant forms 3.1 ± 0.83 new stolons after 4 weeks on WPM plus 0.5 mg BA l^{-1} and 0.7% agar (Table Nerv-1). The new stolons arise from nodes and tips. They can produce additional stolons if subcultured on the same medium (Table Nerv-1). On being subcultured on WPM plus 0.5 mg BA l^{-1} and 0.4% agar (Table Nerv-2), stolons produce leafy shoots and an occasional inflorescence (Fig. Nerv-2F). These shoots form roots in the presence of NAA (Table Nerv-3).

General Comments. This method (Fig. Nerv-2) can be used to produce a large number of plants. However it is necessary to determine whether plants produced through micropropagation contain all compounds necessary for the preparation of ayurvedic drugs. Cytokinins induce flowering in vitro by some orchids and other plants. It is interesting to note that BA has a similar effect on *N. aragoana*.

TABLE NERV-1. **Woody plant (WPM) medium (Lloyd and McCown, 1980) modified for the culture of *Nervilia aragoana* explants for stolon production (Latha, 1999*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400.0	40.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	96.0	9.6 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)$	556.0	55.6 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	Potassium sulfate, K_2SO_4	990.0	99.0 g l^{-1}		
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.25	25.0 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(d)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(e)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
11	6-Benzyladenine (BA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. This medium was originally formulated for the culture of a woody plant, mountain laurel, *Kalmia latifolia*.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe lists $MnSO_4 \cdot H_2O$ which seems to be a typographical error because the formulae and concentrations of all other microelements are the same as in the Murashige–Skoog (MS) medium (Murashige and Skoog, 1962).

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.2, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media. As a rule it is preferable to add heat labile substances after autoclaving. However in this case the cytokinin and vitamins can be added before sterilization.

TABLE NERV-2. **Woody plant (WPM) medium (Lloyd and McCown, 1980) modified for the induction of leaves on stolons produced from *Nervilia aragoana* explants (Latha, 1999*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400.0	40.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	96.0	9.6 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)$	556.0	55.6 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	Potassium sulfate, K_2SO_4	990.0	99.0 g l^{-1}		
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.25	25.0 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(d)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(e)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
11	6-Benzyladenine (BA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	4.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. This medium was originally formulated for the culture of a woody plant, mountain laurel, *Kalmia latifolia*.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe lists $MnSO_4 \cdot H_2O$ which seems to be a typographical error because the formulae and concentrations of all other microelements are the same as in the Murashige–Skoog (MS) medium (Murashige and Skoog, 1962).

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.2, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media. As a rule it is preferable to add heat labile substances after autoclaving. However in this case the cytokinin and vitamins can be added before sterilization.

TABLE NERV-3. **Woody plant (WPM) medium (Lloyd and McCown, 1980) modified for rooting of *Nervilia aragoana* shoots (Latha, 1999*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400.0	40.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	96.0	9.6 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)$	556.0	55.6 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	Potassium sulfate, K_2SO_4	990.0	99.0 g l^{-1}		
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.25	25.0 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(d)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(e)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	α-Naphthaleneacetic acid (NAA)	0.5	5 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. This medium was originally formulated for the culture of a woody plant, mountain laurel, *Kalmia latifolia*.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe lists $MnSO_4 \cdot H_2O$ which seems to be a typographical error because the formulae and concentrations of all other microelements are the same as in the Murashige–Skoog (MS) medium (Murashige and Skoog, 1962).

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.2, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media. As a rule it is preferable to add heat labile substances after autoclaving. However in this case the cytokinin and vitamins can be added before sterilization.

Nigritella

Many of the European terrestrial orchids are not of major horticultural importance (the same is true for some terrestrial species from the Americas and other areas). This is perhaps one reason for the fact that few tissue culture propagation methods have been developed for these species. The danger of extinction and the need to develop rapid propagation methods have led to the establishment of several interesting research projects (Mark Clements at the National Botanic Gardens in Canberra, Australia; Maureen Weatherhead at the University of Hong Kong, and the brothers Hoppe in Hamburg to mention a few). An earlier project (and one of the earliest in this area of orchid research) was that carried out by Dr. Norbert Franz Haas at the Justus Liebig University in Giessen, West Germany (Haas, 1977*a*, 1977*b*).

Plant Material. Tubers collected (with permission) in a nature preserve in the Bolzano-Italy (Bozen-Südtirol) area were used as sources of explants in the original research. Apical and lateral meristems and subtending buds were removed for culture following surface sterilization.

Surface Sterilization. The tubers as removed from the ground are coated with soil and debris that must be washed away before sterilization. Washed tubers are sterilized by immersing them in 0.5% sodium hypochlorite (NaOCl) solution that is equivalent to 10% Clorox (10 ml Clorox diluted to 100 ml with distilled water) for 15 min. Although not mentioned in the original paper it is a good idea to wash the sterilized tubers with sterile distilled water before the meristems are removed. Explants should be sterilized a second time in 0.3% sodium hypochlorite, equivalent to 6% Clorox (6 ml Clorox diluted to 100 ml with sterile distilled water) for 10 min. A distilled water wash following this sterilization is also advisable.

Culture Vessels. Erlenmeyer flasks, 250 ml, containing 80 ml of culture medium were used in the original research. Other culture vessels can also be used.

Culture Conditions. The explants were maintained under 14-h photoperiods of 2000 lx provided by Osram brand cool white fluorescent tubes and 20–22°C.

Culture Media. MS medium, one-third strength of macroelements, other components as given in the original recipe (Table Nla-1), was used in the original research. It is not clear whether hormones were used and if so at what levels. Since this is a north temperate terrestrial orchid it is possible that the media used for *Anacamptis*, *Dactylorchis*, *Dactylorhiza*, and *Orchis* may also be suitable.

Procedure. Shoot apices were excised from new shoots on tubers and placed on the culture medium.

Developmental Sequence. The explants formed tubers at their bases and shoots from their apices. Anthocyanin containing inflorescenses were formed on rare occasions.

TABLE NLA-1. **Murashige–Skoog medium (Murashige and Skoog, 1962) as used for the culture of *Nigritella* (Haas, 1977*a*, 1977*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	550	55 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	147	14.7 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	123.3	12.3 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	633.3	63.3 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	56.7	5.7 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	37.3 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Indoleacetic acid (IAA)	Not clear if any (and if so how much) was added[f]			
	Cytokinin				
11	Kinetin	Not clear if any (and if so how much) was added[f]			
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]If auxins or cytokinin do not dissolve, add a few drops of KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 16), adjust pH as required, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid, hormones (if any), and vitamins (items 8 and 10–14) to hot solution with sterilized pipettes, mix well, and dispense medium into preautoclaved culture vessels. Omit agar if preparing liquid medium.

General Comments. Dr. Norbert F. Haas-von Schmude left the academic world and now lives in the city of Wettenberg, Germany where he and his wife Gudrun operate one of the finest commercial orchid laboratories anywhere. He no longer devotes time to pure research and his more recent tissue culture procedures remain unpublished.

Oberonia

The method developed for *Acampe praemorsa* was used to isolate 12.4×10^4 protoplasts from leaves of *Oberonia santapui* (Seeni and Abraham, 1986).

Odontioda

A hybrid genus (*Cochlioda* × *Odontoglossum*), *Odontioda* is a cool growing orchid well liked by hobby growers. It also has considerable potential as a pot plant due to its rapid growth, numerous cultivars, attractive inflorescences, and colorful flowers.

Micropropagation of *Odontioda* through Callus Cultures

There are reports that excised shoot tips ("meristems") form a large callus consisting of numerous PLBs. Each PLB can form a plantlet. Procedures used for *Cymbidium*, *Odontoglossum*, and *Odontonia* (Morel, 1960, 1963, 1964*a*, 1965*b*, 1970, 1974) can be used for *Odontioda*.

Micropropagation of *Odontioda*

A protocol for micropropagation of *Odontioda* was developed at the Aichi University of Education in Japan by Professor Syoichi Ichihashi and his associates as part of research on tissue culture of the *Odontoglossum* alliance (Ichihashi et al., 1999; Murase et al., 1999, 2002).

Plant Material. Shoot tips from young shoots or lateral buds are cultured.

Surface Sterilization. Shoots should be washed with tap water before trimming excess tissue and removing their leaves. After that they should be immersed in 1% sodium hypochlorite (19 or 17 ml of 5.25% or 6% household bleach respectively diluted to 100 ml with distilled water) for 20 min with moderate shaking and then washed three times with sterile distilled water. Following their excision the tips should be rinsed with 0.5% sodium hypochlorite (50 ml of the solution above diluted to 100 ml with distilled water) and soaked in sterile distilled water.

Culture Vessels. Test tubes (25 × 120 mm), Erlenmeyer flasks, and other containers are suitable.

Culture Conditions. Cultures should be maintained initially at 25°C under constant illumination of 650 lx provided by Plantlux (Toshiba) fluorescent lamps or standard culture room conditions. When PLBs form they should be cultured at 20–22.5°C. The effects of temperature on PLB growth can be significant, as can be seen in

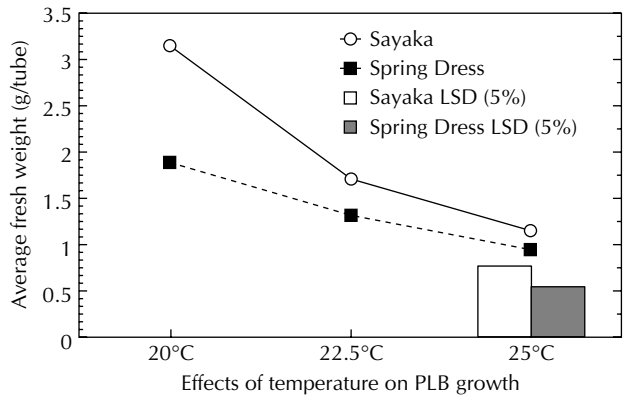


FIG. ODA-1. **Temperature effects on the growth of PLBs of *Odontioda*.**

Fig. Oda-1 which shows the response of *Odontioda* Lovely Morning "Sayaka" and *Odontioda* Shelley "Spring Dress" (Ichihashi et al., 1999).

Culture Media. New *Phalaenospis* (NP) medium (Ichihashi, 1992*a*, 1992*b*) with 20 g sucrose (Table Oda-1) should be used to culture explants and maintain PLBs. NP with banana homogenate (BH; Table Oda-2) is suitable for PLB culture and proliferation and plantlet growth. No medium is suggested for the culture of plantlets to maturation. Plantlets can be cultured on one of the versions of the Knudson C (KC) medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate (BH) or charcoal (see Table Pln-1) or the Vacin and Went (VW) solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If BH is not listed in the recipe for a medium, it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal (AC) should be added to the medium selected for use as suggested for KC that does contain this darkening agent (Table Pln-1, footnote *f*).

Procedure. The explants are cultured on the first medium (Table Oda-1) after surface sterilization. PLBs which form on this medium can be cultured on the same medium to increase their number or should be moved to the second solution (Table Oda-2) for proliferation, growth, and plantlet formation. For further growth the plantlets can be moved to one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, and V-4) with or without BH or AC. If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). AC should be added to the medium selected for use as suggested for KC that does contain this darkening agent (Table Pln-1, footnote *f*).

TABLE ODA-1. **New *Phalaenopsis* (NP) medium (Ichihashi, 1992) for the culture of *Odontioda* explants and protocorm-like bodies (PLBs; Ichihashi et al., 1999; Murase et al., 2002)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	32.0	3.2 g l^{-1}	10	
2	Ammonium sulfate, $(NH_4)_2SO_4$	303.9	30.39 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	637.6	63.76 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	256.4	25.64 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	424.0	42.4 g l^{-1}	10	
6	Monopotassium phosphate, KH_2PO_4	462.7	46.27 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2{\cdot}6H_2O$	0.0125	1.25 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.0125	1.25 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.415	41.5 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[e]	To 1000 ml			
	Solidifier				
17	Gelrite[e,f]	3.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6 ± 0.1, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 15). Add Gelrite as described in the footnote below. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave.

[f]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] (item 17) is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum (Phytagel, Gelrite) Gellan gum must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE ODA-2. **New *Phalaenopsis* (NP) medium (Ichihashi, 1992) for the culture of *Odontioda* plantlets (PLBs; Ichihashi et al., 1999; Murase et al., 2002)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	32.0	3.2 g l^{-1}	10	
2	Ammonium sulfate, $(NH_4)_2SO_4$	303.9	30.39 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	637.6	63.76 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	256.4	25.64 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	424.0	42.4 g l^{-1}	10	
6	Monopotassium phosphate, KH_2PO_4	462.7	46.27 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.0125	1.25 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.0125	1.25 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.415	41.5 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
14	Banana homogenate[e]	75.0 ml	No stock	No stock	Weigh
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Gelrite[f,g]	3.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

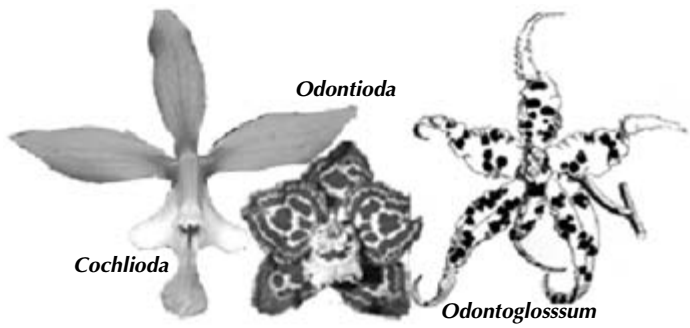
[e]Opinions differ regarding the use of banana in culture media. The predominant opinion is that only homogenized pulp of ripe banana should be used. Other views are that: (1) unripe (green) bananas are preferable; (2) the peel rather than the pulp should be used; (3) unpeeled banana homogenate should be incorporated in media; and (4) slices, ripe or unripe, peeled or unpeeled, rather than homogenate are most effective. Opinions also differ regarding which is the most effective variety (Gros Michel, Raja, Pisang Susu, and others). Since there is no experimental evidence to support any view the best approach is to follow the recommendations in the original reports and use what is suggested there. This is the case here.

[f]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6 ± 0.1, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Add Gelrite as described in the footnote below. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave.

[g]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite (item 17)] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium; it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

Developmental Sequence. PLBs form on the first medium (Table Oda-1). They grow and proliferate on the second medium (Table Oda-2). Plantlets form and grow on the second solution (Table Oda-2) or the other media suggested above.

General Comments. Using *Odontioda* to formulate a medium which could be suitable for the *Odontoglossum* alliance is a good idea because this hybrid genus may have requirements which are similar to those of other genera in the group (cool growing orchids like *Aliceara*, *Belleara*, *Brassia*, *Brassidium*, *Burrageara*, *Cochlioda*, *Colmanara*, *Degarmoara*, *Howeara*, *Maclellanara*, *Milpasia*, *Milpilia*, *Miltassia*, *Miltistonia*, *Miltonia*, *Miltonidium*, *Miltonioda*, *Miltoniopsis*, *Odontioda*, *Odontobrassia*, *Odontocidium*, *Odontoglossum*, *Odontonia*, *Oncidium*, *Rodricidium*, *Rodriglossum*, *Rodriguezia*, *Vuylstekeara*, *Wilsonara*, and others). This procedure is not suitable for *Odontoglossum*. Therefore it should only be used as a starting point in attempts to modify the medium for other members of the alliance.



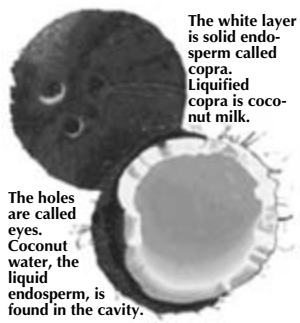
Odontoglossum

Apical and axillary buds of *Odontoglossum* have been cultured (Khaw et al., 1978*a*, 1978*b*). The procedures used for *Cymbidium* (Morel, 1960, 1963, 1964*a*, 1965*b*, 1970, 1974) are suitable.

Odontonia

The procedures employed for shoot-tip culture of *Cymbidium*, *Odontoglossum*, and *Ondontioda* (Morel, 1960, 1963, 1964*a*, 1965*b*, 1970, 1974) are also suitable for *Odontonia*. Protoplasts of *Odontonia* Debutante (*Miltonia warscewiczii* × *Odontoglossum cariniferum*) can be isolated by the procedure used for *Angraecum giryamae*, but the yield after 27 h of incubation is "few" (Price and Earle, 1984; these authors misspell *A. giryamae* as *A. gyrimae*).

Smallest and Largest Seeds: Orchids and Coconuts



Hindu mythology relates that Lord Vishnu brought the coconut tree to earth. According to the Ayurveda coconut water (CW), the clear liquid endosperm of coconuts, has many health promoting qualities for humans. In 1941 Johannes van Overbeek (1908–1988) and his associates showed that CW can also have beneficial effects on the growth of *Datura* embryos (van Overbeek et al., 1941). However, when CW was first added to a medium for *Cattleya* seed germination its effects were deleterious (Mariat, 1951). This did not deter a Hawaiian orchid grower named Herbert C. Shipman from adding "a few teaspoonfuls" to a medium he used to germinate *Dendrobium phalaenopsis*. He reported that the seedlings "began to grow like weeds" (Yamada, 1952). Subsequently CW was shown to have stimulating effects on germination of *Cattleya*, *Paphiopedilum*, and *Vanilla* (Hegarty, 1955), as well as to retard differentiation and enhance proliferation of *Phalaenopsis* seedlings (Ernst, 1967*b*). The first suggestion that CW should be used in orchid tissue culture seems to have been made in 1962 (Murashige, 1962). Copra is not used in orchid seed germination and tissue culture media. Coconut water is often referred to as coconut milk. This is incorrect. Coconut milk is a white milky liquid obtained when copra is ground, diced, squeezed, or simply liquefied in a homogenizer.

Oncidium

The development of attractive *Oncidium* hybrids, the need to propagate some species clonally, and the importance of a number of crosses as cut flowers have led to the development of several tissue culture methods.

Clonal Propagation of *Oncidium* by Means of Shoot-tip Cultures

Not many details are available about this procedure. The context in which it is mentioned (Bertsch, 1966) suggests that methods used for *Cymbidium* (More, 1960, 1963, 1964*a*, 1965*b*, 1970, 1974) can also be employed for *Oncidium*. Other methods used for *Cattleya* (Scully, 1967) and *Cymbidium* (Sagawa et al., 1966) may also be suitable. Modified Vacin and Went medium with 15% (v/v) coconut water cannot support the growth of apical and axillary buds of *Oncidium* (Sagawa and Kunisaki, 1982).

Clonal Propagation of *Oncidium* by Means of Flower-stalk-tip Cultures

Propagation methods involving the culture of flower-stem parts are non-destructive. Therefore several investigators have tried to develop culture methods for flower-stem parts of a number of species. One of these species is *Oncidium papilio* (Fast, 1973*a*).

Plant Material. Flower stalks of *O. papilio* with apical buds that are still dormant are used.

Surface Sterilization. Remove all dry sheaths and rinse sections under running water. Dip in alcohol, pass through a flame, immediately immerse in freshly filtered 5% (w/v) calcium hypochlorite, and soak for 10 min while occasionally shaking.

Culture Vessels. Use 30 × 180 mm test tubes with 10 ml liquid medium, covered with cotton buns or two-hole stoppers with cotton stuffed in their holes or 25–500-ml Erlenmeyer flasks stoppered in the same manner.

Culture Conditions. Maintain cultures under diffuse light at temperature of 20–24°C. When a rotating table is used, set it at 5 rpm.

Culture Media. A modified liquid or solid Knudson C medium (Table Onc-1) is used for callus induction. A modified liquid or solid MS medium (Table Onc-2), or a mixture of this medium and Knudson C in a 1:1 ratio (v/v), is used to increase the callus. The same media in solid form are used for plantlet differentiation. These media enriched with 5–10% (w/v) banana homogenate (medium Onc-2a) or seed-free tomato juice (medium Onc-2b), are used to enhance plantlet growth.

Procedure. Remove the top 2 cm from a flower stalk, take off all dry sheaths, and wash under running water. After surface sterilization (see above), the section must

TABLE ONC-1. **Modified Knudson C medium (Knudson, 1946) for the culture of *Oncidium* (Fast, 1973*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	1000	100 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	500	50 g l^{-1}	10	
3	Potassium chloride, KCl	250	25 g l^{-1}	10	Or weigh
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}	1	One solution
(b)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	1	1 g l^{-1}		
(b)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1	1 g l^{-1}		
(c)	Manganese sulfate, $MnSO_4 * 4H_2O$	0.01	10 mg l^{-1}		
(d)	Cupric sulfate, $CuSO_4 \cdot 5H_2O$	0.03	30 mg l^{-1}	1	One solution
(e)	Aluminium chloride, $AlCl_3$	0.03	30 mg l^{-1}		
(f)	Nickel chloride, $NiCl_2 \cdot 6H_2O$	0.03	30 mg l^{-1}		
(g)	Potassium iodide, KI	0.01	10 mg l^{-1}		
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	12–15 g	No stock	No stock	

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, they must be kept frozen between uses.

[c]Dissolve the iron salt and chelating agent in the same 1 l of water. Stir and/or heat until both are completely dissolved.

[d]Pour all microelements in the same 1 l of water, and stir and/or heat until they are completely dissolved.

[e]Add items 1–7 to 900 ml distilled water (item 9), adjust pH to 5.0–5.5, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When the agar is completely dissolved, dispense medium into culture vessels and autoclave. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary, adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower pH if necessary. Omit agar for liquid medium.

be handled with sterile forceps. Under aseptic conditions, cut off the very tip with a sterile scalpel and discard, remove the 2–3-mm section below and place it with the cut surface on the agar nutrient medium when solid medium is utilized.

Developmental Sequence. Depending on the physiological condition of the bud, a callus and subsequently PLBs will form 4–8 weeks after the start of culture. These bodies are separated and subcultured. This is repeated every 4 weeks depending on the vitality of the explant, the composition of the nutrient medium, and the season. Several thousand protocorms can be obtained 6 months after the start of propagation. Shoots and roots will form on a medium that contains tomato or banana extract. After 6 months the new plantlets should be transferred once more onto the same medium in larger flasks (500-ml Erlenmeyer flasks). This allows growth of strong plants, which are planted and treated like normal seedlings.

TABLE ONC-2. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for the culture of *Oncidium* (Fast, 1973*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	1650	165 g l^{-1}	10	Or weigh
2	Potassium nitrate, KNO_3	1900	190 g l^{-1}	10	Or weigh
3	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
5	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
6	Iron citrate, $FeC_6H_5O_7 \cdot 3H_2O$	25	25 g l^{-1}	10	
7	**Microelements**[c]				
(a)	Boric acid, H_3BO_3	1	1 g l^{-1}		
(b)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	7	7 g l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	0.01	10 mg l^{-1}		
(d)	Cupric sulfate, $CuSO_4 \cdot 5H_2O$	0.03	30 mg l^{-1}	1	One solution
(e)	Aluminum chloride, $AlCl_3$	0.03	30 mg l^{-1}		
(f)	Nickel chloride, $NiCl_2 \cdot 6H_2O$	0.03	30 mg l^{-1}		
(g)	Potassium iodide, KI	0.01	10 mg l^{-1}		
	Vitamins[d–f]				
8	Niacin (nicotinic acid)	1.22	100 mg 100 ml^{-1} 95% ethanol	1	
9	Thiamine (vitamin B_1)	0.5	50 mg 100 ml^{-1} 95% ethanol	1	Or combine
10	Pyridoxine·HCl (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol	1	
	Auxin[d,f,g]				
11	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol	1	
	Cytokinin[d,f,g]				Or combine
12	Kinetin	0.05	5 mg 100 ml^{-1} 95% ethanol	1	
	Complex additive				
13	Peptone[d]	1 g	No stock	No stock	Weigh
14	**Tomato or banana pulp**[h]	50–100 g	No stock	No stock	Weigh
	Sugar[i]				
15	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[i]	To 1000 ml			
	Solidifier				
17	Agar[i]	10–12 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, they must be kept frozen between uses.

[c]Add all microelements to the same 1 l, stir and/or heat until dissolved. Add 1 ml per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[d]Add only to medium Onc-2a.

[e]All vitamins may be combined into the same 100 ml of 95% ethanol; use 1 ml per liter of culture medium. Refrigerate or freeze between uses.

[f]All vitamins and both hormones may be combined into the same 100 ml of 95% ethanol; use 1 ml per liter of culture medium. Refrigerate or freeze between uses.

[g]Both hormones may be combined into the same 100 ml of 95% ethanol; use 1 ml per liter of culture medium. Refrigerate or freeze between uses.

[h]Add banana homogenate to make medium Onc-2a and seed-free tomato juice for solution Onc-b.

[i]Mix items 1–7, 13, and 14 with 800 ml distilled water (item 19). Adjust pH to 5.0–5.5, add sugar (item 15), and bring volume to 1000 ml with more distilled water (item 16). Add agar (item 17), slowly while stirring to the gently boiling solution. When agar is fully dissolved, autoclave the medium under standard conditions. Use agar only if a solid medium is desired. Combine vitamins (items 8–10), auxin (item 11), and cytokinin (item 12), and add after medium has been autoclaved (and before it solidifies if agar had been included), using sterile glassware and under aseptic conditions. Mix well, and distribute medium into sterile culture vessels.

General Comments. *Oncidium papilio* is a very pretty orchid that can be propagated from seed or through tissue culture rather than collected from the forest and thereby brought to near extinction.

Clonal Propagation of *Oncidium* through Flower-stalk Buds

A method for the clonal propagation of *Oncidium* was developed at the Singapore Botanic Gardens as a means of causing minimal damage to existing plants (Lim-Ho and Lee, 1987).

Plant Material. Dormant buds taken from the bases of flower stalks should be cultured (Table Onc-3).

Surface Sterilization. The stems should be washed with an antiseptic soap before the bracts covering the buds are removed. After that it is best to cut the stems so that 3–6 mm are left above the bud and 2 mm remain below it. The difference in length prevents inverted planting of the buds. These stem sections should be immersed in 20% Clorox (20 ml Clorox diluted to 100 ml with distilled water) for 30 min. There is no mention in the original paper of subsequent washing with sterile distilled water, but this is advisable.

Culture Vessels. Test tubes were used in the original research (Lim Ho and Lee, 1987), but other containers can also be employed.

Culture Conditions. Cultures were maintained under 12-h photoperiods of approximately 1000 lx provided by Sylvania Gro Lux lamps and 25°C. Different illumination can also be provided.

Culture Media. Vacin and Went medium (Vacin and Went, 1949) with two auxins and one cytokinin (Table Onc-4) is suitable for plants and/or callus induction. Unmodified Vacin and Went medium (Table Onc-5) should be used for differentiation and protocorm formation.

TABLE ONC-3. **Culture of flower-stalk buds of *Oncidium* (Lim-Ho and Lee, 1987)**

Species or hybrid	Response[a]	Time to leaf formation, months
Oncidium ampliatum	Few plants, some callus	5
[(Oncidium baueri × *Oncidium ampliatum)* × *Oncidium flexuosum]*	Few plants, no callus	3
Oncidium cebolleta	Few plants, some callus	5
Oncidium Dr. Schragen	Few plants, no callus	4
Oncidium Golden Sunset "Sunspot"	Many plantlets and PLBs	1.5
Oncidium Purple Envy	Many plantlets and PLBs	1
Oncidium sphacelatum	Few plants, some callus	6

[a]Buds were cultured on several modifications of the Murashige–Skoog medium, but formed callus, protocormlike bodies (PLBs), and/or plantlets on a modification containing 0.5 ppm 2,4-dichlorophenoxyacetic acid (2,4-D), 2 ppm benzyladenine (BA), and 0.5 ppm naphthaleneacetic acid (NAA; see Table Onc-4). Unmodified Vacin and Went medium should be used for plantlet regeneration.

TABLE ONC-4. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for the culture of *Oncidium* flower-stalk buds (Lim-Ho and Lee, 1987)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxins				
10	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[f,g]	1	
11	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.5	50 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
12	Benzyl adenine (BA)	2	200 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably will have little effect.

[e]Keep frozen between uses.

[f]If auxins or cytokinin do not dissolve, add a few drops of KOH or HCl respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml of distilled water (item 17); set pH as required, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid, hormones, and vitamins (items 8, 10–15) to hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE ONC-5. **Vacin and Went medium for the germination of orchid seeds (Vacin and Went, 1949) as used for the culture of flower-stalk buds of *Oncidium* (Lim-Ho and Lee, 1987)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml of water, stir and/or heat until it dissolves. Add the other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Add items 1, 3–7 to the 500 ml of distilled water (item 9) that contains item 2, set pH as required, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar for liquid medium.

Procedure. The stem sections should be inserted into the medium with their long side above the agar. This positions them right side up.

Developmental Sequence. Plants, callus, and/or PLBs form after periods ranging from 1 to 6 months.

General Comments. It is interesting to note that the buds fail to develop on media that contain only 0.5 mg 2,4-D l^{-1}, or 2,4-D and 2 mg BA l^{-1}. Plantlets, callus masses, and PLBs form only on a medium that contains these hormones plus 0.5 mg l^{-1} NAA. It would be interesting to determine the effects of a medium that contains NAA and BA only.

Mericloning of *Oncidium* through the Culture of Apical-shoot Explants

A procedure for the culture of apical shoot explants of *Oncidium* developed by Helen Nair and her associates at the Botany Department, University of Malaya, Kuala Lumpur (Khaw et al., 1978*a*, 1978*b*) utilizes a "model nutrient medium . . ." (Table Onc-6). Excision, sterilization, and culture conditions should be similar to those employed in other procedures for the same genus and for *Cattleya*, *Cymbidium*, *Odontoglossum*, *Miltonia*, and *Dendrobium*.

TABLE ONC-6. **A "model medium" [actually a modified Vacin and Went solution (Vacin and Went, 1949)] for the initiation of protocorm-like bodies from apical or axillary meristems (Khaw et al., 1978*a*, 1978*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $CaH_4(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3	525	52.5 g l^{-1}	10	
6	**Iron**[d]				
(a)	Ferrous sulfate, $Fe_2(SO_4)_3$	27.8	2.78 g l^{-1}	10	One solution
(b)	Chelating agent, Na_2EDTA	37.3	37.3 g l^{-1}		
7	**Microelements**[e]				
(a)	Aluminum chloride, $AlCl_3$	0.014	14 mg l^{-1}	10	One solution
(b)	Ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 7H_2O$	0.148	148 mg l^{-1}		
(c)	Boric acid, H_3BO_3	9.99	9.99 g l^{-1}		
(d)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.03	30 mg l^{-1}		
(e)	Nickel chloride, $NiCl_2 \cdot 6H_2O$	0.031	31 mg l^{-1}		
(f)	Potassium iodide, KI	0.38	38 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1	1 g l^{-1}		
(h)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}		
	Complex additive				
8	Coconut water	150 ml	No stock	No stock	
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve and it is not possible to prepare a stock solution. To facilitate the preparation of medium 200 mg of the salt should be placed in 500 ml of water, stirred and/or heated until it dissolves. The other components of the medium should be added after that.

[d]The original recipe of the Vacin and Went medium contains 28 mg l^{-1} ferric citrate $Fe_3(C_6H_4O_6)_3 \cdot 2H_2$.

[e]The original papers (Khaw et al., 1978*a*, 1978*b*) do not list the chemical formulae of all microelements. Those given here are the ones most commonly used in culture media and/or found in catalogs. Add all microelements to the same 1 l of water, mix well and dispense as needed.

[f]Add items 1, 3–8 to 500 ml of distilled water (item 10) which contain the calcium phosphate (item 2); set pH as required; add sugar (item 9), adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to cold solution which is then brought to a boil and stirred. When dissolved, distribute into culture vessels and autoclave. Omit agar when preparing liquid medium.

Clonal Propagation of *Oncidium* through the Culture of Apical Buds

Apical buds of two *Oncidium* species and an equal number of hybrids were cultured on modifications of the Vacin and Went (see Table, Onc-5, Arach-5, and Arach-6) and Knudson C media by procedures similar to those used for *Arachnis*, *Aranda*, *Aranthera*, and *Dendrobium* (Lim-Ho, 1981).

Isolation of *Oncidium* Protoplasts

The method developed for *Acampe praemorsa* was used to isolate 7.3×10^4 protoplasts from leaves of *Oncidium ampliatum* (Seeni and Abraham, 1986).

Plant Production through Somatic Embryos Formation on Leaf Explants

Oncidium hybrids are of major importance to orchid cut flower and pot plant producers as well as to hobby plant growers. Both require the production of plants through mass rapid clonal propagation. Somatic embryogenesis is of basic interest for developmental biologists and tissue culture researchers. Practical and basic aspects are combined in a method for the micropropagation of *Oncidium* (Chen et al., 1999).

Plant Material. In vitro grown 9-month-old plantlets of *Oncidium* Gower Ramsey (Fig. Onc-1G), produced from flower-stalk buds, were sources of explants. The explants were leaf segments, 5 mm long taken, from leaves, 2–4 cm and 5–7 cm long.

Surface Sterilization. There is no need to surface-sterilize explants taken from plantlets which are growing in vitro. However they must be washed with sterile distilled water to remove medium residues if any.

Culture Vessels. Test tubes, 20×150 mm were used in the original research. Erlenmeyer flasks and other containers filled with medium to 25–30% of their capacity are also suitable.

Culture Conditions. Cultures should be kept in the dark at 26 ± 2°C for 20–30 days to bring about the formation of PLBs and somatic embryos (SEs). PLBs and SEs produced in the dark should be maintained at 26 ± 2°C under 16-h photoperiods of 28–36 μmol m^{-2} s^{-1} provided by 40-W daylight fluorescent tubes (FL-30D/29, China Electric Co., Taipei, Taiwan). Standard culture room conditions will probably also be suitable for the PLBs and SEs and for shoot and root production.

Culture Media. For PLB and SE formation explants should be cultured on modified half-strength MS medium (Murashige and Skoog, 1962) containing 1 mg TDZ l^{-1} (Table Onc-7) in the dark. Once formed, PLBs and SEs should be cultured on the same medium (Table Onc-7) under illumination. Shoots which are produced on this medium should be cultured on half-strength MS containing 0.5 mg NAA l^{-1} (Table Onc-8) to induce root production. SEs and PLBs can also be cultured directly on

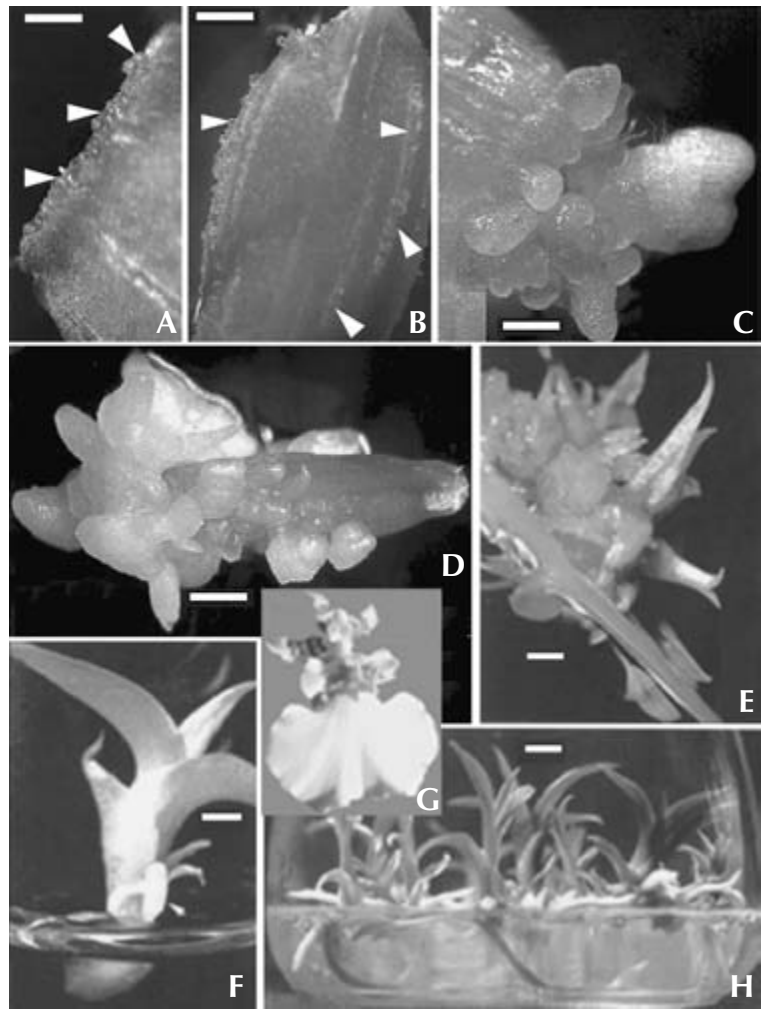


FIG. ONC-1. **Development of *Oncidium* Gower Ramsey leaf explants. A. Embryogenic nodular masses (arrowheads) emerging from wound surfaces (scale bar = 500 µm). B. Embryogenic nodular masses (arrowheads) on epidermal cell layers on explants from 5–7-mm-long leaves (scale bar = 550 µm). C. More than 20 embryos formed on the surface of a section of leaf explants (scale bar = 800 µm). D. Nodular masses on tips of leaf explants (scale bar = 500 µm). E. PLBs with shoots (scale bar = 100 mm). F. Root being produced by a plant (scale bar = 100 mm). G. Flower of *Oncidium* Gower Ramsay. Sepals and petals are yellow. Markings are dark orange to brown. H. Plantlets ready for transfer to pots (scale bar = 100 mm). (Sources: A–F, H, modified from Chen et al., 1999; G, digital photograph by Joseph Arditti.)**

TABLE ONC-7. **Modified half-strength Murashige–Skoog ($^1/_2$MS) medium (Murashige and Skoog, 1962) for the culture of leaf explants, protocorm-like bodies (PLBs) and shoots of *Oncidium* Gower Ramsey (Chen et al., 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2{\cdot}2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2{\cdot}6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
11	Thidiazuron (TDZ)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
12	Peptone	1.0 g	No stock	No stock	Weigh
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Gelrite[g,h]	2.5 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4{\cdot}4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4{\cdot}7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]Available from www.sigmaaldrich.com.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.2, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Add the gellan gum to the cold solution as indicated in footnote *h*, distribute the medium to culture vessels, and autoclave. It is preferable not to autoclave hormones and vitamins, but in this medium they can be autoclaved for 15 min at 121°C.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE ONC-8. **Modified half-strength Murashige–Skoog ($^1/_2$MS) medium (Murashige and Skoog, 1962) for plantlet production from protocorm-like bodies (PLBs) and root induction on shoots of *Oncidium* Gower Ramsey (Chen et al., 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
12	Peptone	1.0 g	No stock	No stock	Weigh
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Gelrite[g,h]	2.5 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve add a few drops of 0.1 N KOH.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.2, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Add the gellan gum to the cold solution as indicated in footnote *h*, distribute the medium to culture vessels and autoclave. It is preferable not to autoclave hormones and vitamins, but in this medium they can be autoclaved for 15 min at 121°C.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

this medium (Table Onc-8) for plantlet formation. When plantlets produced on this medium become large enough they should be potted in sphagnum moss and grown in a greenhouse.

Procedure. Explants are placed on the first medium and cultured in the dark for 20–30 days until PLBs and SEs are formed (Fig. Onc-1A–E). These should be moved to the same medium under illumination for shoot production. The shoots must be moved to the auxin-containing medium for rooting and plantlet production (Fig. Onc-1F, H).

Developmental Sequence. Structures described as "nodular masses" form on wound surfaces and leaf tips during the first 20 days (Fig. Onc-1A, B) of culture. SEs are formed during the subsequent 6–10 days. These give rise to PLBs (Fig. Onc-1C, D) and shoots (Fig. Onc-1E). Roots (Fig. Onc-1F) and individual plantlets (Fig. Onc-1H) form when shoots are transferred to the NAA-containing medium (Fig. Onc-2; Table Onc-8).

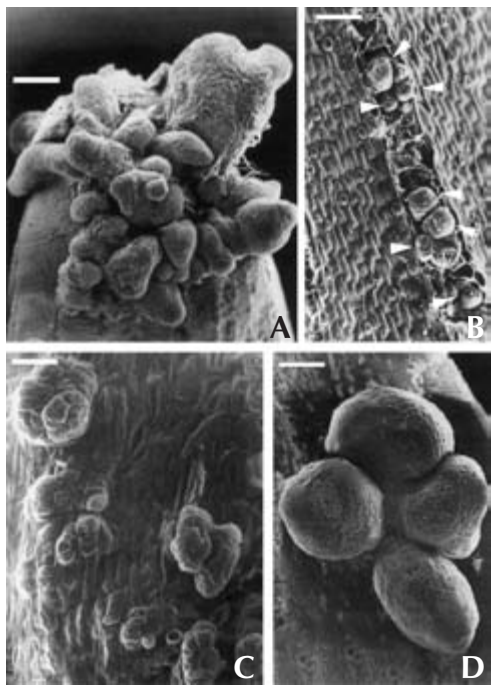


FIG. ONC-2. **Scanning electron photographs of somatic embryos (SEs) and protocorm-like bodies (PLBs) of *Oncidium* Gower Ramsey. A. Cluster of SEs at the tips of a leaf explant (scale bar = 500 μm). B. Small globular SEs (arrowheads) which formed from mesophyll cell located under the leaf surface (scale bar = 110 μm). C. Small PLBs which formed from SEs on the surface of a leaf explant (scale bar = 75 μm). D. A cluster of four PLBs which formed from SEs (scale bar = 300 μm). (Modified from Chen et al., 1999.)**

General Comments. This method is innovative and very interesting because it involves the production of SEs, which give rise to PLBs and plantlets (Fig. Onc-2). It is also very efficient because several explants can be taken from one plantlet in vitro and every explant produces many SEs and PLBs. Other favorable aspects of this procedure are the use of low hormone levels and elimination of excessive proliferation, both of which reduce the likelihood of mutations. Follow up research showed that the auxins IAA, IBA, 2,4-D, and NAA inhibited somatic embryogenesis whereas the cytokinins BA, 6-furfurylaminopurine (kinetin), 6-[4-hydroxy-3-methylbut-2-enylaminopurine (zeatin), N^6-[2-isopentenyl]-adenine (2iP), and TDZ enhanced it. TDZ at 1 mg l^{-1} was the most effective cytokinin (Chen and Chang, 2001).

Effects of Ancymidol and Paclobutrazol on Somatic Embryogenesis in Leaf Explants of *Oncidium*

Attempts to increase the production of somatic embryos by leaf explants of *Oncidium* Gower Ramsey showed that ancymidol (ANC) and paclobutrazol (PAC) can bring about higher yield (Chen and Chang, 2003). Except as indicated below the protocol is the same as in the preceding method.

Plant Material. Tips, approximately 1 cm long, were taken from the same explant sources as in the preceding procedure.

Surface Sterilization. There is no need to surface-sterilize explants taken from plantlets which are growing in vitro. However they must be washed with sterile distilled water to remove medium residues if any.

Culture Vessels. Test tubes, 20×150 mm were used in the original research. Erlenmeyer flasks and other containers filled with medium to 25–30% of their capacity are also suitable.

Culture Conditions. Cultures should be kept in the dark at 26 ± 2°C for 20–30 days to bring about the formation of PLBs and somatic embryos (SEs). PLBs and SEs produced in the dark should be maintained at 26 ± 2°C under 16-h photoperiods of 28–36 μmol m^{-2} s^{-1} provided by 40-W daylight fluorescent tubes (FL-30D/29, China Electric Co., Taipei, Taiwan). Standard culture room conditions will probably also be suitable for the PLBs and SEs and for shoot and root production.

Culture media. Two different initial media can be used:

1 Thidiazuron (TDZ) in Table Onc-7 is replaced with 2.5 mg ANC l^{-1} which must be added to the medium after autoclaving. Dissolve 2.5 mg ANC in 2 ml distilled water, bring up to 5 ml with 95% ethanol, shake vigorously and add to the autoclaved medium, mix well and distribute to preautoclaved culture vessels.
2 PAC, 10 mg l^{-1} is used to replace TDZ in Table Onc-7 and added as ANC above.

TABLE ONC-9. **Effects of ancymidol and paclobutrazol on somatic embryo formation on leaf explants of *Oncidium* Gower Ramsey (Chen and Chang, 2003)**

	Percent embryogenesis				
Substance	Leaf tip	Adaxial side	Abaxial side	Cut end	Embryos dish^{-1}
Ancymidol, 2.5 mg l^{-1}	87.5	57.5	0	27.5	154.8
Paclobutrazol, 10 mg l^{-1}	80.0	52.5	0	62.5	193.2

A large number of embryos were obtained with these substances in the original research (Table Onc-9). Both ANC and PAC are available from www.sigmaaldrich.com. PLBs and shoots formed from these embryos should be cultured on the same medium (Table Onc-8) as in the preceding method.

Procedure. The procedure is the same as that in the preceding method. Explants are cultured on the first medium (Table Onc-7 modified as in point 1 or 2 above). Somatic embryos, PLBs, and shoots obtained on the first medium are treated as in the preceding method and cultured on the second medium (Table Onc-8).

Developmental Sequence. As in the preceding method.

General Comments. Caution is necessary because it is not known whether ANC and PAC can induce undesirable mutations.

Somatic Embryogenesis in Callus Cultures of *Oncidium*

Several micropropagation methods were developed for *Oncidium* hybrids using a variety of explants. Experiments at the Institute of Botany, Academia Sinica, Taipei, Taiwan led to the establishment of totipotent calli which can be proliferated and used to produce somatic embryos (SEs) and plants (Chen and Chang, 2000*a*).

Plant Material. Stem internodes, 5 mm long; leaves, 2–4 cm and 5–7 cm long; and root tips, 1 cm long, were taken from in vitro growing plants derived from flower-stalk buds and were cultured to produce callus.

Surface Sterilization. Explants taken from plants growing in vitro do not require surface sterilization. However, they should be rinsed with sterile distilled water to remove medium residues.

Culture Vessels. Culture tubes, 20 × 150 mm were used in the original research. Other containers filled with medium to 25–30% of their volume can also be used.

Culture Conditions. To induce callus formation cultures should be kept in the dark at 26 ± 1°C. Cultures for somatic embryogenesis (SEG) were maintained at 26 ± 1°C under 16-h photoperiods of 28–36 μmol m^{-2} s^{-1} provided by daylight fluorescent tubes (FL-30D/29, 40 watt, China Electric Co., Taipei, Taiwan). Standard culture room conditions are also suitable for SEs.

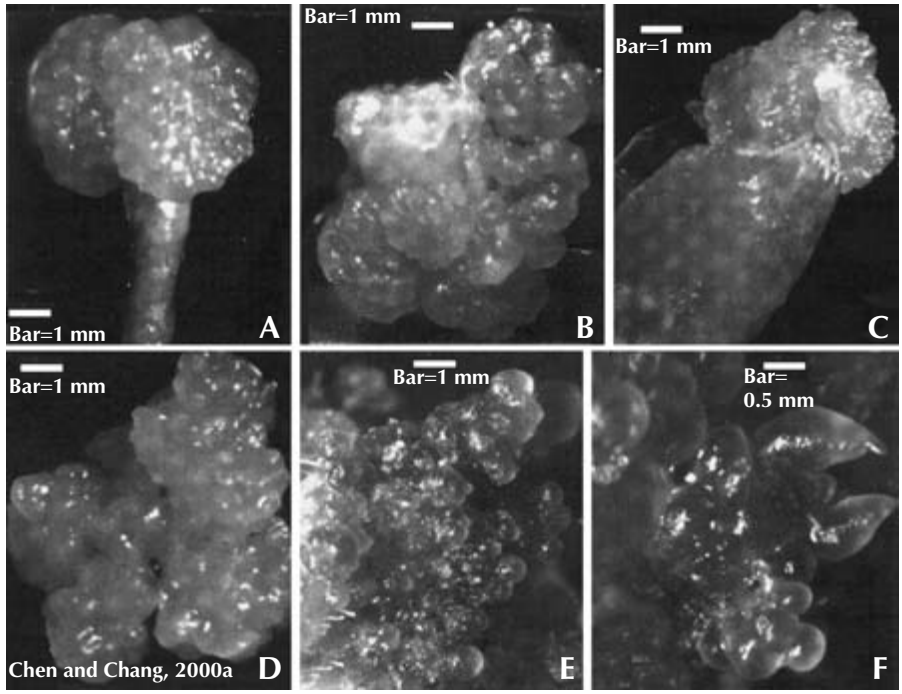


FIG. ONC-3. **Callus induction and somatic embryogenesis in *Oncidium* Gower Ramsey. A. Callus production by tip of root explant. B. Cut surfaces of stem explant producing callus. C. Callus arising from cut surface of leaf explant. D. Compact yellow callus after five subcultures. E. Callus turns green and produces somatic embryos in the light. F. Somatic embryos enlarge, elongate, and produce protocorm-like bodies. (Chen and Chang, 2000*a*.)**

Culture Media. Several media must be used depending on explant and intended results. Stem and root explants produce callus (Fig. Onc-3A, B, D, E) when placed on modified half-strength MS medium (Murashige and Skoog, 1962) supplemented with 3 mg each of 2,4-D and TDZ per liter (Table Onc-10). The callus should be subcultured on the same medium for proliferation. Leaf explants produce callus (Fig. Onc-3C) on half-strength MS containing 10 mg 2,4-D l^{-1} and 3 mg TDZ l^{-1} (Table Onc-11). The same medium (Table Onc-11) should be used to proliferate the leaf callus. Root and leaf callus forms SEs (Fig. Onc-3A, C–F) on half-strength MS with 0.1 mg NAA l^{-1} and 3 mg TDZ l^{-1} (Table Onc-12). SEs form on stem-derived callus on half-strength MS without hormones (Table Onc-13). Each callus produces PLBs and plantlets (Fig. Onc-4A–E, G) on the SE-inducing medium (Table Onc-12 for root and leaf callus and Table Onc-13 for leaf callus). Plantlets can be grown to deflasking in these media or on solutions using to grow plantlets of other species and hybrids (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Den-2, Den-12, Den-21, and Onc-5). Plantlets should be taken out of the culture vessels and potted in sphagnum moss.

TABLE ONC-10. **Modified half-strength Murashige–Skoog (½MS) medium (Murashige and Skoog, 1962) for the culture of stem and root explants of *Oncidium* Gower Ramsey (Chen and Chang, 2000*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	2,4-Dichlorophenoxyacetic acid (2,4-D)	3.0	300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
12	Thidiazuron (TDZ)	3.0	300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
13	Peptone	1.0 g	No stock	No stock	Weigh
	Vitamins				
14	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
16	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses. If the auxin or cytokinin does dissolve add a few drops of 1 N KOH and HCl respectively.

[f]Available from www.sigmaaldrich.com.

[g]Add items 1–16 to 900 ml of distilled water (item 18), adjust pH to 5.2, add sugar (item 17), and raise volume to 1000 ml with distilled water (item 18). Add the gellan gum to the cold solution as indicated in footnote *h*, distribute the medium to culture vessels and autoclave. It is preferable not to autoclave hormones and vitamins, but in this medium they can be autoclaved for 15 min at 121°C.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE ONC-11. **Modified half-strength Murashige–Skoog (½MS) medium (Murashige and Skoog, 1962) for the culture of leaf explants of *Oncidium* Gower Ramsey (Chen and Chang, 2000*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	2,4-Dichlorophenoxyacetic acid (2,4-D)	10.0	1000 mg 400 ml^{-1} 95% ethanol[e,f]	4	
	Cytokinin				
12	Thidiazuron (TDZ)	3.0	300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
13	Peptone	1.0 g	No stock	No stock	Weigh
	Vitamins				
14	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
16	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses. If the auxin or cytokinin does dissolve add a few drops of 1 N KOH and HCl respectively.

[f]Available from www.sigmaaldrich.com.

[g]Add items 1–16 to 900 ml of distilled water (item 18), adjust pH to 5.2, add sugar (item 17), and raise volume to 1000 ml with distilled water (item 18). Add the gellan gum to the cold solution as indicated in footnote *h*, distribute the medium to culture vessels and autoclave. It is preferable not to autoclave hormones and vitamins, but in this medium they can be autoclaved for 15 min at 121°C.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE ONC-12. **Modified half-strength Murashige–Skoog (½MS) medium (Murashige and Skoog, 1962) for the induction of somatic embryos on callus derived from leaf and root explants of *Oncidium* Gower Ramsey (Chen and Chang, 2000*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	Naphthaleneacetic acid (NAA)	0.1	10 mg 400 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
12	Thidiazuron (TDZ)	3.0	300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
13	Peptone	1.0 g	No stock	No stock	Weigh
	Vitamins				
14	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
16	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses. If the auxin or cytokinin does dissolve add a few drops of 1 N KOH and HCl respectively.

[f]Available from www.sigmaaldrich.com.

[g]Add items 1–16 to 900 ml of distilled water (item 18), adjust pH to 5.2, add sugar (item 17), and raise volume to 1000 ml with distilled water (item 18). Add the gellan gum to the cold solution as indicated in footnote *h*, distribute the medium to culture vessels and autoclave. It is preferable no to autoclave hormones and vitamins, but in this medium they can be autoclaved for 15 min at 121°C.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE ONC-13. **Modified half-strength Murashige–Skoog (½MS) medium (Murashige and Skoog, 1962) for the induction of somatic embryos on callus derived from stem explants of *Oncidium* Gower Ramsey (Chen and Chang, 2000*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Complex additive				
11	Peptone	1.0 g	No stock	No stock	Weigh
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]Available from www.sigmaaldrich.com.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.2, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Add the gellan gum to the cold solution as indicated in footnote *h*, distribute the medium to culture vessels and autoclave. It is preferable not to autoclave hormones and vitamins, but in this medium they can be autoclaved for 15 min at 121°C.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

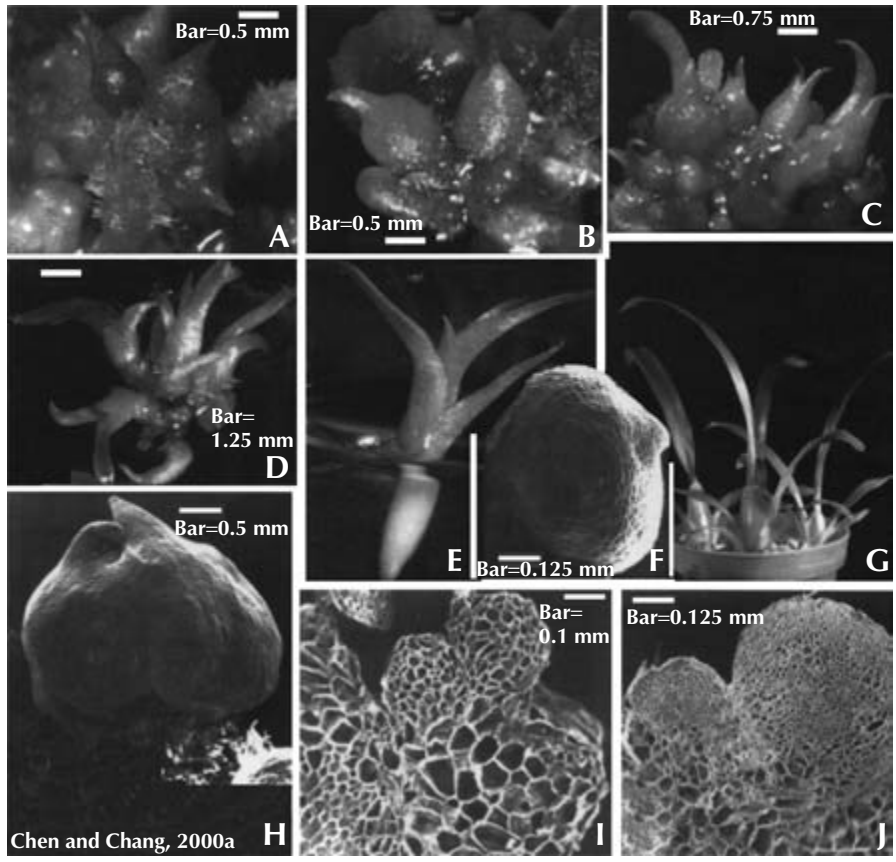


FIG. ONC-4. **Somatic embryogenesis and plantlet formation in *Oncidium* Gower Ramsey. A. Cluster of somatic embryos (SEs) and PLBs with shoot-tip-like protrusions (top) and rhizoids (bottom). B. Two young SE derived PLBs with sheath leaves. C. Several SE derived PLBs with expanding leaves. D. SE derived PLBs in the process of producing shoots. E. Rooted plantlet. F. SE derived PLBs. G. Potted plantlets with pseudobulbs. H. Young SE derived PLBs with leaf initials. I. Two SEs starting to develop on the outer layer of a callus mass. J. Two SEs with cells which are smaller than those of the callus from which they originated. (Chen and Chang, 2000*a*.)**

Procedure. Explants should be paced on callus-inducing media (Table Onc-10 for stem and root explants and Table Onc-11 for leaf explants) and cultured in the dark for induction and proliferation. Once there is enough callus sections should be moved to SE-inducing medium (Table Onc-12 for root and leaf callus and Table Onc-13 for leaf callus) in the light. There the yellowish callus will turn green and produce PLBs, SEs, and plantlets (Figs Onc-2 and Onc-3). Plantlets should be moved to fresh medium of the same type or one of the other solutions listed above.

Developmental Sequence. Explants give rise to callus on the first media (Table Onc-10 for stem and root explants and Table Onc-11 for leaf explants) in the dark. This callus is yellow-white, compact, and embryogenic. The highest percentage of

callus production (25% of explants) is by root explants. Root callus is also the best growing and has the highest proliferation rate. Stem and leaf explants have a lower percentage of callus production (5–10%) and do not proliferate as well. Almost all of the SEs produced plantlets which grew well with nearly 100% survival when potted in sphagnum moss and grown in a greenhouse.

General Comments. This is an efficient and uncomplicated procedure which should prove to be very useful in commercial laboratories. However, the high levels of 2,4-D in two media (Tables Onc-10 and Onc-11) are a reason for concern because they can cause undesirable mutations.

Effects of Culture Conditions and Explant Characteristics on Somatic Embryogenesis of *Oncidium* Gower Ramsay

A considerable amount of research was carried out at the Institute of Botany, Academia Sinica in Taiwan on micropropagation, tissue culture, and somatic embryogenesis of *Oncidium* Gower Ramsay (Chen and Chang, 2002). This research included studies on the effects of culture conditions, some medium components, and explant characteristics (Chen and Chang, 2002). The findings are with leaf sections but they may apply to other explants cultured by these investigators (Chen and Chang, 2000*a*, 2000*b*, 2001, 2002, 2003; Chen et al., 1999) as shown in the tables and parts of the abstract (taken from Chen and Chang, 2002).

Plant material. Explants were taken from 2-month-old plantlets grown in vitro.

Surface Sterilization. Explants taken from plantlets growing in vitro do not require surface sterilization. However, they should be washed with sterile distilled water to remove medium residues.

Culture Vessels. Culture tubes, 20×150 mm, were used in the original research. Other containers are also suitable.

Culture Conditions. Cultures should be maintained at $26 \pm 1°C$ in the dark or under 16-h photoperiods of 28–36 $\mu mol\ m^{-2}\ s^{-1}$ produced by 40-W daylight fluorescent tubes FL-30D/29 manufactured by the China Electric Co. (Taipei, Taiwan). The following quotes are taken from the abstract in Chen and Chang (2002):

> Embryo formation was significantly affected by explant position. Leaf tip segments [table 1 in box] had a significantly higher embryogenic response than other segments of leaves. Adaxial-side-up orientation significantly promoted embryogenesis in comparison with abaxial-side-up orientation [table 2 in box]. . . . There was no significant effect of sucrose in a range of concentrations (10–60 $g\ l^{-1}$) [table 3 in box]. [*Comment*: The most suitable concentrations are 10 or 20 g sucrose l^{-1}.]
>
> Modified $^1/_2$MS medium (containing 85 $mg\ l^{-1}$ KH_2PO_4) supplemented with 170 $mg\ l^{-1}$ NH_2PO_4 significantly promoted direct somatic embryogenesis [table 4 in box].

Culture Medium. The results of this research suggest an optimal medium for leaf explants (Table Onc-14).

Tables of relevance to quotes from the abstract in Chen and Chang (2002)

TABLE 1. **Effect of explant position on the donor leaf on direct somatic embryogenesis from leaf explants of *Oncidium* "Gower Ramsey"**[a]

Explant position	% embryogenesis	Embryos explant^{-1}
S1 (leaf tip segment)	65 a	11.2
S2	5 b	5.0
S3	0 b	0
S4	10 b	10.8
S5	10 b	9.8
S6 (leaf base segment)	5 b	11.0

[a]Six 5-mm-long leaf segments from tip (segment 1. S1) to base (S6) were excised from 3-cm-long leaves of *in vitro* plantlets. Explants were placed adaxial side up on the surface of medium. Data were scored after 40 days in culture. Means of 20 replicates (explants) with the same letters are not significantly different at $p < 0.05$ (Duncan, 1955).

TABLE 2. **Effect of explant orientation on direct somatic embryogenesis on leaf explants of *Oncidium* "Gower Ramsey"**[a]

	% embryogenesis				
Orientation	T	Ad	Ab	C	Embryos. explant^{-1}
Ad	75 a	40 a	0 a	15 a	10.7
Ab	15 b	15 b	0 a	10 a	8.0

[a]1-cm-long *in vitro* leaves were used as explants and placed with either adaxial (Ad) or abaxial (Ab) side up. The percentage of embryo-produced explants and average embryos per embryo-producing explants were scored on various regions of leaf explants, including leaf tip (T), adaxial side (Ad), abaxial side (Ab) and cut end (C). Data were scored after 40 days in culture. Means of 20 replicates (explants) with the same letters are not significantly different at $p < 0.05$ (Duncan, 1955).

TABLE 3. **Effect of sucrose on direct somatic embryogenesis on leaf explants of *Oncidium* "Gower Ramsey"**[a]

	% embryogenesis				
Sucrose (g l^{-1})	T	Ad	Ab	C	Embryos. explant^{-1}
0	0 b	0 b	0 a	0 a	0
10	75 a	35 a	0 a	15 a	10.9
20	75 a	40 a	0 a	15 a	10.7
30	65 a	35 a	0 a	20 a	9.4
60	70 a	35 a	0 a	10 a	6.2

[a]1-cm-long *in vitro* leaves were used as explants and placed with either adaxial (Ad) or abaxial (Ab) side up. The percentage of embryo-produced explants and average embryos per embryo-producing explants were scored on various regions of leaf explants, including leaf tip (T), adaxial side (Ad), abaxial side (Ab) and cut end (C). Data were scored after 40 days in culture. Means of 20 replicates (explants) with the same letters are not significantly different at $p < 0.05$ (Duncan, 1955).

TABLE 4. **Effect of NaH_2PO_4 on direct somatic embryogenesis on leaf explants of *Oncidium* "Gower Ramsey"**[a]

	% embryogenesis				
NaH_2PO_4 (mg l^{-1})	T	Ad	Ab	C	Embryos explant^{-1}
0	45 b	25 a	0 a	5 a	9.3
17	50 ab	30 a	0 a	10 a	10.0
42.5	45 b	30 a	0 a	10 a	9.4
85	75 a	35 a	0 a	10 a	10.3
170	75 a	40 a	0 a	15 a	10.7

[a]1-cm-long *in vitro* leaves were used as explants and placed with either adaxial (Ad) or abaxial (Ab) side up. The percentage of embryo-produced explants and average embryos per embryo-producing explants were scored on various regions of leaf explants, including leaf tip (T), adaxial side (Ad), abaxial side (Ab) and cut end (C). Data were scored after 40 days in culture. Means of 20 replicates (explants) with the same letters are not significantly different at $p < 0.05$ (Duncan, 1955).

TABLE 5. **Effects of casein hydrolysate (C. H.), peptone and glutamine on direct somatic embryogenesis on leaf explants of *Oncidium* "Gower Ramsey"**[a]

Medium composition		% embryogenesis				Embryos explant^{-1}
		T	Ad	Ab	C	
Control		50 bc	35 a	0 a	5 a	9.8
C. H.	0.1 g l^{-1}	50 bc	35 a	0 a	15 a	9.8
	0.5	55 abc	40 a	0 a	10 a	9.0
	1	70 ab	40 a	0 a	10 a	9.7
Peptone	0.1	75 ab	40 a	0 a	15 a	9.6
	0.5	80 a	40 a	0 a	15 a	10.0
	1	75 ab	40 a	0 a	15 a	10.7
Glutamine	0.1	55 abc	30 a	0 a	10 a	9.3
	0.5	55 abc	25 a	0 a	15 a	8.4
	1	40 c	25 a	0 a	5 a	9.0

[a]1-cm-long *in vitro* leaves were used as explants and placed with either adaxial (Ad) or abaxial (Ab) side up. The percentage of embryo-produced explants and average embryos per embryo-producing explants were scored on various regions of leaf explants, including leaf tip (T), adaxial side (Ad), abaxial side (Ab) and cut end (C). Data were scored after 40 days in culture. Means of 20 replicates (explants) with the same letters are not significantly different at $p < 0.05$ (Duncan, 1955).

TABLE ONC-14. **Modified half-strength Murashige–Skoog (½MS) medium (Murashige and Skoog, 1962) optimized for the induction of somatic embryos on leaf section explants of *Oncidium* Gower Ramsey (Chen and Chang, 2000*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
11	Thidiazuron (TDZ)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
12	Peptone	0.5 g	No stock	No stock	Weigh
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
16	Sucrose	10.0–20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Gelrite[g,h]	2.2–2.5 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses. If the cytokinin does dissolve add a few drops of 1 N HCl.

[f]Available from www.sigmaaldrich.com.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.2, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Add the Gelrite (item 18) to the cold solution as indicated in footnote *h*, distribute the medium to culture vessels and autoclave. It is preferable not to autoclave hormones and vitamins, but in this medium they can be autoclaved for 15 min at 121°C.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

> The best response on direct embryo formation was obtained on the modified 1/2MS medium supplemented with 10–20 g l^{-1} sucrose, 170 mg l^{-1} NaH_2PO_4 and 0.5 g l^{-1} peptone. . . . Peptone at 0.5 mg l^{-1} gave significantly higher embryogenic response (80%) on leaf tips than [the] control treatment (50%) [table 5 in box]. [*Comment*: The effects of 1 g casein hydrolysate (CH) l^{-1} are nearly the same as those of 0.5 g peptone l^{-1}.]

Procedure. Explants should be placed on the optimal medium (Table Onc-14) to bring about production of somatic embryos (SEs). Once there are enough SEs they should be cultured as in the other procedures by these authors in this section.

Developmental Sequence. SEs form on the explants as described in previous procedures for *Oncidium* by the same authors(Chen and Chang, 2000*a*, 2000*b*, 2001, 2002, 2003; Chen et al., 1999).

General Comments. The optimal medium (Table Onc-14) was developed with/for leaf sections but may be suitable for other explants and should be tested not only with *Oncidium* but also related orchids. Most tissue culture and micropropagation media are developed empirically. The authors should be commended for the systematic studies of explant sources and positioning as well as the nature and concentrations of some components (their tables 1–5, as shown in the box).

Plantlet Production from Flower-stalk Explants of *Oncidium* Sweet Sugar via Embryo and Bud Formation

Culture of leaf explants taken from plantlets in vitro is an efficient means of clonal propagation for *Oncidium*, but removal of leaves from small plantlets can damage them. The culture of shoot tips may endanger an entire plant. Explants from flower stalks do not have such drawbacks and can be used for mass rapid clonal propagation (Chen and Chang, 2000*b*).

Plant Material. Explants, 5 mm in length (Fig. Onc-5), are taken from internodes of flower stalks (approximately 15–20 cm long) produced by 3–4-year-old potted plants of *Oncidium* Sweet Sugar (Figs Onc-6C and Onc-7H) when the flower buds are fully formed but before the appearance of flower color.

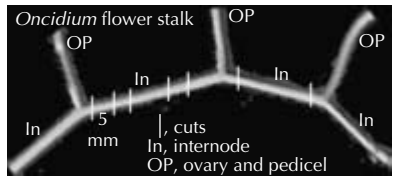


FIG. ONC-5. **Part of an *Oncidium* flower stalk showing internodes, remnants of pedicels and ovaries, and location of cuts (not to scale). (Photograph by J. Arditti.)**

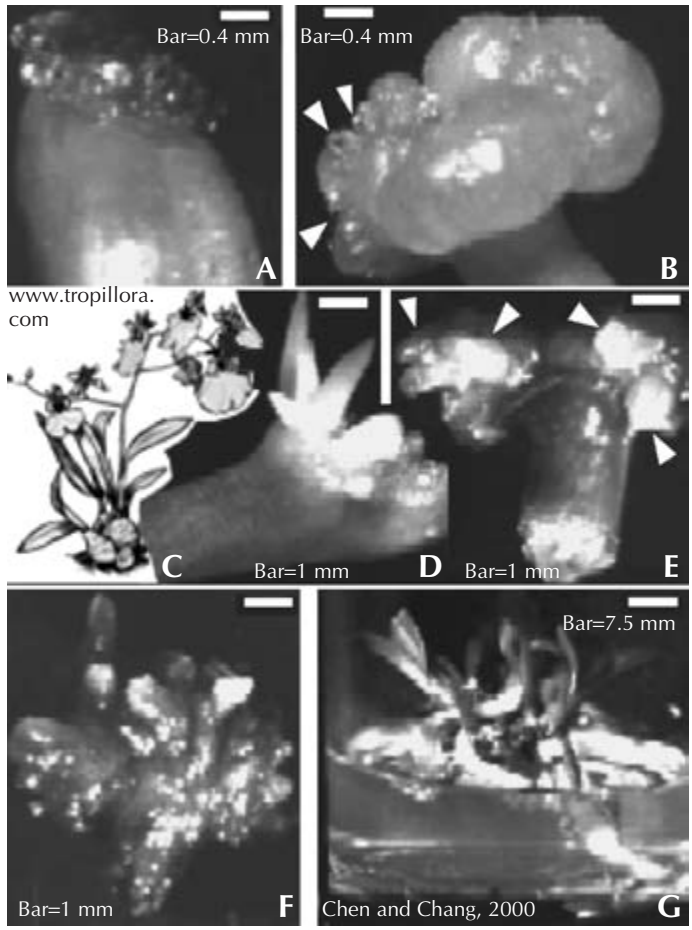


FIG. ONC-6. **Formation of somatic embryos (SEs), vegetative buds (VBs), leaves, and plantlets from flower-stalk explants of *Oncidium* Sweet Sugar. A. SEs on the wound surface of a flower-stalk explant. B. SEs (wedges) formed via nodular masses in a cut surface of a flower-stalk explant. C. *Oncidium* Sweet Sugar. D. VBs on the cut surface of a flower-stalk explant. E. VB (wedges) on the wound surfaces of a flower-stalk explant formed via nodular masses. F. SEs still on the explant forming abnormal nodular masses. G. Leaves and roots produced from the flower-stalk explant. (Sources: A, B, D–G, Chen and Chang, 2000*b*; C, www.tropillora.com.)**

Surface Sterilization. The explants should be washed with running water and a mild detergent, rinsed with water, immersed in 0.25% (w/v) mercuric chloride (www.fishersci.com, www.vwr.com, www.thomassci.com, www.sigmaaldrich.com) for 5 min (this a toxic substance which must be handled with care) and rinsed three times with sterile distilled water.

Culture Vessels. Test tubes, 20×120 mm and other culture vessels filled with medium to 20–30% of their volume are suitable.

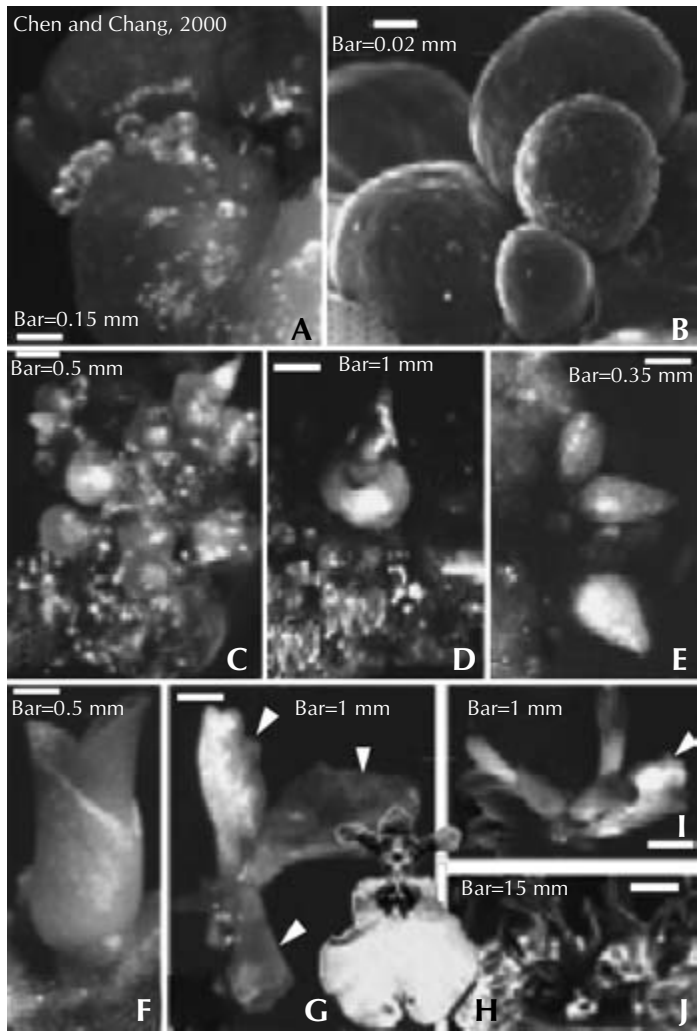


FIG. ONC-7. **Production of plantlets (P), PLBs, vegetative buds (VBs), callus, leaves, abnormal tissues, and roots by explants of *Oncidium* Sweet Sugar in vitro. A. Somatic embryos (SEs) on callus. B. Scanning electron micrograph of SEs on callus. C. Enlarged green SEs cultured under illumination. D. SEs derived from PLBs with two sheaths. E. VB on C. F. VBs with leaves. G. A yellow abnormal tissue with three petal-like structures (wedges). H. *Oncidium* Sweet Sugar flower. I. Petal-like structure (wedge) adjacent to two VBs on callus. J. Rooted plantlets with pseudobulbs ready for planting in sphagnum. (Sources: A–G, I, J, Chen and Chang, 2000*b*; H, photograph by Joseph Arditti.)**

Culture Conditions. Explants should be cultured at 26 ± 1°C in the dark for 20–30 days to induce the formation of somatic embryos (SEs) and vegetative buds (VBs). SEs and VBs should be cultured at 26 ± 1°C under 16-h photoperiods of 26–36 μmol m^{-2} s^{-1} [40-W daylight fluorescent tubes FL-30D/29 produced by China Electric Co. (Taipei, Taiwan) were used in the original research]. Standard culture room conditions should also prove to be suitable.

Culture Media. To bring about the formation of SEs and/or VBs (Figs Onc-6A, B, D, E and Onc-7E) explants should be cultured on a modified half-strength MS medium (Murashige and Skoog, 1962) containing 1 mg TDZ l^{-1} (Table Onc-15). SBs form on wound surfaces, produce leaves, and eventually form plantlets (Fig. Onc-6G) when cultured on half-strength MS containing 0.5 mg NAA l^{-1} (Table Onc-16). Tissues from the first medium (Table Onc-15) produce SEs (Fig. Onc-7A, B), callus, PLBs (Fig. Onc-7C), and eventually leaves (Fig. Onc-7D) when subcultured on a solution containing 1 mg TDZ l^{-1} and 1 mg NAA l^{-1} (Table Onc-17). PLBs form plantlets on a hormone free half-strength MS (Table Onc-18). VBs with two leaves produce roots on half-strength MS, the second medium (Table Onc-16). Plantlets (Fig. Onc-7J) taken out of flasks should be potted in sphagnum moss.

Developmental Sequence. Most developmental steps are outlined in the preceding section. If not cultured on an appropriate medium for 1.5–2 months the SEs and VBs form green abnormal structures (Fig. Onc-6F) which turn brown eventually and die. VBs produce leaves on the first medium (Fig. Onc-7F; Table Onc-15). Yellowish abnormal structures (Fig. Onc-7G, I) form if TDZ is present in the medium at 3 mg l^{-1}. SE-, VB-, and PLB-derived plants grow well on appropriate media and produce pseudobulb-bearing plantlets (Fig. Onc-7J) after 2–3 months of culture. When potted in sphagnum and grown in a greenhouse these plantlets survive very well (100%).

To Autoclave or Not to Autoclave

A frequently asked question is whether vitamins, hormones, and other organic additives can be autoclaved. A little known, but excellent publication by van Bragt et al. (1971), "Effects of sterilization on components in nutrient media," contains many answers. The information below is from it.

Vitamins: "addition of vitamins to media before [autoclaving] is not advisable."
Abscisic acid: Both the *cis* and *trans* isomers are not affected by autoclaving.
Auxins: "Sterilization by autoclaving at 110°–120° [for] 15–60 min does not destroy" NAA and 2,4-D. "IAA is [destroyed] under . . . low pH. . . ." Therefore it may not be necessary to cold-sterilize NAA and 2,4-D, but IAA should not be autoclaved.
Cytokinins: BA, isopentenyl adenosine, kinetin, and zeatin can be autoclaved.
Gibberellins: "Autoclaving reduced . . . activitiy of GA_3 by more than 90%." In other words, gibberellins should not be autoclaved.
Precautions: Despite this advice it is wise to test autoclaved media which contain these substances before using them for explants from rare and/or expensive orchids.

These substances should be added to research solutions after autoclaving during initial experiments and only subsequently before sterilization.

As a precaution the recommendations for many media in this book are to add hormones, vitamins, and other organic substances after autoclaving. They can be added following sterilization only after preliminary tests.

TABLE ONC-15. **Modified half-strength Murashige–Skoog (½MS) medium (Murashige and Skoog, 1962) optimized for the culture of flower-stalk explants of *Oncidium* Sweet Sugar (Chen and Chang, 2000*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
11	Thidiazuron (TDZ)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
12	Peptone	1.0 g	No stock	No stock	Weigh
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses. If the cytokinin does dissolve add a few drops of 1 N HCl.

[f]Available from www.sigmaaldrich.com.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.2, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Add the gellan gum to the cold solution as indicated in footnote *h*, distribute the medium to culture vessels and autoclave. It is preferable not to autoclave hormones and vitamins, but in this medium they can be autoclaved for 15 min at 121°C.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE ONC-16. **Modified half-strength Murashige–Skoog (½MS) medium (Murashige and Skoog, 1962) for the production of vegetative buds and plantlets from flower-stalk explants of *Oncidium* Sweet Sugar (Chen and Chang, 2000*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	Naphthaleneacetic acid (NAA)	0.5	50 mg 400 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
12	Peptone	1.0 g	No stock	No stock	Weigh
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses. If the auxin or cytokinin does dissolve add a few drops of 1 N KOH and HCl respectively.

[f]Available from www.sigmaaldrich.com.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.2, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Add the gellan gum to the cold solution as indicated in footnote *h*, distribute the medium to culture vessels and autoclave. It is preferable not to autoclave hormones and vitamins, but in this medium they can be autoclaved for 15 minutes at 121°C.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE ONC-17. **Modified half-strength Murashige–Skoog (½MS) medium (Murashige and Skoog, 1962) for the induction of callus and somatic embryos from flower-stalk explants of *Oncidium* Sweet Sugar (Chen and Chang, 2000*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	Naphthaleneacetic acid (NAA)	1.0	100 mg 400 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
12	Thidiazuron (TDZ)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
13	Peptone	1.0 g	No stock	No stock	Weigh
	Vitamins				
14	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
16	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses. If the auxin or cytokinin does dissolve add a few drops of 1 N KOH and HCl respectively.

[f]Available from www.sigmaaldrich.com.

[g]Add items 1–16 to 900 ml of distilled water (item 18), adjust pH to 5.2, add sugar (item 17), and raise volume to 1000 ml with distilled water (item 18). Add the gellan gum to the cold solution as indicated in footnote *h*, distribute the medium to culture vessels and autoclave. It is preferable not to autoclave hormones and vitamins, but in this medium they can be autoclaved for 15 min at 121°C.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE ONC-18. **Modified half-strength Murashige–Skoog (½MS) medium (Murashige and Skoog, 1962) for plantlets from protocorm-like bodies (PLBs) produced by somatic embryos derived from flower-stalk explants of *Oncidium* Sweet Sugar (Chen and Chang, 2000*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Complex additive				
11	Peptone	1.0 g	No stock	No stock	Weigh
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]Available from www.sigmaaldrich.com.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.2, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Add the gellan gum to the cold solution as indicated in footnote *h*, distribute the medium to culture vessels and autoclave. It is preferable not to autoclave hormones and vitamins, but in this medium they can be autoclaved for 15 min at 121°C.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

In Vitro Production of Plantlets by Proliferation of Protocorm-like Bodies of *Oncidium* Gower Ramsay

Graceful inflorescences with golden "dancing lady" flowers make *Oncidium* Gower Ramsey a very desirable cut flower orchid. Demand for plants is high. Therefore a method for mass rapid clonal propagation was developed at the C. C. Shroff Institute in Bombay, India (Badge and Sharon, 1997).

Plant Material. Apical meristems should be taken from healthy mature plants.

Surface Sterilization. Plants or parts from which explants will be taken should be washed with running water and a mild detergent (2% Teepol available from www.sigmaaldrich.com was used in the original research) until all debris, loose particles, and potting mix are removed and then rinsed three times with sterile distilled water. This was done during the original research, but rinsing with sterile distilled water prior to treatment with a surface sterilizing agent seems unnecessary – tap water is sufficient. After the rinse explants should be immersed for 15 min in 2% sodium hypochlorite (38 or 33 ml of 5.25 or 6% Clorox, respectively, made to 100 ml with distilled water) with stirring or agitation and rinsed with sterile distilled water three times (the use of sterilized water is a must at this stage).

Culture Vessels. Culture tubes with the medium dried on a slant were used in the original research (size was not indicated; 20 × 150 mm tubes are suitable). Other containers are also suitable.

Culture Conditions. Explants should be maintained at 22 ± 2°C and 65 ± 5% relative humidity in the dark for the first 48 h and after that under 16-h photoperiods of 3000 lx. Standard culture room conditions are also suitable after the initial dark period.

Culture Media. Explants should be placed on modified Vacin and Went (VW) medium (Vacin and Went, 1949) containing 150 ml coconut water (Table Onc-19) for the induction of callus. The callus should be subcultured on the same medium (Table Onc-19) for proliferation and formation and multiplication of PLBs. The PLBs produce plantlets when cultured on modified MS medium (Murashige and Skoog, 1962) containing 200 mg casein hydrolysate (CH) l^{-1} (Table Onc-20). Plantlets which form on the second medium can either be cultured on it or moved to another solution (see Tables Aranda-6 to Aranda-8, Anth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Den-2, Den-12, Den-21, Odnta-1, Onc-5, Pln-1, and V-4) for growth (this step is not part of the original paper).

Procedure. Explants are taken after the initial washing of the plants, sterilized and cultured on the first medium (Table Onc-19). When callus forms it is subcultured on the same medium (Table Onc-19) to proliferate it and to produce PLBs. The PLBs can be multiplied on this medium (Table Onc-19) or moved to modified MS (Table Onc-20) for plantlet production.

TABLE ONC-19. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of *Oncidium* Gower Ramsay meristems, callus proliferation, and protocorm-like body (PLBs) formation and multiplication (Badge and Sharon, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Complex additive				
9	Coconut water[e]	150.0 ml	No stock	No stock	Measure
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to the culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Add items 1 and 3–7 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 3), bring volume to 900 ml with distilled water (item 10), adjust pH to 5.1, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

Developmental Sequence. The explants form callus on the first medium (Table Onc-19). When subcultured on the same medium (Table Onc-19) the callus proliferates and forms PLBs. The PLBs increase in number on this medium (Table Onc-19). On the second medium (Table Onc-20) PLBs continue to proliferate and older ones (1–2 months in age) form plantlets. Once formed the plantlets grow well on the second medium (Table Onc-20) or one of the other media (see Tables Aranda-6 to Aranda-8, Anth-5C-3, C6, C-19, Cym-1 to Cym-3, Cym-5, Den-2, Den-12, Den-21, Onc-5, Odnta-1, Pln-1, and V-4).

TABLE ONC-20. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for plantlet production from protocorm-like bodies of *Oncidium* Gower Ramsey (Badge and Sharon, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Complex additive				
10	Casein hydrolysate[g]	200.0	No stock	No stock	Weigh
	Auxin[f]				
	Cytokinin[f]				
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[h]	To 1000 ml			
	Solidifier				
16	Agar[h]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]MS usually contains auxin and/or cytokinin, but there is no indication that this is the case for this modification.

[g]Add items 1–13 to 700 ml of distilled water (item 15), adjust pH to 5.7, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. As a rule amino acids and hormones should be added after a medium has been autoclaved, but in this case there is no need to do so.

General Comments. Root and leaf explants were also cultured as part of this research. Leaf explants produced callus on VW but subsequent growth was poor. Root explant cultures were overrun by internal contaminants. This is not surprising because orchid roots which grow in a potting medium or come into contact with other surfaces contain mycorrhizal fungi. Therefore root explants should be taken from aerial roots which have not come into contact with potting mix or any other surface. Such roots will probably be free of mycorrhiza and have a chance of developing in culture. A great advantage of this protocol is that it makes possible propagation of outstanding cultivars because the explants are taken from mature plants.

Plantlet Production of *Oncidium bifolium* from Leaf Explants

Oncidium bifolium, one of the southernmost members of its genus, is a relatively small orchid. A method for plantlet production was developed at the Faculdad de Ciencia Agrarias, Universidad Nacional Del Nordeste in Argentina.

Plant Material. Sections, 4×4 mm, were taken from expanded leaves of seedlings growing in vitro.

Surface Sterilization. Explants from seedlings growing in vitro do not require surface sterilization. However, they should be rinsed with sterile distilled water to remove medium residues.

Culture Vessels. Culture tubes, Erlenmeyer flasks and other containers filled with medium to 20–30% of their capacity are suitable.

Culture Conditions. Standard culture room conditions are appropriate.

Culture Media. PLBs and plantlets develop from the explants on half-strength MS medium (Murashige and Skoog, 1962) supplemented with 1 mg TDZ l^{-1} (Table Onc-21). Plantlets which form on this medium can be grown on one of several media (see Tables Aranda-6 to Aranda-8, Anth-5C-3, C6, C-19, Cym-5, Cym-1 to Cym-3, Den-2, Den-12, Den-21, Onc-5, Odnta-1, Pln-1, and V-4) until they are large enough to be potted in community pots.

Procedure. Explants should be cultured on the first medium (Table Onc-21) until PLBs are formed. These should be moved to fresh medium (Table Onc-21) for plantlet formation. The plantlets can be moved to one of the other media (see Tables Aranda-6 to Aranda-8, Anth-5C-3, C6, C-19, Cym-5, Cym-1 to Cym-3, Den-2, Den-12, Den-21, Onc-5, Odnta-1, Pln-1, and V-4).

Developmental Sequence. PLBs and plantlets form on the first medium (Table Onc-21) after 120 days of culture. Plantlets grow further on one of the other media.

General Comments. This protocol was developed with seedlings. Therefore it cannot be used to propagate selected forms. The method should be tested with explants from young leaves of mature plants.

TABLE ONC-21. **Modified half-strength Murashige–Skoog (½MS) medium (Murashige and Skoog, 1962) for culture of *Oncidium bifolium* leaf explants (Flachsland et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Thidiazuron (TDZ)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Gelrite[g,h]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses. If the cytokinin does dissolve add a few drops of 1 N HCl.

[f]Available from www.sigmaaldrich.com.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.2, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Add the Gelrite (item 16) to the cold solution as indicated in footnote *h*, distribute the medium to culture vessels and autoclave. It is preferable not to autoclave hormones and vitamins, but in this medium they can be autoclaved for 15–20 min at 121°C.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

Effects of Basal Medium and Banana and Sugar Concentrations on the Growth of *Oncidium* Protocorm-like Bodies in Vitro

Oncidium PLBs grow best on MS medium (Murasghige and Skoog, 1962) containing 40 g sucrose and 100 g banana pulp l^{-1} (Kusumoto et al., 1998). This report and the one below are not in agreement regarding sucrose.

Effects of Basal Medium and Banana and Sugar Concentrations on the Growth of *Oncidium* Plantlets in Vitro

Oncidium plantlets grow best on MS medium (Murasghige and Skoog, 1962) containing 30 g sucrose and 100 g banana pulp l^{-1} (Kusumoto and Takeda, 1997). This report and the one above are not in agreement regarding sucrose.

Plantlet Production from *Oncidium varicosum* Root Tips

Professor G. B. Kerbauy in Brasil is interested in culturing orchid root tips due to the "obvious advantages offered by this source of explants in comparison with shoots" (Kerbauy, 1984*b*). His interest led him to develop a micropropagation method for root tips of *Oncidium varicosum* (Kerbauy, 1984*b*, 1993*a*, 1993*b*).

Plant Material. Root tips, approximately 5 ± 1 mm long, were taken from 4–6-cm-tall seedlings of *Oncidium varicosum* Rogersii grown asymbiotically on Knudson C (KC) medium (Knudson, 1946) with 27.8 mg Fe-EDTA l^{-1} instead of $FeSO_4$, supplemented with 60 g ripe banana (RB) l^{-1} (presumably homogenized), 1 g activated charcoal (AC) l^{-1}, and solidified with 8 g agar l^{-1}.

Surface Sterilization. Explants taken from plants growing in vitro do not require surface sterilization. If roots from mature plants are excised a number of precautions must be taken because root meristems are protected only by a thin root cap and are therefore vulnerable to injury during surface sterilization (unpublished method for roots of *Oncidium*, *Cattleya*, *Epidendrum*, and *Catasetum* and related species developed by Professor Kerbauy and kindly described in an e-mail message). Roots which will serve as sources of explants must not touch the substrate or any other surface. To prevent these roots from touching the substrate or surfaces the plants should be removed from their pots and suspended in the air in a greenhouse. High temperatures and low relative humidities must be avoided. As new roots develop they must be sprayed weekly with a fungicide and a bactericide. Professor Kerbauy warns against the use of systemic fungicides because "they are very harmful to orchids." Roots should be harvested when they reach a length of nearly 2.0 cm. The segments should be placed in a small sieve and washed with tap water. After being washed the root segments should be immersed in approximately 30 ml of sterile water which contains a few drops of mild household detergent, agitated gently for 20 min, and then rinsed with sterile distilled water. The washed roots should be dipped for 20 s

in 70% ethyl alcohol (73–74 ml 95% ethanol diluted to 100 ml with sterile distilled water), submerged with agitation for 20 min in 1% sodium hypochlorite (19 or 17 ml of 5.25 or 6% Clorox diluted to 100 ml with distilled water plus a few drops of a mild detergent), and then rinsed 3–5 times with sterile distilled water. Root-tip explants, 5 mm long, are taken from the sterilized roots and placed in culture.

Culture Vessels. Culture tubes can be used to culture explants on solid medium. Erlenmeyer flasks, 125-ml capacity, should be used for liquid medium and plantlet production.

Culture Conditions. In the original research explants, PLBs and plantlets were maintained at 25 ± 1°C under 16-h photoperiods of 500 lx provided by Gro-Lux tubes. Standard culture room conditions will probably also prove to be suitable. Liquid cultures should be "gently shaken" (Kerbauy, 1993*a* does not define "gently, but 30–50 rpm should be appropriate).

Culture Media. A modified Vacin and Went medium (Vacin and Went, 1949) is used for PLB induction and multiplication (Table Onc-22; personal communication from Professor Kerbauy). Bud formation and plantlet formation take place on what is described as "semi-solid" medium without an indication how much agar was used to solidify it. The amount suggested here (Table Onc-23) is an estimate which may require adjustment and modifications. When plantlets reach a height of 2–3 cm they should be moved to the KC medium used for seed germination (Table Onc-24).

Procedure. Explants are cultured on the first medium (Table Onc-22) until PLBs are formed. These PLBs are multiplied by subculturing them on the same medium (Table Onc-22). Once there are enough PLBs they should be moved to the second medium (Table Onc-23) for bud and plantlet formation. The plantlets should be grown to deflasking size on KC (Table Onc-24).

Developmental Sequence. BA has an inhibitory effect on callus formation and growth by root-tip explants which were "intensely green" in the presence of this hormone. Auxin had an enhancing effect even in the presence of BA. Peptone and thiamine had growth promoting effects which were not as pronounced as those of NAA. Increasing concentrations of NAA enhanced callus formation. However, high levels of this hormone could induce undesirable mutations. The plantlets "had a normal appearance similar to that of mericlones regenerated from shoot tip cultures," but mutations can show up the flowers and/or inflorescences. Therefore use of NAA and other hormones requires caution and lower concentrations are preferable. Coconut water has been shown to induce callus and/or proliferation without inducing mutation and should be used in preference to hormones whenever possible. Buds and shoot develop on a medium described as "semi-solid." Some investigators refer to all agar media as "semi-solid." Others reserve the term for a medium which is solidified with a low concentration of agar. Since the basal medium is solidified with 7 g agar l^{-1} the suggestion here is to solidify the "semi-solid" substrate with 3–5 g l^{-1}. This may or may not be appropriate. Users should try other agar concentrations (perhaps 0.5, 0.8, 1.0, or 2.0 g l^{-1}) and use the one which proves to be most effective. Plantlets

TABLE ONC-22. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of root tips of *Oncidium varicosum* (Kerbauy, 1984*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	1.0 mg	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	Benzyladenine (BA)	0.05 mm	5 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 11) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_3(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH or 0.1 N HCl respectively.

[f]Keep frozen between uses.

[g]Add items 1, 3–9 to the 500–700 ml of distilled water (item 11) which contain the calcium phosphate (item 2), raise volume to 900 ml with distilled water (item 11), adjust pH to 5.5, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

grow well on banana-containing KC medium. The accepted practice is to incorporate into culture media homogenized pulp of ripe bananas.

General Comments. The use of root tips as explants for micropropagation is an excellent idea because removing a few roots will not damage the donor plants. Explants from plants in vitro do not require surface sterilization. This is an advantage during initial research because it eliminates a step that could damage explants badly enough to prevent growth even on a suitable medium. However explants from seedlings

TABLE ONC-23. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for induction of buds and shoots from protocorm-like bodies (PLBs) derived from callus produced by root tips of *Oncidium varicosum* (Kerbauy, 1984*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water[e]	150.0 ml	No stock	No stock	Weigh
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	4.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Add items 1, 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 2), raise volume to 900 ml with distilled water (item 10), adjust pH to 5.5, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the Murashige–Skoog (MS) medium will probably be suitable. The original paper states that the medium is "semi-solid," but does not indicate how much agar to use. That is why the recommendation here is for 4 g agar l^{-1}. This amount may or may not be appropriate. Those who use this procedure may have to experiment with different agar levels if 4 g l^{-1} prove to be inappropriate.

TABLE ONC-24. **Knudson C (KC) medium (Knudson, 1946) modified for the culture of plantlets produced by protocorm-like bodies (PLBs) derived from callus produced by root-tip explants of *Oncidium varicosum* (Kerbauy, 1984*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement[d]				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
7	Banana homogenate[e]	60.0 g	No stock	No stock	Weigh
	Darkening agent				
8	Activated charcoal[f]	1.0 g	No stock	No stock	Weigh
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier				
11	Agar[e]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. Knudson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$ l^{-1}, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored.

[d]There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of other nutrients would be of benefit. If incorporation of additional microelements becomes necessary those used in the Murashige–Skoog (MS) medium are suitable.

[e]Opinions differ regarding the use of banana in culture media. The predominant opinion is that only homogenized pulp of ripe banana should be used. Other views are that: (1) unripe (green) bananas are preferable; (2) the peel rather than the pulp should be used; (3) unpeeled banana homogenate should be incorporated in media; and (4) slices, ripe or unripe, peeled or unpeeled, rather than homogenate are most effective. Opinions also differ regarding which is the most effective banana variety (Gros Michel, Raja, Pisang Susu, and others). Since there is no experimental evidence to support any view the best approach is to follow the recommendations in the original reports and/or use what is suggested there.

[f]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite which are very different in nature should be used only if required by a specific procedure or except under special circumstances.

[g]Pour 800 ml distilled water (item 10) in a homogenizing container, add items 1–7 and homogenize until the banana pulp is completely dispersed, adjust pH to 5.5, add sugar (item 9), and bring volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour in the darkening agent (item 8) while stirring vigorously. When the charcoal becomes distributed evenly pour the medium into culture vessels and autoclave. There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of other nutrients would be of benefit. If the incorporation of additional microelements is necessary those used in the Murashige–Skoog (MS) medium are suitable.

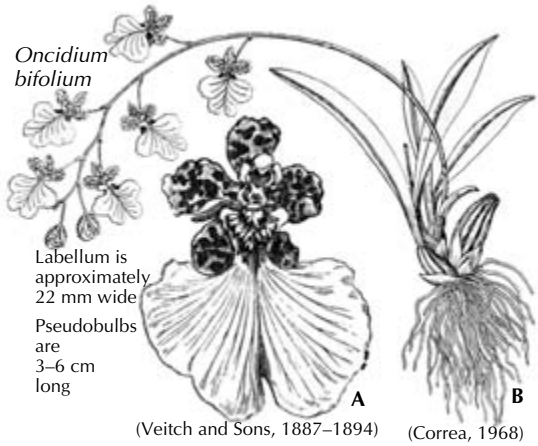
preclude selection of outstanding cultivars because the nature of each plant is not known. Therefore procedures which utilize explants from seedlings are more useful as model systems than as practical methods. Use of explants from roots of mature plants allows for selection of desirable form. However roots which have come into contact with potting mix or other surfaces may contain mycorrhizal fungi which will grow out of the explants and smother cultures. Therefore Professor Kerbauy's method for surface sterilization of roots of mature plants and the culture of explants taken from them is very valuable.

In Vitro Morphogenesis of *Oncidium*

Embryogenic callus was induced from root, stem, leaf, and flower-stalk internodes. The callus eventually produced plantlets. Direct somatic embryogenesis was induced on leaf segments cultured on modified half-strength MS medium (Chang et al., 2001).

Effects of Salt Concentration and Organic Additives on in Vitro Growth of Protocorm-like Bodies and Plantlets of *Oncidium* Gower Ramsey

PLBs of *Oncidium* Gower Ramsey "Hamana" grew best on full and half-strength MS medium (Murashige and Skoog, 1962) supplemented with banana (50 or 100 g l^{-1}), potato, and tryptone (3 g l^{-1}). Growth was also good on 2 g Hyponex l^{-1} (Chen and Chen, 1998).



(Veitch and Sons, 1887–1894) (Correa, 1968)

Ophrys

The genus *Ophrys* is well known for its flowers, which resemble flies, wasps, and bees, and for its pollination mechanisms, which involve pseudocopulation. Some *Ophrys* species have been cultured.

Tissue Culture of *Ophrys apifera*

The threat of extinction of this, "one of the most beautiful and endangered orchids in Germany," led two brothers, high school students at the time, to develop a tissue culture procedure in their home laboratory (Hoppe and Hoppe, 1987*a*, 1987*b*, 1988).

Plant Material. Seedlings (grown in vitro at the Kiel Botanical Garden) that were 2 mm long (white tubers with green tips) and 30 mm long (with two green leaves also 30 mm long), 2 and 8 months old, served as sources of explants for the original research. The shoots of the seedlings were broken off, and the remaining portions (tuber and shoot base) were cultured.

Surface Sterilization. There is no need to surface-sterilize seedlings grown in vitro.

Culture Vessels. Test tubes (30 × 200 mm) containing 20 ml medium and covered with caps fitted with cotton-filled glass tubes were used in the original research. Other containers should prove equally suitable.

Culture Conditions. The brothers Hoppe maintained their cultures on a rotary shaker at 12 rpm under continuous illumination of 2500 lx (provided by four 40-W Sylvania Gro Lux tubes) at 21 ± 1.5°C. Their shaker was built so that the direction of rotation changed every 15 s.

Culture Media. A liquid medium (P) should be used for initial culture of the smaller seedlings (Table Oph-1). A different medium (S; Table Oph-2) is more appropriate for larger seedlings. Proliferation occurs in medium P (Table Oph-1). A third medium (D) supports differentiation (Table Oph-3).

Procedure. Place explants in the appropriate liquid medium (P for smaller seedlings, S for larger ones), and allow them to form callus and PLBs. The latter are subcultured in medium P for proliferation. For plantlet production the PLBs are moved to medium D.

Developmental Sequence. Callus derived from smaller seedlings is white, covered with rhizoids. When subcultured this callus produces PLBs. These proliferate every time they are subcultured but never turn green. The larger seedlings form callus at the end of a pencil-like growth. This callus produces PLBs in medium P. The PLBs produce plantlets on medium D, which develop like normal seedlings.

TABLE OPH-1. **Culture medium P for the culture of *Ophrys* explants (Hoppe and Hoppe, 1987*a*, 1987*b*, 1988)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
2	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
3	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37	37 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	28	2.8 g l^{-1}		
4	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	10	1.0 g l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.25	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	25	2.5 g l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	10	1 g l^{-1}		
	Amino acid				
5	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
6	*myo*-inositol	1100	No stock	No stock	Weigh
	Auxin				
7	Indoleacetic acid	0.5	100 mg 100 ml^{-1} 95% ethanol[f,g]	0.5	
	Cytokinin				
8	Kinetin	0.5	10 mg 50 ml^{-1} 95% ethanol[g]	0.5	
	Vitamins[h]				
9	Biotin	0.063	12.6 mg	0.5	One solution
10	Cyanocobalamine (vitamin B_{12})	0.001	0.2 mg		
11	Niacin (nicotinic acid)	5	100 mg		
12	Pantothenic acid	0.75	150 mg		
13	Pyridoxine·HCl (vitamin B_6)	0.5	100 mg		
14	Riboflavin·PO_4	0.5	100 mg		
15	Thiamine (vitamin B_1)	0.125	25 mg		
	Complex additive				
16	Casamino acids[i]	1.0 g	No stock	No stock	Weigh
	Sugar				
17	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[j]	To 1000 ml			

[a]This medium contains components from several media. The microelements are from Murashige–Skoog solution.

[b]Amounts are given in mg unless indicated otherwise.

[c]Add chelating agent (item 3a) and iron salt (item 3b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements (items 4a–f) to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin does not dissolve, add a few drops of KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Dissolve all vitamins in the same 100 ml of 70% ethanol, and add 0.5 ml of this solution per liter of medium.

[i]Cat. no. 2245, Merck & Co., Darmstad, Germany.

[j]Add items 1–4, 6, and 16 to 900 ml distilled water (item 18). Adjust pH to 5–5.5, add sugar (item 17) and adjust volume to 1000 ml with distilled water (item 18). Pour solution into a 2-l flask, and autoclave. Add amino acid, hormones, and vitamins (items 5, 7–15) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium into preautoclaved culture vessels.

TABLE OPH-2. **Culture medium S for the culture of *Ophrys* explants (Hoppe and Hoppe, 1987*a*, 1987*b*, 1988)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
2	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
3	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37	37 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	28	2.8 g l^{-1}		
4	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	10	1.0 g l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.25	25 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	25	2.5 g l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	10	1 g l^{-1}		
	Amino acid				
5	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
6	*myo*-inositol	1100	No stock	No stock	Weigh
	Auxin				
7	Indoleacetic acid	0.5	100 mg 100 ml^{-1} 95% ethanol[f,g]	0.5	
	Cytokinin				
8	Kinetin	0.5	10 mg 50 ml^{-1} 95% ethanol[g]	0.5	
	Vitamins[h]				
9	Biotin	0.063	12.6 mg	0.5	One solution
10	Cyanocobalamine (vitamin B_{12})	0.001	0.2 mg		
11	Niacin (nicotinic acid)	5	100 mg		
12	Pantothenic acid	0.75	150 mg		
13	Pyridoxine·HCl (vitamin B_6)	0.5	100 mg		
14	Riboflavin·PO_4	0.5	100 mg		
15	Thiamine (vitamin B_1)	0.125	25 mg		
	"Wound hormone"				
16	Traumatic acid, *trans*-2-dodecene-1, 12-dioic acid	0.33	66 mg 100 ml^{-1} 95% ethanol[g]	0.5	
	Complex additive				
17	Casamino acids[i]	1.0 g	No stock	No stock	Weigh
	Sugar				
18	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[j]	To 1000 ml			

[a]This medium contains components from several media. The microelements are from Murashige–Skoog solution.

[b]Amounts are given in mg unless indicated otherwise.

[c]Add chelating agent (item 3a) and iron salt (item 3b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements (items 4a–f) to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin does not dissolve, add a few drops of KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Dissolve all vitamins in the same 100 ml of 70% ethanol, and add 0.5 ml of this solution per liter of medium.

[i]Cat. no. 2245, Merck & Co., Darmstad, Germany.

[j]Add items 1–4, 6, and 17 to 900 ml distilled water (item 19). Adjust pH to 5–5.5, add sugar (item 18), and adjust volume to 1000 ml with distilled water (item 19). Pour solution into a 2-l flask and autoclave. Add amino acid, hormones, and vitamins (items 5, 7–16) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium into preautoclaved culture vessels.

TABLE OPH-3. **Culture medium D for the differentiation of *Ophrys* explants (Hoppe and Hoppe, 1987*a*, 1987*b*)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
2	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
3	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37	37 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	28	2.8 g l^{-1}		
4	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	10	1.0 g l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.25	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	25	2.5 g l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	10	1 g l^{-1}		
	Amino acid				
5	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
6	*myo*-inositol[f]	1100	No stock	No stock	Weigh
	Vitamins[g]				
7	Biotin	0.063	12.6 mg	0.5	One solution
8	Cyanocobalamine (vitamin B_{12})	0.001	0.2 mg		
9	Niacin (nicotinic acid)	5	100 mg		
10	Pantothenic acid	0.75	150 mg		
11	Pyridoxine·HCl (vitamin B_6)	0.5	100 mg		
12	Riboflavin·PO_4	0.5	100 mg		
13	Thiamine (vitamin B_1)	0.125	25 mg		
	Complex additive				
14	Casamino acids[h]	1.0 g	No stock	No stock	Weigh
	Polysaccharide				
15	Glycogen[i]	1.0 g	No stock	No stock	Weigh
	Green banana homogenate	5 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[j]	To 1000 ml			
	Solidifier				
18	Agar[j]	10–15g	No stock	No stock	

[a]This medium contains components from several media. The microelements are from Murashige–Skoog solution.

[b]Amounts are given in mg unless indicated otherwise.

[c]Add chelating agent (item 3a) and iron salt (item 3b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements (items 4a–f) to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]Also known as *meso*-inositol, *i*-inositol, or inositol.

[g]Dissolve all vitamins in the same 100 ml of 70% ethanol, and add 0.5 ml of this solution per liter of medium.

[h]Cat. no. 2245, Merck & Co., Darmstad, Germany.

[i]From animal liver.

[j]Add items 1–4, 6, 14, 15, and 16 to 700 ml distilled water (item 17). Adjust pH to 5–5.5, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. Pour hot solution into a 2-l flask and autoclave. Add amino acid and vitamins (items 5, 7–13) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium into preautoclaved culture vessels. Omit agar if preparing liquid medium. To prepare the green banana homogenate homgenize 5 g of pulp from an unripe (green) banana with 150 ml of distilled water.

General Comments. This procedure was developed with seedlings, but it may prove suitable for explants from mature plants.

Production and Fusion of Protoplasts of *Ophrys lutea* and *Ophrys bombylifera*

A procedure for the isolation of *Ophrys* protoplasts and fusion between them and those of *Barlia robertiana (B. longibracteata)* was developed at the Escola Polytechnica in Lisbon, Portugal (Pais et al., 1983).

Plant Material. The youngest leaves of *Ophrys lutea* and *Ophrys bombylifera* are used.

Surface Sterilization. See *Barlia robertiana* (see p. 258, Vol. I; Pais et al., 1982).

Culture Vessels. Petri dishes are suitable.

Culture Conditions. See *Barlia robertiana* (Pais et al., 1982).

Culture Media. The medium for *B. robertiana* (Pais et al., 1982) is the same, except that the molarity of sorbitol should be 0.55 and the incubation time is 3 h. Fusion medium consists of the following (per liter): 4.09 g (70 mmol) sodium chloride, NaCl; 6.15 g (75 mmol) sodium acetate, CH_3CO_2Na; 222 mg (2 mmol) calcium chloride, $CaCl_2$; 64 mg (0.35 mmol) sorbitol; and 50 g sodium alginate (a fusion agent). Total osmolarity of the medium should be 550 mOsm, and the pH is adjusted to 5.5. The percentage of fusion is observed after staining the protoplasts with a 1% solution of fluorescein diacetate in acetone.

Procedure. Protoplast suspensions, 0.5 ml containing 10^8 protoplasts, are added to 0.1 ml fusion medium and "gently soaked" for about 30 min. Staining with the fluorescein solution is for 1–5 min.

Developmental Sequence. Fusion starts immediately after the protoplasts are added to the fusion medium. Fused protoplasts were not cultured.

General Comments. This procedure should prove useful as a starting point for the development of fusion methods that may lead to viable hybrids.

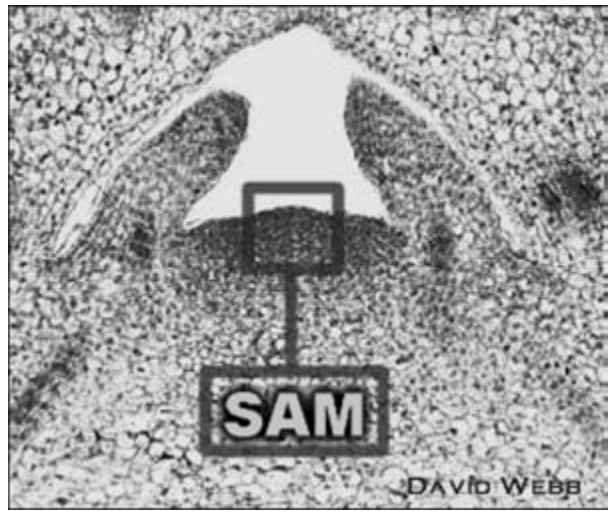
Culture of *Ophrys* Tuber Sections

Tuber sections (cubes 5 mm on each side) of *Ophrys fuciflora* and *Ophrys apifera* were taken during the rest periods of these species in July, cultured on a modified MS medium, and formed callus masses that produced PLBs (Champagnat and Morel, 1972; Morel, 1974). The exact composition of the medium is not given (Champagnat and Morel, 1972). It is only described as "*la solution minerale de Skoog*

additionnée de 3% de glucose et contenant de l'acide naphtyle acétique et de la kinétine à la concentration de 5.10^{-7} g/l." Such lack of detail is not uncommon in papers from Morel's laboratory and one can only assume that the culture medium is some sort of modification of the MS medium and contains 5.10^{-7} g each of NAA and kinetin per liter.

Orchis

A procedure for the culture of meristems of buds taken from tubers of *Orchis coriophora* has been described (Allenberg, 1976), but not in sufficient detail for inclusion here. This procedure has been criticized on several grounds (von Ramin, 1976).



Shoot apical meristem (SAM in image) of an orchid with two leaf primordia above it. Despite the common term "meristem culture," what is commonly cultured is an explant which includes the meristem and surrounding tissue. (Courtesy Professor David T. Webb's World Wide Web page, www.botany.hawaii.edu/faculty/webb/default.htm.)

Otochilus

Described by some as "a very peculiar genus of three known" epiphytic species, *Otochilus* is found from the Himalayas to Thailand and Vietnam. Its name is derived from the Greek ουσ (*ous*) or οτοσ (*otos*) meaning ear and χηειλοσ (*cheilos*) meaning lip. The reference is to auriculate (ear like) lateral lobes on the labellum which clasp the base of the gynostemium (Schultes and Pease, 1963; Bose and Bhattacharjee, 1980).

A Quick Method for the Micropropagation of *Otochilus alba*

An epiphyte described as a "bizarre orchid," *Otochilus alba* is found in the Khasia and Naga Hills and Manipur in India at altitudes of 1000–1500 m (Bose and Bhattacharjee, 1980) and as high as 2000 m (Mukhopadhyay and Roy, 1994). Its flowers (Fig. Otch-1I) are whitish pink, 1.5 cm across, and appear in June. They are

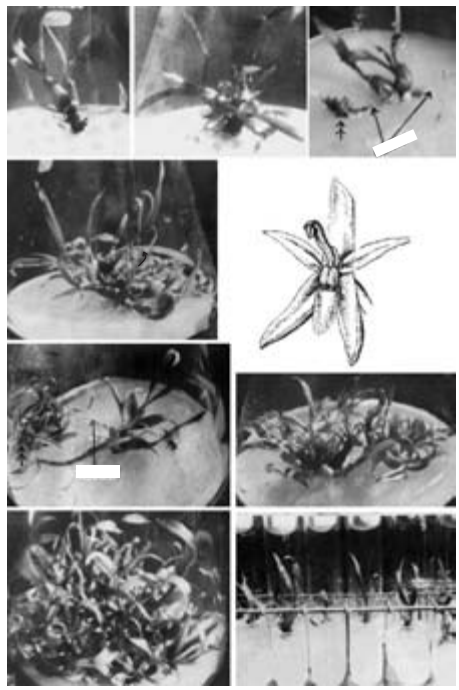


FIG. OTCH-1. **Tissue culture of *Otochilus*. A. Five-week-old shoot-derived pseudobulb. B. Multiplication of pseudobulbs on IAA-containing medium. C. Multiplication of pseudobulbs on NAA-containing medium. D. Induction of runners on Phytamax medium. Arrow points to bulbous tissue at tip. E. Development and multiplication of pseudobulbs from bulbous tissue at tip of runners (arrow). F. Multiplication of pseudobulbs on Phytamax medium. G. Many pseudobulbs after 50 days of culture. H. Root induction. I. Flower. (A–H, Mukhopadhay and Roy, 1994; I, Bose and Bhattacharjee, 1980.)**

produced on pendulous racemes which arise from the terminal pseudobulbs. A "runner mediated" micropropagation method described as "effective as well as astounding" was developed at the Botany Department, University of Calcutta, India (Mukhopadhyay and Roy, 1994).

Plant Material. Apical shoot explants, 5–6 mm long, were taken from *Otochilus alba* plants collected at an altitude of 1800–2000 meters and maintained in a greenhouse at 20 ± 2°C. Terminal leaves should be removed following surface sterilization and the apical region (2–3 mm long) is excised and cultured.

Surface Sterilization. The explants should be surface sterilized by immersing them for 6 min with gentle agitation in a 0.1% solution of mercuric chloride [1 g $HgCl_2$ dissolved in 100 ml distilled water (this is a very toxic substance which must be handled with great care); available from www.fishersci.com], and then washing them five times with sterile distilled water. Explants are removed after the last wash.

Culture Vessels. Erlenmeyer flasks (250 ml capacity containing 60 ml medium) and culture tubes with 25 ml substrate should be used. Other containers are also suitable.

Culture Conditions. Cultures should be maintained at 22–24°C under 60% relative humidity and 16-h photoperiods of 3000 lx provided by fluorescent lamps (type not described). Standard culture room conditions are also appropriate.

Culture Media. Explants should be cultured initially on half-strength MS medium (Murashige and Skoog, 1962) containing 0.2 mg NAA l^{-1} and 10% (v/v) coconut water (Table Otch-1). Pseudobulbs which form on this medium should be transferred after 6 weeks to a medium containing 2 mg N^6-(2-isopentenyl)-adenosine (2iP) l^{-1} and 0.5 mg NAA l^{-1} for pseudobulb (Table Otch-2) and shoot (Table Otch-3) formation. To bring about the formation of runners which produce multiple plantlets the 6-week-old pseudobulbs should be cultured on the Phytamax medium (catalog number P-6393 www.sigmaaldrich.com), a commercial preparation (Table Otch-4). Pseudobulbs with leaves should be cultured on a modification (see Table Aranda-8) of the Knudson C medium (Knudson, 1946).

Procedure. Explants should be cultured on the first medium (Table Otch-1) to induce pseudobulb formation (Fig. Otch-1A). When these pseudobulbs are 6 weeks old and have between two and four terminal leaves they should be moved to a secondary medium (Tables Otch-2 and Otch-3) to induce multiplication (Fig. Otch-1B, C). To bring about the formation of runners (Fig. 1D–G), the 6-week-old pseudobulbs should be cultured on the Phytamax medium (Table Otch-4). Leaf-bearing pseudobulbs should be moved to the Knudson C medium (see Table Aranda-8) for rooting (Fig. Otch-1H).

Developmental Sequence. Single small pseudobulbs develop from the explants within 2 weeks of the start of culture on the first medium (Table Otch-1). A pair of leaves appear on these "pseudobulbs almost simultaneously." When these

TABLE OTCH-1. **Modified half-strength Murashige–Skoog (HMS) medium (Murashige and Skoog, 1962) for the culture of *Otochilus alba* shoot-tip explants (Mukhopadhyay and Roy, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
9	Naphthaleneacetic acid (NAA)	0.2	20 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin-like substance				
10	Adenine sulfate	50.0	No stock	No stock	Weigh
	Vitamins				
11	Niacin (nicotinic acid)	5.0	500 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
13	Coconut water[g]	100.0 ml	No stock	No stock	Measure
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[h]	To 1000 ml			
	Solidifier				
16	Agar[h]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[h]Add items 1–13 to 750 ml of distilled water (item 15), adjust pH to 5.6, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave.

TABLE OTCH-2. **Modified half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of shoot-tip derived leaf bearing pseudobulbs (Mukhopadhyay and Roy, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
9	Naphthaleneacetic acid (NAA)	0.2	20 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
10	N^6-(2-isopentenyl)-adenosine (2iP)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	5.0	500 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[g]	To 1000 ml			
	Solidifier				
15	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or 1 N HCl respectively.

[g]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH to 5.6, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave.

TABLE OTCH-3. **Phytamax brand (www.sigmaaldrich.com) modified half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for induction of runners on pseudobulbs derived from shoot-tip explants of *Otochilus alba* (Mukhopadhyay and Roy, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Buffer				
8	(*N*-morpholino) ethane sulfonic acid (MES)	1.0	No stock	No stock	Weigh
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
15	Peptone	2.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or 0.1 N HCl respectively.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.6, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media.

TABLE OTCH-4. **Modified Knudson C (KC) medium (Knudson, 1946) for rooting of *Otochilus alba* pseudobulbs (Mukhopadhyay and Roy, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement[d]				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
7	Indolebutyric acid (IBA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[f]	To 1000 ml			
	Solidifier				
10	Agar[f]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. Kundson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$ l^{-1}, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored.

[d]There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If incorporation of additional microelements becomes necessary those used in the Murashige–Skoog (MS) medium are suitable.

[e]If the auxin does not dissolve add a few drops of 0.1 N KOH. Keep frozen between uses.

[f]Add items 1–7 to 900 ml of distilled water (item 9), adjust pH to 5.6, add sugar (item 8), and raise volume to 1000 ml with distilled water (item 9). Bring the solution to a gentle boil and add the agar (item 10) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If the incorporation of additional microelements is necessary those used in the Murashige–Skoog (MS) medium are suitable.

pseudobulbs reach 5–10 mm in length, between two and four new pseudobulbs form on each one (Fig. Otch-1A). The original explants turn brown gradually and die. Shoots multiply better on a medium containing NAA and 2iP (Fig. Otch-1B) than on IAA and 2iP (Fig. Otch-1C). Runners which produce pseudobulbs (Fig. Otch-1D–G) develop on the Phytamax medium. Rooting (Fig. Otch-1H) occurs on Knudson C (see Table Aranda-8).

General Comments. This is an interesting, clever, and apparently effective procedure. The Phytamax medium (Table Otch-4) differs from the other media used to produce pseudobulbs (Tables Otch-1 and Otch-3) by the presence of pyridoxine sulfate, peptone, and BA and the absence of adenine sulfate. Production of runners on this medium could be due to these differences. The authors have done well to list the composition of the Phytamax medium.

Pachystoma

A terrestrial tuber-producing species, *Pachystoma senile* is found in India, Sri Lanka, Malabar, Malay islands, and South China. A tissue culture procedure for it was developed at the Botany Department, Panjab University, Chandigarh, India (Vij et al., 1983).

Plant Material. Tubers of 6-month-old seedlings growing in vitro were sliced in 3- to 5-mm-thick segments.

Surface Sterilization. Seedlings growing in vitro do not require surface sterilization.

Culture Vessels. Erlenmeyer flasks, culture tubes, and other standard containers are suitable.

Culture Conditions. Cultures should be maintained under 12-h photoperiods of 3500 lx (provided in the original research by two 40-W Philips fluorescent tubes) and 25 ± 2°C.

Culture Media. Several modifications of the Mitra, Prasad, and Roychowdhury medium (Table Pach-1) were used in the original research. For callus formation the medium should contain (per liter) 1 g peptone and 1 mg NAA, or only 1 mg 2,4-D. PLBs in large numbers form in the presence of 1 g yeast extract l^{-1} (Table Pach-2). Plantlets develop from these PLBs when the basal medium contains 1 mg IAA l^{-1} (Table Pach-3).

Procedure. The explants are placed on the medium, allowed to develop, and subcultured as required.

Developmental Sequence. Some explants form PLBs, while others develop roots, shoots, plantlets, and/or callus. Callus can be formed on a medium that contains 1 mg l^{-1} 2,4-D. PLBs develop

> . . . from the cut surface of explants in the presence of either indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA), kinetin (KN) or gibberellic acid (GA). . . . Regeneration frequency was enhanced by adding yeast extract. NAA caused profuse rhizogenesis. In peptone (P) and casein hydrolysate . . . supplemented combinations, shoot buds were directly initiated and their formation was enhanced in the presence of IAA or KN individually and in combination. Callusing was also noticed when NAA was used along with P. . . . (Vij et al., 1983)

General Comments. These "studies indicate that . . . tuber segments of *P. senile* can be successfully used as explants for regeneration . . . [that is] . . . dependent on . . . hormones. . . . Differentiated plantlets . . . have been successfully transferred to community pots" (Vij et al., 1983).

TABLE PACH-1. **Modified Mitra–Prasad–Roychowdhury medium (Mitra et al., 1976) for callus induction from tuber slices of *Pachystoma senile* (Vij et al., 1983)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100	10 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	200	20 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180	18 g l^{-1}	10	
5	Monosodium phosphate, NaH_2PO_4	150	15 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40 mg l^{-1}		
(e)	Potassium iodide, Kl	0.03	3 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Hormone				
14	2,4-Dichlorophenoxyacetic acid (2,4-D)	1	400 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Sugar				
15	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto	9 g	No stock	No stock	Weigh
	Darkening agent				
18	Activated charcoal	2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original recipe used 3 ml $Na_2FeEDTA$, a preparation that may not be readily available. The substitute listed here is used very commonly in orchid tissue culture media. Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium.

[e]Keep refrigerated or frozen between uses.

[f]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, add darkening agent (item 18) slowly with vigorous stirring; after it has been dispersed completely, dispense solution into culture vessels and autoclave. Omit agar for liquid medium. Media that contain hormones and vitamins should not be autoclaved unless prior experimentation has determined that this will have no deleterious effects. This is such a case (Mitra, 1971; Mitra et al., 1976), so the medium may be autoclaved.

TABLE PACH-2. **Modified Mitra–Prasad–Roychowdhury medium (Mitra et al., 1976) for the induction of protocorm-like bodies from tuber slices of *Pachystoma senile* (Vij et al., 1983)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100	10 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot H_2O$[b]	200	20 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180	18 g l^{-1}	10	
5	Monosodium phosphate, NaH_2PO_4	150	15 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Complex additive				
14	Yeast extract	1 g	No stock	No stock	Weigh
	Sugar				
15	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto	9 g	No stock	No stock	Weigh
	Darkening agent				
18	Activated charcoal	2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original recipe used 3 ml $Na_2FeEDTA$, a preparation that may not be readily available. The substitute listed here is used very commonly in orchid tissue culture media. Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium.

[e]Keep refrigerated or frozen between uses.

[f]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, add darkening agent (item 18) slowly with vigorous stirring; after it has been dispersed completely, dispense solution into culture vessels and autoclave. Omit agar for liquid medium. Media that contain hormones and vitamins should not be autoclaved unless prior experimentation has determined that this will have no deleterious effects. This is such a case (Mitra, 1971; Mitra et al., 1976), so the medium may be autoclaved.

TABLE PACH-3. **Modified Mitra–Prasad–Roychowdhury medium (Mitra et al., 1976) for the induction of plantlets from protocorm-like bodies derived from tuber slices of *Pachystoma senile* (Vij et al., 1983)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100	10 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$[b]	200	20 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	250	25 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180	18 g l^{-1}	10	
5	Monosodium phosphate, NaH_2PO_4	150	15 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2{\cdot}6H_2O$	0.05	5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.05	5 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2{\cdot}4H_2O$	0.4	40 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.05	5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	0.05	5 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Hormone				
14	Indoleacetic acid (IAA)	1	400 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Sugar				
15	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[f]	9 g	No stock	No stock	Weigh
	Darkening agent				
18	Activated charcoal	2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original recipe used 3 ml $Na_2FeEDTA$, a preparation that may not be readily available. The substitute listed here is used very commonly in orchid tissue culture media. Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium.

[e]Keep refrigerated or frozen between uses.

[f]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, add darkening agent (item 18) slowly with vigorous stirring; after is has been dispersed completely, dispense solution into culture vessels and autoclave. Omit agar for liquid medium. Media that contain hormones and vitamins should not be autoclaved unless prior experimentation has determined that this will have no deleterious effects. This is such a case (Mitra, 1971; Mitra et al., 1976), so the medium may be autoclaved.

Paphiopedilum

All genera of the subfamily Cypripedioideae (*Cypripedium*, *Paphiopedilum*, *Phragmipedium*, and *Selenipedium*; and a more recent fifth genus, *Mexipedium*) present a special challenge in respect to culture in vitro. Their seeds are still more difficult to germinate than those of tropical epiphytes even now, and may require special media. And, these plants are still recalcitrant in tissue culture.

Selenipedium and *Mexipedium* explants seem not to have been cultured in vitro as yet. One report regarding the culture of *Phragmipedium* (Stokes et al., 1975) raises grave doubts (such a content-free, badly written, and highly questionable paper should not have been published at all). A second report (Fast, 1979) is of high caliber and respectable. *Cypripedium* explants were cultured in vitro only recently (Tomita, 1998; Jo et al., 2001).

Reports regarding the culture of *Paphiopedilum* vary in quality. What seems to have been the first report (Bubeck, 1973) was highly touted at the time by Dr. O. Wesley Davidson of Rutgers University as a major advance. However dissertations which contain important advances are usually published in peer reviewed journals as research paper(s). This dissertation was not published in such a manner. A second paper published (several times in different forms) shortly after that (Stewart and Button, 1975, 1976*b*, 1976*c*) raises questions. The procedure never became popular despite the fact that one of its authors (Joyce Stewart) rose to prominence in her reincarnations as an orchid taxonomist and a successful participant in international orchid petty politics, and received several gratuitous awards.

Methods for the culture of shoots and leaf tips of *Paphiopedilum* (Allenberg, 1976) did not become practically important either. One report (Holdgate, 1977), despite not being very impressive, led to highly questionable advertising claims about commercial tissue culture of *Paphiopedilum* by a heavily promoted UK laboratory. These claims had to be withdrawn because they could not be sustained.

A method developed at the University of California, Riverside under the guidance of Professor Toshio Murashige, co-formulator of the MS medium (Huang, 1988), is scientifically sound, but did not become widely used.

The slow progress in the development of micropropagation procedures for *Paphiopedilum* is due in part to the very high value of plants which means that not enough are available for experiments. Difficulties in developing an appropriate surface sterilization procedure may be another reason. Slow development of plantlets could be a third reason.

A warning: *Paphiopedilum* sap is allergenic and can cause severe rashes (Hausen, 1984). Precautions must be taken to avoid contact with the sap.

Meristem Culture of *Paphiopedilum*

What seems to be the first effort to culture *Paphiopedilum* was carried out as a Ph.D. research project. It was reported in a dissertation but apparently never published otherwise (Bubeck, 1973).

Plant Material. Explants were taken from *Paphiopedilum lawrenceanum* × *Paphiopedilum* Maudiae, *Paphiopedilum callosum, Paphiopedilum curtisii* Sanderae, *Paphiopedilum* Emerald × *Paphiopedilum* Alma Gevaert, *Paphiopedilum insigne* Harefield Hall, *Paphiopedilum villosum* × *Paphiopedilum insigne* Maulei, *Paphiopedilum nitens* Sallieri × *Paphiopedilum oenantum, Paphiopedilum callosum* × *Paphiopedilum lawrenceanum* var. *hyeanum, Paphiopedilum insigne* × *Paphiopedilum spicerianum, Paphiopedilum* Concobelatum × *Paphiopedilum niveum,* and *Paphiopedilum* Gwen Hannen × *Paphiopedilum* Phantasy. Young, actively growing shoots are taken from the plants and their leaves are removed before surface sterilization. Apices of terminal (i.e., apical) and of axillary buds are removed from the sterilized shoots.

Surface Sterilization. The shoots and explants are sterilized by placing them in a solution (per liter) of three to four drops of Tween 20 [polyoxyethylene-(20)-sorbitan monolaurate], 100 g Carbowax 4000 (polyethylene glycol 4000), and 50 ml Clorox. Tween 20 and Carbowax 4000 are dissolved in distilled water and the solution is autoclaved. Clorox is added to the autoclaved solution for the sterilization step only. Shoots are immersed in this solution for 2 min, placed in sterile distilled water for an equal period, then rinsed again, and drained on sterile paper towels.

Culture Vessels. During the original research the explants were cultured in 125-ml Erlenmeyer flasks containing 50 ml medium, but other containers can also be used.

Culture Conditions. Cultures should be maintained under illumination of 18-h photoperiods of approximately 4500 lx provided by cool white fluorescent tubes, and at a temperature of 20°C.

Culture Media. Explants are started on a modified MS medium (Table Paph-1). When they start to grow, they should be transferred to a bud-induction medium (Table Paph-2). Yet another modification (Table Paph-3) is used for root induction.

Procedure. Take explants while the shoot is immersed in the sterile Tween 20 and Carbowax 4000 solution (no Clorox). Place them on solid medium and later transfer to the bud-induction medium. When the adventitious buds that form on each explant reach 5 mm in length, excise and move them to the rooting medium. Treat plantlets that form in the last medium like seedlings.

Developmental Sequence. The explants are white when first placed in culture. They may turn yellow or pale green within 1–3 days. However, they can also remain yellow for several weeks and then turn white, brown, or green. In most cases surviving cultures can be identified in 3 weeks, but in some cases a brownish black explant may develop a green shoot after 60–90 days.

> A few cell layers of callus developed outside the area wounded during excision. However the major amount of growth took place in the area above the wound. Buds and roots and buds differentiated from vascular bundles in the area above the wound.

Root induction was fortuitous.

TABLE PAPH-1. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for the culture of *Paphiopedilum* explants (Bubeck, 1973)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[c]	1650	165 g l^{-1}	0.5	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	0.5	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	0.5	
4	Potassium nitrate, KNO_3[c]	1900	190 g l^{-1}	0.5	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	0.5	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[e]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[f]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
13	Coconut water[f,h]	50 ml	No stock	No stock	Weigh
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[i]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]The original publication is not clear in that it states that 5% or 10% of the Murashige–Skoog salts were used, but it gives a recipe for this medium that includes more than salts (Bubeck, 1973). Those who wish to use this medium should prepare two versions, one omitting items 1–7 and another containing all components. It is also not clear whether only the macroelements were used at low concentrations. We have assumed this to be the case. This recipe is given solely to provide as complete an account as possible of all micropropagation procedures for orchids; however, we do not recommend its use.

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add the salts to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference will probably have little effect.

[f]Keep frozen between uses.

[g]Keep refrigerated between uses.

[h]The Murashige–Skoog medium normally includes auxins and cytokinins, but none seem to have been used for this procedure except for those that may be present in the coconut water.

[i]Add items 1–7, 9, and 13 to 800 ml of distilled water (item 15). Adjust pH to 5.2–5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid and vitamins (items 8, 10–12) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

TABLE PAPH-2. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for bud induction on *Paphiopedilum* explants (Bubeck, 1973)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[c]	1650	165 g l^{-1}	1.0	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	1.0	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	1.0	
4	Potassium nitrate, KNO_3[c]	1900	190 g l^{-1}	1.0	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	1.0	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[e]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[f]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Purine[g]				
10	Adenine	4.0	400 mg 100 ml^{-1} 95% ethanol	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[h]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[h]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[h]	1	
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[i]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]The original publication is not clear in that it states that 5% or 10% of the Murashige–Skoog salts were used, but the recipe it gives for this medium includes more than salts (Bubeck, 1973). Those who wish to use this medium should prepare two versions, one omitting items 8–13, and another containing all components. It is also not clear whether only the macroelements were used at low concentrations. We have assumed this to be the case. This recipe is given solely to provide as complete a listing as possible of all procedures; we do not recommend it.

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference will probably have little effect.

[f]Keep frozen between uses.

[g]Murashige–Skoog medium normally includes auxins and cytokinins, but none seem to have been used for this procedure. Adenine is a purine and therefore a cytokinin-related substance.

[h]Keep refrigerated between uses.

[i]Add items 1–7, and 9 to 800 ml of distilled water (item 15), adjust pH to 5.2–5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid, purine, and vitamins (items 8, 10–13) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE PAPH-3. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for root induction on *Paphiopedilum* explants (Bubeck, 1973)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[c]	1650	165 g l^{-1}	1.0	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	1.0	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	1.0	
4	Potassium nitrate, KNO_3[c]	1900	190 g l^{-1}	1.0	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	1.0	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[e]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	0.225	45 mg 100 ml^{-1} 95% ethanol[f]	0.5	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Purine[g]				
10	Adenine	4.0	400 mg 100 ml^{-1} 95% ethanol	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[h]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[h]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[h]	1	
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[i]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]The original publication is not clear in that it states that 5% or 10% of the Murashige–Skoog salts were used, but the recipe it gives for this medium includes more than salts (Bubeck, 1973). Those who wish to use this medium should prepare two versions, one omitting items 8–13, and another containing all components. It is also not clear whether only the macroelements were used at low concentrations. We have assumed this to be the case. "Root induction was fortuitous – no case could be made for any additive." This recipe is given solely for the purpose of providing as complete a listing as possible; we do not recommend it.

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[f]Keep frozen between uses.

[g]Murashige–Skoog medium normally includes auxins and cytokinins, but none seem to have been used for this procedure. Adenine is a purine and therefore a cytokinin-related substance.

[h]Keep refrigerated between uses.

[i]Add items 1–7 and 9 to 800 ml of distilled water (item 15), adjust pH to 5.2–5.5, add sugar (item 14) and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid, hormones, and vitamins (items 8, 10–13) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

General Comments. Like other culture methods for *Paphiopedilum* this one seems to work, but the number of surviving explants is low. This fact renders the procedure too risky for very expensive clones since it involves the removal of a shoot that would be lost if the micropropagation attempt were to fail.

Tissue Culture of *Paphiopedilum*

Initial success with the culture of *Paphiopedilum* was very limited (Lecoufle, pers. comm. cited in Morel, 1974; Stewart and Button, 1975, 1976*c*). Apices "of many *Paphiopedilum* hybrids" form plantlets on the Thomale medium (Thomale, 1954, 1957), and callus forms in the presence of 1 ppm 2,4-D (Morel, 1974). PLBs develop from the callus, and when deprived of 2,4-D they form plantlets (Morel, 1974). These experiments were not completed probably due to Morel's death, but other research projects were carried out elsewhere. One such project was initiated in 1972 at the Department of Botany, University of Natal, Pietermaritzburg, Natal, South Africa. The results were published in 1975 and 1976 (Stewart and Button, 1975, 1976*b*, 1976*c*).

Plant Material. In the original research, explants were taken from *Paphiopedilum villosum, Paphiopedilum fairieanum, Paphiopedilum insigne*, and several hybrids. Plants are taken out of the pots, cleaned (Fig. Paph-1A), and divided into shoots (Fig. Paph-1B). The expanded parts of the leaves are removed leaving a stump about 2 cm long (Fig. Paph-1C). The stem apex is exposed by removing the leaves (Fig. Paph-1D), and the stump is surface-sterilized. Tissue that is damaged by sterilization is removed (Fig. Paph-1E), leaving an approximately $5 \times 5 \times 5$ mm cube ready for culturing (Fig. Paph-1F).

Surface Sterilization. Stems are first washed with water and a detergent. After that they are sterilized in saturated calcium hypochlorite solution (10 g calcium hypochlorite in 100 ml water stirred several times at 5-min intervals and decanted or filtered before use) for 10 min with gentle agitation and rinsed several times with sterile distilled water. Leaves are removed one by one after that. The stump is rinsed with calcium hypochlorite and sterile distilled water following the removal of each leaf. At the end of this process damaged tissue is removed (Fig. Paph-1E), and the remainder (Fig. Paph-1F) is cultured.

Culture Vessels. Test tubes 80×25 containing 10 ml medium and sealed with Cap-O-Test lids were used in the original research. Similar tubes and lids or other containers can also be used.

Culture Conditions. Explants should be maintained under 12-h photoperiods (light intensity and source not given, but probably as below) at 23–27°C and then moved to the dark at 25°C until the start of proliferation. After proliferation the cultures are moved to 16-h photoperiods of 1000 lx provided by Sylvania Gro Lux tubes and 23–27°C. Explants should be moved to fresh medium every 8 weeks.

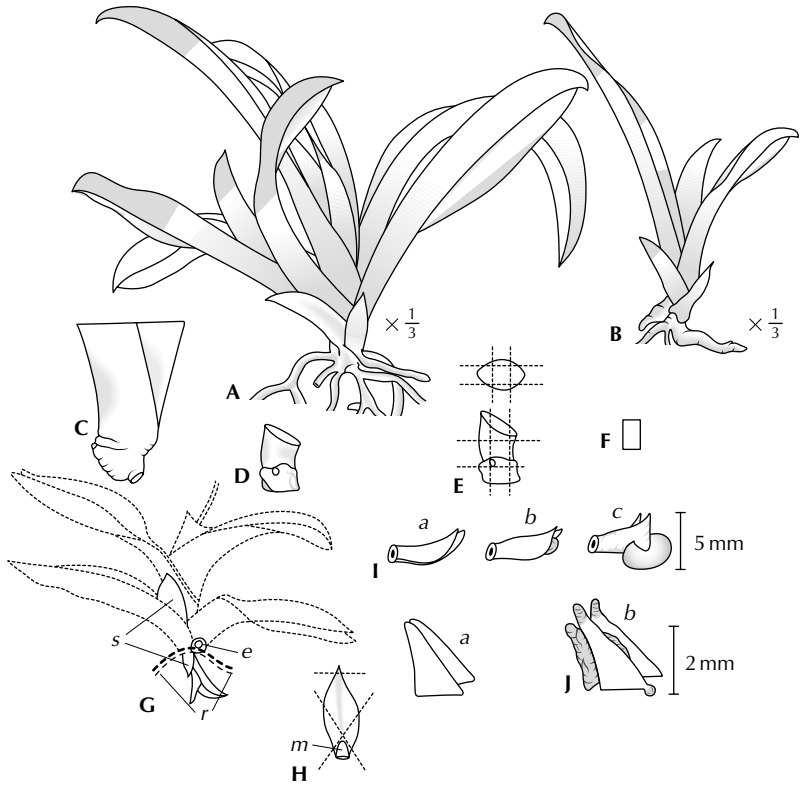


FIG. PAPH-1. **Tissue culture of *Paphiopedilum*. A. Flowering-size plant that serves as a source of explants. B. Shoot taken from the plant. C. The shoot following trimming of leaves and roots. D. After further trimming the explant is ready for surface sterilization. E. The cuts (*dotted lines*) necessary to remove tissue that was damaged by surface sterilization. F. Cubical explant ready for culture. G. Plant (*dotted lines*) with young shoots (s; *solid lines*) that will be removed for culture; e; cut remaining after removal of shoot; r; root. H. Excision (*crossing dashed lines*) of a shoot tip with meristem (m). I. Formation of protocorm-like bodies: a, start of culture; b, 19 days later; c, 24 days after b. J. Callus formation on leaf tips: a, start of culture; b, 3 months later. (A–F, Stewart and Button, 1975; G–I, Allenberg, 1976.)**

Culture Media. Modified Heller's medium (Table Paph-4) is used to culture the explants. Callus formed in this medium is "expected to continue to proliferate as long as 2,4-D is present in the medium." The subsequent treatment of this callus is not clear from the original paper. One possibility is to transfer parts of it to the same medium plus a cytokinin (Table Paph-5) to induce formation of PLBs. Other sections can be moved to Thomale GD medium plus 2,4-D (Table Paph-6) for further growth. Development of the PLBs may occur on unmodified Thomale GD medium (Table Paph-7).

Procedure. See Plant material section above.

TABLE PAPH-4. **Heller's medium (Heller, 1953) for initial culture of *Paphiopedilum* explants (Stewart and Button, 1975, 1976*b*, 1976*c*)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium chloride, $CaCl_2 \cdot 2H_2O$	75	7.5 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25.7 g l^{-1}	10	
3	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	125	12.5 g l^{-1}	10	
4	Potassium chloride, KCl	750	75 g l^{-1}	10	
5	Sodium nitrate, $NaNO_3$[c]	600	60 g l^{-1}	10	
6	**Micronutrients**[d]				
(a)	Aluminum chloride, $AlCl_3$	0.03	3 mg l^{-1}		
(b)	Boric acid, H_3BO_3	1	100 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.03	3 mg l^{-1}		
(d)	Ferric chloride, $FeCl_3 \cdot 6H_2O$	1	100 mg l^{-1}		
(e)	Manganese sulfate, $MnSO_4 \cdot H_2O$	0.1	10 mg l^{-1}	10	One solution
(f)	Nickel chloride, $NiCl_2 \cdot 6H_2O$	0.03	3 mg l^{-1}		
(g)	Potassium iodide, Kl	0.01	1 mg l^{-1}		
(h)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1	100 mg l^{-1}		
	Auxin				
7	2,4-Dichlorophenoxyacetic acid (2,4-D)	1	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
8	Sucrose[f]	20 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[g]	To 1000 ml			
	Solidifier				
10	Agar, Difco Bacto[f]	12–15 g	No stock	No stock	Weigh

[a]This recipe is included for the purpose of providing as complete a listing of methods as possible; we do not recommend its use.

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. It may be advisable to replace $FeCl_3$ (item 6d) with chelated iron. To prepare a chelated iron solution add 3.73 g Na_2EDTA (chelating agent) and 2.78 g $FeSO_4 \cdot 7H_2O$ to the same 1 l of distilled water, and stir and/or heat until both are dissolved. Add 10 ml of this solution to 1 l of culture medium.

[e]Keep refrigerated or frozen between uses.

[f]Recommended by subsequent investigators.

[g]Add items 1–6 to 900 ml of distilled water (item 9), adjust pH to 5.3, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add auxin (item 7) to hot, still liquid solution under sterile conditions with a sterile pipettes, mix well, and dispense solution into preautoclaved culture vessels.

Developmental Sequence. The explants produce callus within 3 months. Depending on the medium being used this callus can be expected to (1) grow, (2) produce PLBs, and/or (3) regenerate plantlets.

General Comments. It is a pity that some information seems to be lacking from the original papers. Nevertheless, it is important to keep in mind that contamination was a major problem with these experiments and the number of successful cultures was small.

A somewhat different procedure involves the addition of a cytokinin to the medium and the utilization of another, vitamin-containing modification of Heller's solution. Of the 18 explants used for this procedure, 15 remained uncontaminated

TABLE PAPH-5. **Heller's medium (Heller, 1953) for callus induction from *Paphiopedilum* explants (Stewart and Button, 1975, 1976*b*, 1976*c*)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium chloride, $CaCl_2 \cdot 2H_2O$	75	7.5 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25.7 g l^{-1}	10	
3	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	125	12.5 g l^{-1}	10	
4	Potassium chloride, KCl	750	75 g l^{-1}	10	
5	Sodium nitrate, $NaNO_3$[c]	600	60 g l^{-1}	10	
6	**Micronutrients**[d]				
(a)	Aluminum chloride, $AlCl_3$	0.03	3 mg l^{-1}	10	One solution
(b)	Boric acid, H_3BO_3	1	100 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.03	3 mg l^{-1}		
(d)	Ferric chloride, $FeCl_3 \cdot 6H_2O$	1	100 mg l^{-1}		
(e)	Manganese sulfate, $MnSO_4 \cdot H_2O$	0.1	10 mg l^{-1}		
(f)	Nickel chloride, $NiCl_2 \cdot 6H_2O$	0.03	3 mg l^{-1}		
(g)	Potassium iodide, Kl	0.01	1 mg l^{-1}		
(h)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1	100 mg l^{-1}		
	Auxin				
7	2,4-Dichlorophenoxyacetic acid (2,4-D)	1	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin				
8	Benzyladenine (BA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
9	Sucrose[f]	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[g]	To 1000 ml			
	Solidifier				
11	Agar, Difco Bacto[f]	12–15 g	No stock	No stock	Weigh

[a]This recipe is included for the purpose of providing as complete a listing of methods as possible. We do not recommend its use.

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. It may be advisable to replace the $FeCl_3$ with chelated iron. To prepare a stock solution add 3.73 g Na_2EDTA (chelating agent) and 2.78 g $FeSO_4 \cdot 7H_2O$ to the same 1 l of distilled water, and stir and/or heat until both are dissolved. Add 10 ml of this solution to 1 l of culture medium.

[e]Keep refrigerated or frozen between uses. The auxin or the cytokinin can be solubilized with a few drops of dilute or HCl.

[f]Recommended by subsequent investigators.

[g]Add items 1–6 to 900 ml of distilled water (item 10), adjust pH to 5.3, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add hormones (items 7 and 8) to the hot, still liquid solution under sterile conditions with sterile pipettes, and dispense solution into preautoclaved culture vessels.

but only four survived. One explant produced a PLB that developed into a plantlet. The other three each produced a small callus that gave rise to a PLB that developed into a plantlet. Two explants became contaminated and the third formed plantlets that were separated. These plantlets produced a yellowish growth that enlarged and after 3 months "took up most of the space in the tube." Anatomical observations showed it to be "a root of unusual proportions." On a medium without auxin or cytokinin the plantlet developed normally.

Both procedures could serve as starting points for future research but should not be used to propagate valuable clones.

TABLE PAPH-6. **Modified Thomale GD medium (Thomale, 1954, 1957) for the culture of *Paphiopedilum* shoot-tip explants (Stewart and Button, 1975, 1976*b*, 1976*c*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	60	6 g l^{-1}	10	
2	Ammonium nitrate, NH_4NO_3[b]	370	37 g l^{-1}	10	
3	Magnesium nitrate, $Mg(NO_3)\cdot 6H_2O$[b]	110	11 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	300	30 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	400	40 g l^{-1}	10	
	Iron[c]				
6	Ferrous sulfate, $FeSO_4\cdot 7H_2O$	20	2.0 g l^{-1}	10	
	Vitamin[d]				
7	Niacinamide (nicotinamide)	10	1000 mg 100 ml^{-1} 95% ethanol[e]	1	
	Auxin				
8	2,4-Dichlorophenoxyacetic acid (2,4-D)	1	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Growth factors				
9	Wuchsstoff "66F"[f]	1	As purchased	As purchased	As purchased
	Sugars				
10	Fructose	10 g	No stock	No stock	Weigh
11	Glucose	10 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[g]	To 1000 ml			
	Solidifier				
13	Agar[g]	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. The original recipe does not include chelated iron probably because it was not available at the time. To prepare a stock solution, dissolve 3.73 g of chelating agent (Na_2EDTA) and 2.78 g of ferrous sulfate ($FeSO_4\cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[d]This is often omitted. It can be replaced with an equal amount of niacin.

[e]Keep refrigerated or frozen between uses.

[f]This proprietary growth stimulant is available from Chemische Fabrik H. Stähler, Stade bei Hamburg, Germany.

[g]Add items 1–6 and 9 to 900 ml of distilled water (item 12), adjust pH to 5.3, add sugars (items 10 and 11), and adjust volume to 1000 ml with distilled water (item 12). Bring solution to a gentle boil, and add agar (item 13) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add the vitamin (item 7) and auxin (item 8), swirl or stir well to mix completely, and dispense medium into preautoclaved culture vessels. Alternately, add items 1–9 to 900 ml of distilled water (item 12) before pH is set, adjust volume, add sugars, and dissolve agar. Then dispense medium into culture vessels and autoclave. Omit agar for liquid medium.

TABLE PAPH-7. **Thomale GD medium for the germination of *Paphiopedilum* seeds (Thomale, 1954, 1957) and development of protocorm-like bodies from shoot-tip explants (Stewart and Button, 1975, 1976*b*, 1976*c*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	60	6 g l^{-1}	10	
2	Ammonium nitrate, NH_4NO_3[b]	370	37 g l^{-1}	10	
3	Magnesium nitrate, $Mg(NO_3)\cdot 6H_2O$[b]	110	11 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	300	30 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	400	40 g l^{-1}	10	
	Iron[c]				
6	Ferrous sulfate, $FeSO_4\cdot 7H_2O$	20	2.0 g l^{-1}	10	
	Vitamin				
7	Niacinamide (nicotinamide)[d]	10	1000 mg 100 ml^{-1} 95% ethanol	1	
	Growth factors				
8	Wuchsstoff "66F"[e]	1	As purchased	As purchased	As purchased
	Sugars				
9	Fructose	10 g	No stock	No stock	Weigh
10	Glucose	10 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Agar[f]	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. The original recipe does not include chelated iron probably because it was not available at the time. To prepare a stock solution, dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4\cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[d]This is the amide of the more frequently used vitamin niacin (nicotinic acid).

[e]This proprietary growth stimulant is available from Chemische Fabrik H. Stähler, Stade bei Hamburg, Germany.

[f]Add items 1–6 and 8 to 900 ml of distilled water (item 11), adjust pH to 4.9–5.2, add sugars (items 9 and 10), and adjust volume to 1000 ml with distilled water (item 11). Bring solution to a gentle boil, and add agar (item 12) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add vitamin (item 7), swirl or stir well to mix completely, and dispense medium into preautoclaved culture vessels. Alternately, add items 1–8 to 900 ml of distilled water (item 11) before pH is set, adjust volume, add sugars, and dissolve agar. Then dispense medium into culture vessels and autoclave. Omit agar if preparing liquid medium.

Asexual Multiplication of *Paphiopedilum* in Vitro

Since "efforts to develop tissue culture ('meristemming') techniques have been without much success" (Bubeck, 1973; Stewart and Button, 1975) a procedure was developed at the University of California, Riverside, under the guidance of Toshio Murashige, coformulator of the Murashige–Skoog medium (Huang, 1988).

Plant Material. Shoot apices are isolated from emerging laterals of flowering-age plants. After the surface-sterilization procedure the apices should be taken from the 0.005% sodium hypochlorite solution and trimmed by removing (1) all remaining leaves (with a Bard Parker No. 7 handle and disposable No. 11 blade) and (2) basal tissue except for 1 mm. The explant should be 2–3 mm tall (Fig. Paph-2A) with no vascular tissue or brown spots.

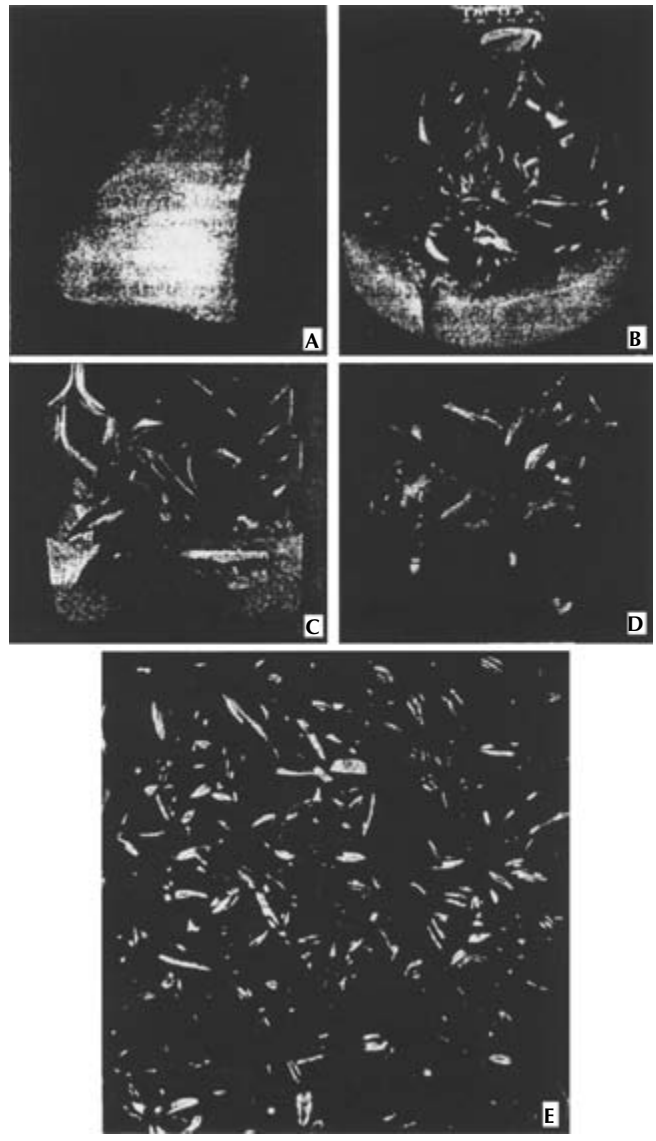


FIG. PAPH-2. **Culture of *Paphiopedilum* shoots. A. Shoot-tip explant ready for initial culture; (×50). B. Axillary shoots after 8 weeks in the second medium (Table Paph-9). Three clusters, each consisting of three shoots, were subcultured in a 250-ml Erlenmeyer flask containing 100 ml medium. C, D. Rooted shoots in the third medium (Table Paph-10). Plantlets grow better in (C) gelrite than in (D) agar. E. Tissue culture derived plantlets in the greenhouse. (Huang, 1988.)**

Surface Sterilization. Laterals are severed from the plant and washed with pHisoHex solution (an antimicrobial hexachlophene-containing detergent available in drugstores and pharmacies) or household detergent. This is followed by a rinse with distilled water and the removal of all leaves except two small upper ones that

encase the shoot apex. Most of the stem is discarded after that. To retard browning the trimmed shoots should be stored in a solution (per liter) of 100 mg ascorbic acid (do not use pills of vitamin C for human consumption because they may contain damaging additives) and 150 mg citric acid. The trimmed apices should be surface-sterilized by immersing them in 0.5% sodium hypochlorite (10 ml Purex or Clorox diluted to 100 ml with distilled water and 2 drops of Tween 20) for 15 min. At the end of this period the solution should be decanted and replaced with 0.005% sodium hypochlorite (1 ml Purex or Clorox diluted to 1000 ml with distilled water). No rinse is required after this solution.

Culture Vessels. Initial culture should be in 25 × 150 mm glass culture tubes containing 25 ml medium and capped with polypropylene closures. The medium should cool at a 30° slant. Subsequent culturing can be carried out in 250-ml Erlenmeyer flasks, Magenta GA7 vessels, or other containers containing at least 100 ml culture medium solidified horizontally.

Culture Conditions. The culture conditions vary in each of three steps.

Step 1. New cultures should be maintained in the dark and 27°C for the first passage. After 4 weeks the explants must be subcultured onto the same medium and moved to 16-h photoperiods of 1000 lx provided by Sylvania Gro Lux lamps. Explants must be subcultured every 4 weeks until they reach a length of 1.5–2 cm (two to four passages).

Step 2. Move the 1.5–2-cm-long explants to another medium (also 25 ml in 25 × 150 mm culture tubes) and to 16-h photoperiods of 3000 lx (also provided by Sylvania Gro Lux lamps) and 27°C. Shoots will multiply considerably (Fig. Paph-2B), and tissues should be subcultured every 8 weeks. After the first subculturing in this step use Erlenmeyer flasks of 250-ml capacity or magenta GA7 vessels.

Step 3. Move shoots to a third medium (in magenta GA7 vessels or Mason jars containing 100 ml medium) for rooting and place under 16-h photoperiods of 10,000 lx provided by Sylvania Gro Lux VHO tubes (Fig. Paph-2C, D). After a month the plantlets can be moved to a potting mix (Fig. Paph-2E).

Culture Media. The medium used in step 1 is a codification of the MS solution (Table Paph-8). This medium is modified for step 2 (Table Paph-9) and step 3 (Table Paph-10). Since disinfection of *Paphiopedilum* does not exclude all bacteria and fungi, antibiotics must be included in the medium for step 1. Carbenicillin and Cefotaxime, each at 100–500 mg l^{-1} (depending on the severity of infection), can be used to inhibit bacteria. Cefotaxime is inactivated by light, which is why the cultures must be kept in the dark. Benomyl will inhibit fungi according to our experience (see section on anticontaminants in Chapter 2; Thurston et al., 1979). The author of this procedure (Huang, 1988) also recommends its use but with some reservation. In our experience 50 mg l^{-1} benlate (and double that amount of commercial preparations that contain only 50% active principle) is sufficient to inhibit many fungi. That is why the recipe for the initial medium (Table Paph-8) contains benomyl (benlate).

Procedure. Since the procedure in this case is associated very closely with changing culture conditions, the section, Culture Conditions above, outlines the procedure

TABLE PAPH-8. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for *Paphiopedilum* explant culture (Huang, 1988)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin and related substance				
11	N^6-Isopentenyl adenine (2iP)	3	300 mg 100 ml^{-1} 95% ethanol[f,g]	1	
12	Adenine	30	No stock	No stock	Weigh
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
16	Coconut water	150 ml	No stock	No stock	Measure
	Anticontaminants				
17	Carbenicillin[h]	350	No stock	No stock	Weigh
18	Benomyl[h]	100	No stock	No stock	Weigh
	Sugar				
19	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
20	Water, distilled[i]	To 1000 ml			
	Solidifier				
21	Gelrite[j]	2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin do not dissolve, add a few drops of dilute KOH or HCL, respectively.

[g]Keep refrigerated between uses.

[h]Benomyl preparations obtained from commercial nurseries usually contain 50% active principle. The remainder is insoluble and inert. Carbenicillin is available from Sigma Chemical Co., (PO Box 14508, St. Louis, MO 63178, USA). To sterilize the anticontaminants, place them togenther in 2 ml 95% ethanol in a vial, shake vigorously, allow to stand for 5 min , and shake again. Repeat this shaking–resting cycle three to four times. Add the mixture to hot, still liquid medium after a vigorous shaking. Wash any powder that remains in the vial into the medium first with another 2 ml portion of 95% ethanol and then several times with sterile distilled water.

[i]Add items 1–6 to 800 ml of distilled water (item 20), adjust pH to 5.7, add sugar (item 19), and adjust volume to 1000 ml with distilled water (item 20). Bring solution to a gentle boil, and add Gelrite (item 21) slowly while stirring. Gelrite can also be added to the cold solution, which is then brought to a boil and stirred. When the Gelrite is completely dissolved, pour solution into a 2-l flask and autoclave. Add anticontaminants (items 17 and 18) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar or Gelrite when preparing liquid medium. Culture media that contain organic additives such as hormones and/or vitamins should not be autoclaved unless prior experimental evidence indicates (as it does in this case) that it can be done without deleterious effects.

[j]Agar or Gelrite are added to solid media only. *Paphiopedilum* explants grow better on media solidified with 2 g Gelrite (Scott Laboratories, Carson, CA 90746, USA).

TABLE PAPH-9. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for the culture of *Paphiopedilum* explants (Huang, 1988)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin and related substance				
11	N^6-isopentenyl adenine (2iP)	3	300 mg 100 ml^{-1} 95% ethanol[g]	1	
12	N^6-benzyladenine	100[h]	No stock	No stock	Weigh
13	Adenine	30	No stock	No stock	Weigh
	Vitamins				
14	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
15	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
16	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
17	Coconut water	150 g	No stock	No stock	Measure
	Sugar				
18	Sucrose	75 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[i]	To 1000 ml			
	Solidifier				
20	Gelrite[j]	2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinins do not dissolve, add a few drops of dilute KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]According to Huang (1988) 100 mg l^{-1} of this cytokinin would be toxic for most plants, but not for *Paphiopedilum*.

[i]Add items 1–17 to 800 ml of distilled water (item 19), adjust pH to 5.7, add sugar (item 18), and adjust volume to 1000 ml with distilled water (item 19). Bring solution to a gentle boil, and add Gelrite (item 20) slowly while stirring. Gelrite can also be added to cold solution, which is then brought to a boil and stirred. When Gelrite is completely dissolved, dispense solution into culture vessels and autoclave. Agar or Gelrite are not added to liquid media. Culture media which contain organic additives such as hormones and/or vitamins should not be autoclaved unless prior experimental evidence indicates (as it does in this case) that it can be done without deleterious effects.

[j]*Paphiopedilum* explants grow better on media solidified with 2 g Gelrite (Scott Laboratories, Carson, CA 90746, USA).

TABLE PAPH-10. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for the rooting of plantlets derived from *Paphiopedilum* explants (Huang, 1988)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.3	30 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin-related substance				
11	Adenine	30	No stock	No stock	Weigh
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Gelrite[i]	2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of dilute KOH.

[g]Keep refrigerated between uses.

[h]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.7, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add Gelrite (item 17) slowly while stirring. Gelrite can also be added to the cold solution, which is then brought to a boil and stirred. When Gelrite is completely dissolved, dispense solution into culture vessels and autoclave. Culture media that contain organic additives such as hormones and/or vitamins should not be autoclaved unless previous experimental evidene indicates (as it does in this case) that it can be done without deleterious effects.

[i]*Paphiopedilum* explants grow better on media solidified with 2 g Gelrite (Scott Laboratories, Carson, CA 90746, USA).

in detail. In brief, this procedure utilizes three steps: Step 1 establishes aseptically growing explants; if contamination is heavy the levels of antibiotics must be increased and time periods between subcultures decreased in step 1. The second step is the multiplication of shoots by enhanced axillary branching, while rooting of shoots and hardening of the plants is the third step.

Developmental Sequence. Explants grow to a length of 1.5–2 cm during step 1 and are freed of contaminants if any. In step 2 the explants branch and multiply. They root in step 3.

General Comments. *Paphiopedilum* sap is allergenic and can cause severe rashes (Hausen, 1984). Therefore plants must be handled with caution. The first few hundred plants produced by this method have flowered and show no abnormalities. It is not clear at present whether this procedure can be used for *Cypripedium*, *Phragmipedium*, and *Selenipedium*.

Culture of *Paphiopedilum* Shoot and Leaf Tips

Shoots of *Paphiopedilum callosum* (Fig. Paph-1G) were removed and surface-sterilized by immersion in 10% Clorox (10 ml Clorox diluted to 100 ml with distilled water) for 12 min. All except the two innermost chlorophyll-free leaves were excised after that (Fig. Paph-1H), and the shoot apex was apparently cultured in a modification of the Thomale GD medium (Table Paph-7). The author (Allenberg, 1976) refers to a "GD-D medium" without giving a reference or composition. Some of the apices cultured in this manner produced PLBs (Fig. Paph-1I*a–c*).

Leaf tips placed on GD-D medium containing l mg IAA and an equal amount of kinetin produced PLBs (Fig. Paph-1J). It is not clear whether the leaf tips were taken from seedlings in vitro or mature plants, and if the latter how they were surface-sterilized.

Induction of Lateral Buds and Plantlets on *Paphiopedilum*

This book deals with in vitro propagation procedures, but since *Paphiopedilum* plants are difficult to propagate in this manner a possible different approach will be mentioned here.

Paphiopedilum plants have been reported to produce shoots and plantlets (keikis) from their roots (Fig. Paph-3; Northen, 1982) and flower stems (Nieman, 1980; Sampolinski, 1983). Attempts to culture *Paphiopedilum* flower-stem tips have failed (Nieman, 1980; unpublished results from our laboratory), but there is no reason to believe that this cannot be done.

Efforts to induce lateral bud development on *Paphiopedilum* plants that have flowers with BA have produced some results (Stewart and Button, 1977*a*, 1977*b*). Single applications of 1 mg BA l^{-1} to leaf axils in "maximal volume . . . without permitting run off onto the supporting medium" induced 1.4 lateral shoots per plant (in an experimental population of five plants) as did four applications of a 10-mg BA l^{-1}

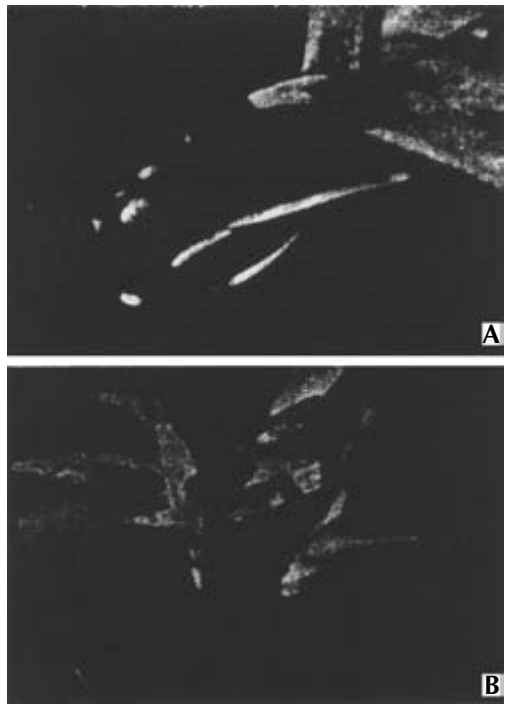


FIG. PAPH-3. **Plantlets on the roots of (A) *Paphiopedilum appletonianum* and (B) *Paphiopedilum callosum*. (Northen, 1982.)**

solution. The control produced only 0.4 shoots. Other treatments brought about, in number of shoots per plant: 1 (four applications of 1 mg BA l^{-1} and one application of 10 mg BA l^{-1}), 1.25 (two applications of 1 mg BA l^{-1}), 1.5 (two applications of 10 mg BA l^{-1}). Statistics are not presented, and it is not clear whether the differences between these numbers are significant.

It is also unclear whether the results are due to factors inherent in the plants or to the BA concentration. The highest concentration, 10 mg l^{-1} (Stewart and Button, 1977*a*) is 250–500 times weaker than the levels employed for *Phalaenopsis* (Abdullah and Arditti, 1983). Another problem with this method (Stewart and Button, 1977*a*) could be the use of an aqueous solution; BA is not water-soluble especially at the recommended levels (Stewart and Button, 1977*b*). A third difficulty may be the autoclaving of the stock solution (Stewart and Button, 1977*b*), which could affect the hormone. On purely speculative grounds it is possible to suggest that a BA paste like the one formulated by K. Zimmer of the University of Hanover and his associates (Abdullah and Arditti, 1983) could be useful.

A paste containing 3750 mg BA l^{-1} can be prepared as follows: Dissolve 440 g Vaseline (which can be purchased at any drugstore) by placing it in a 2-l beaker that has been placed in a 54°C water bath. Add to the Vaseline 5 g chemically pure parafin oil (liquid petrolatum), 9 g stearyl alcohol, and 20 g Tween 20. (These three ingredients can be obtained from Sigma Chemical Co., St. Louis, MO 63178, USA.) Stir all components well.

After that bring the mixture to a volume of 1 liter with distilled water while stirring vigorously and again mixing well. Dissolve 3.75 g (3750 mg) BA in 10–50 ml ethyl alcohol; add this to the mixture, which must be stirred well during the entire preparation. Pour the liquid mixture into containers, and store in a refrigerator at 4°C. BA concentrations can be varied by changing the amount added to the Vaseline–parafin oil–stearyl alcohol–Tween-20 mixture. This paste can be applied to flower stems, axillary buds, and roots, but we are not sure if it will be effective. More research is needed in this area. It may be wise to carry out experiments with lower concentrations at first (e.g., 1, 10, 100, 250, 500, 1000, 1500, and 2000 mg l^{-1}) before using concentrations of 3000, 3500, 3750 mg l^{-1} or more. These experiments should not be carried out with rare and/or expensive plants.

Protoplast Isolation from *Paphiopedilum villosum*

"Very few" protoplasts were isolated from yellow brown petals of *Paphiopedilum villosum* by the method used for *Angraecum eburneum (Angraecum giryamae*) and *Dendrobium* (Price and Earle, 1984).

Isolation of *Paphiopedilum* Protoplasts

The method developed for *Acampe praemorsa* was used to isolate 0.4×10^4 protoplasts from leaves of *Paphiopedilum drurii* (Seeni and Abraham, 1986).

Isolation of Mesophyll Protoplasts of *Paphiopedilum insigne*

Procedures developed for *Calanthe*, *Cymbidium*, *Dendrobium*, *Epidendrum*, and *Phalaenopsis* can be used for *Paphiopedilum insigne* (Yasugi et al., 1986).

Plant Production from Callus Cultures of *Paphiopedilum*

Since "seed setting and germination . . . of many [*Paphiopedilum*] cultivars and hybrids are extremely low" and "efforts to develop tissue culture methods have been without much success (Stewart and Button, 1975 . . . [because] a few plantlets occasionally regenerate from shoot apex-derived callus in an undefined medium, but the calli are difficult to maintain and eventually fail to survive during subcultures (Stewart and Button, 1975) . . . development of protocols for rapid and large scale clonal multiplication of selected elites is of considerable commercial value" (Lin et al., 2000). Such "a repeatable procedure" for the production of "normal plants of a *Paphiopedilum* hybrid from sub-cultured protocorm-derived calli" was developed at the Institute of Botany, Academia Sinica, Taipei, Taiwan (Lin et al., 2000).

Plant Material. Paphiopedilum seeds of the cross *Paphiopedilum callosum* "Oakhill" × *Paphiopedilum lawrenceanum* (Fig. Paph-4I) "Tradition" (this is actu-

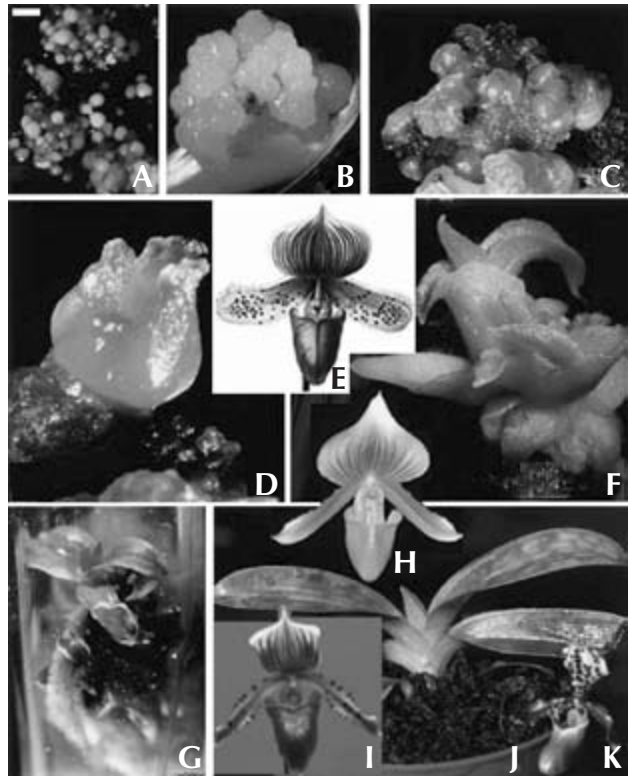


FIG. PAPH-4. **Tissue culture of *Paphiopedilum*. A. Three-month-old protocorms (scale bar = 7.2 mm). B. Subcultured protocorm-derived green callus. C. Green PLBs. D. PLBs starting to form a plantlet. E. Multicolored *Paphiopedilum* Maudiae. F. Plantlet. G. Plantlet with roots and several leaves. H. Green-colored *Paphiopedilum* Maudiae. I. *Paphiopedilum lawrenceanum*. J. Plant in sphagnum. K. *Paphipedilum henryanum*. (Sources: A–D, F, G, J, Lin et al., 2000; E, H, I, K, World Wide Web.)**

ally *Paphiopedilum* Maudiae, Fig. Paph-4E, H) from immature ("green") capsules were germinated on half-strength MS medium (Murashige and Skoog, 1962) containing 20 g sucrose and solidified with 2.5 g Gelrite l^{-1}. The cultures were maintained for 3 months at 25 ± 1°C under 10 μmol m^{-2} s^{-1} (photoperiods not described) provided by 40-W fluorescent tubes FL-30D/29 (China Electric Co., Taipei). Protocorms derived from seeds in these cultures were used to induce callus formation. Stems, root tips, and green leaves of in vitro seedlings of *Paphiopedilum henryanum* (Fig. Paph-4K) no. 1 × *Paphiopedilum philippinense* from a commercial source were also used as explant sources but they failed to produce callus.

Surface Sterilization. Protocorms and explants taken from seedlings in vitro do not require surface sterilization. However, they should be washed with sterile distilled water to remove medium residues.

Culture Vessels. Culture tubes, 20 × 150 mm containing 10 ml medium were used in the original research. Other containers filled with medium to 20–30% of their capacity are also suitable.

Culture Conditions. In the original research the cultures were maintained at 26 ± 1°C in darkness or under 16-h photoperiods of 28–36 μmol m^{-2} s^{-1} provided by 40-W daylight fluorescent tubes FL-30D/29 produced by China Electric Co., Taipei.

Culture Media. Protocorms form callus on modified half-strength MS medium (Murashige and Skoog, 1962) containing 0.1 mg TDZ l^{-1} and 10 mg 2,4-D l^{-1} (Table Paph-11). This callus should be subcultured and maintained on half-strength MS supplemented with 1 mg TDZ l^{-1} and 5 mg 2,4-D l^{-1} (Table Paph-12). PLBs and eventually plantlets will also be produced on this medium. Plantlets which reach a height of 10 mm should be moved to basal half-strength MS supplemented with potato mash (Table Paph-13). When plantlets grow to 4–5 cm in height they should be planted in sphagnum and "acclimated in a greenhouse."

Procedure. Protocorms (Fig. Paph-4A) are placed on the first medium (Table Paph-11) and cultured in the dark for 3 months. Callus produced on this medium is subcultured in the light on the second medium (Table Paph-12; Fig. Paph-4B). PLBs (Fig. Paph-4C) are cultured on the same medium until plantlets are formed (Fig. Paph-4F, G). These plantlets are moved to sphagnum (Fig. Paph-4J).

Developmental Sequence. Protocorms (Fig. Paph-4A) form callus after 3 months in the dark on the first medium (Table Paph-11). This callus turns green (Fig. Paph-4B) and forms PLBs (Fig. Paph-4C), vegetative buds (Fig. Paph-4D), and plantlets (Fig. Paph-4F, G) on the second medium (Table Paph-12). Plantlets develop normally (Fig. Paph-4J) on the third medium (Table Paph-13).

General Comments. This procedure is a major step forward in tissue culture of *Paphiopedilum*. It may serve as a starting point for methods for adult plants, but cannot be used to propagate cultivars because seed derived protocorms are used to start the cultures.

The active principle in household bleaches (Clorox, Purex, Domestos, and other brand names) is sodium hypochlorite. Its concentration varies with the brand. Clorox is probably most commonly used to surface-sterilize explants. At one time it contained 5.25% sodium hypochlorite, but the concentration was increased to the current 6% a few years ago.

TABLE PAPH-11. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for callus production by *Paphiopedilum* protocorms (Lin et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	2,4-Dichlorophenoxyacetic acid (2,4-D)	10.0	1 g 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
12	Thidiazuron (TDZ)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
16	Peptone	1.0 g	No stock	Weigh	
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively.

[g]Add items 1–8, 10, and 16 to 900 ml of distilled water (item 18), adjust the pH to 5.2, add sugar (item 17), and raise volume to 1000 ml with distilled water (item 18). Add the Gelrite (item 19) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 9), hormones (items 11, 12), and vitamins (items 13–15) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels.

TABLE PAPH-12. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for *Paphiopedilum* callus maintenance and plantlet production (Lin et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	2,4-Dichlorophenoxyacetic acid (2,4-D)	5.0	500 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
12	Thidiazuron (TDZ)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
16	Peptone	1.0 g	No stock	Weigh	
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively.

[g]Add items 1–8, 10, and 16 to 900 ml of distilled water (item 18), adjust the pH to 5.2, add sugar (item 17), and raise volume to 1000 ml with distilled water (item 18). Add the Gelrite (item 19) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 9), hormones (items 11, 12), and vitamins (items 13–15) to the hot solution under sterile conditions with sterilized pipettes, mix well and distribute the medium to preautoclaved culture vessels.

TABLE PAPH-13. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for culture of *Paphiopedilum* plantlets (Lin et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2{\cdot}2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2{\cdot}6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	4.3	430.0 mg l^{-1}		
	Complex additive				
9	Potato mash[e]	20.0 g	No stock	Weigh	
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Gelrite[g]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4{\cdot}4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4{\cdot}7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]The original paper only states "fresh potato mash." Therefore it is not clear if this is freshly mashed cooked (probably peeled) potatoes or freshly homogenized (probably peeled) raw potatoes.

[f]Add items 1–8, to 850 ml of distilled water (item 11) in a homogenizer, dice the potatoes into cubes measuring $1 \times 1 \times 1$ cm, and add them to the other components, homogenize the mixture until the potatoes completely mashed, adjust the pH to 5.2, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Add the Gelrite (item 12) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave.

[g]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite from several sources] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels.

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[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels.

Method for Clonal Propagation of *Paphiopedilum*

It would not be unreasonable to assume that after the brouhaha (or perhaps fiasco is a better word) which followed the attempt to patent orchid micropropagation (see Chapter 1) no one would try a patent again. But, Tian Su Zhou (of Nitta-machi, Japan) and Michio Tanaka (of Kagawa-ken, Japan) as inventors for Sapporo Breweries Limited, Tokyo Japan patented an "invention [which] provides a method for clone-multiplication of a *Paphiopedilum*" (United States Patent 6,060,313). Frankly, it is hard to see how such a patent can be enforced. The method is presented here strictly and only for information and not as an inducement or a suggestion, or even an implication, that attempts should be made to utilize it without permission from the patent owners.

Plant Material. Statements in the patent paper are vague, broad, or not very clear. That is why it is necessary to use direct quotes in presenting information. The "starting material is a scape terminal bud derived from a blooming stock or a bud before blooming or a young axillary shoot . . . A scape terminal bud enclosed in a floral bud before blooming is preferably selected as a starting material since it exhibits a higher survival rate after sterilization of the surface . . . A scape terminal bud or an axillary shoot obtained by a non-aseptic method as mentioned above is employed as a starting material after sterilizing the surface, while [sic, whereas is the proper word to use here] an aseptic shoot obtained by an aseptic cultivation is employed as it is . . . Cross-sectional pieces can be prepared from an aseptic shoot obtained from the scape terminal bud or axillary shoot cultivation . . . A scape terminal bud of a blooming stock of *Paphiopedilum* Sharmain and a bud of a stock before blooming were taken . . . Cross-sectional pieces from an aseptic shoot, a viable plant of a hybrid ***Paphiopedilum*** . . . which had been grown by an aseptic cultivation to the stage of 3 to 5 developed leaves . . . cutting as aseptic shoot containing a number of axillary buds." There is also a claim which refers to "a scape terminal bud or an axillary shoot . . . or an aseptic shoot . . . scape terminal bud . . . derived from a floral bud." And, the disclosure mentions "scape terminal bud or an axillary shoot . . . or an aseptic shoot . . . cut into cross sectional pieces." These statements seem to suggest the use of: (1) apical meristems taken from inflorescences or shoots; (2) perhaps flower buds; and (3) cross sections of shoots from plants growing in pots and in vitro.

Surface Sterilization. Floral buds should be washed with running water and a mild detergent and rinsed with distilled water. Following the rinse the buds should be washed three times with 70% ethanol (73–74 ml of 95% ethanol made up to 100 ml with distilled water), submerged in 70% ethanol for 10 min, and rinsed several times with sterile distilled water. After that the buds should be shaken for 10 min in saturated calcium hypochlorite (for preparation see the Liquids section under Sterilization in Chapter 2; this sterilant is also known as Wilson's solution) which contains 0.1% Tween 20 (www.sigmaaldrich.com, www.fishersci.com, www.vwr.com, www.thomassci.com, www.duchefa.com) or a few drops of baby shampoo and washed a few times with sterile distilled water. This process will probably damage the outer tissue layers, but it will not affect the inside of the bud.

Culture Vessels. Culture tubes 2.5 × 15 cm containing 8 ml medium, are listed in an example given in the patent. Rockwool (www.hummert.com, www.bghydro.com are two possible sources; additional sources can be found by using a search on the World Wide Web) plugs, 20 × 3 mm, should be placed in the test tubes before pouring medium into them. Erlenmeyer flasks and other containers, also containing Rockwool support of appropriate size, filled to 25–30% of their capacity with medium are also suitable.

Culture Conditions. Explants should be maintained at 25 ± 2°C under 16-h photoperiods (the acceptable range is 16–24 h) of 1700 lx (1000–4000 lx can be used). The patent does not specify a light source. Standard culture room conditions are also suitable.

Culture Media. Half-strength modified MS medium (Murashige and Skoog, 1962) containing 0.5 mg NAA l^{-1}, 3 mg BA l^{-1}, 10 mg adenine l^{-1}, 800 mg polyvinylpyrrolidone (PVP) l^{-1}, 20 g sucrose l^{-1}, and 15% coconut water (CW) solidified with 2 g Gellan gum l^{-1} (Table Paph-14) should be used for scape terminal buds and "bud of a stock before blooming" (this probably means a young flower bud, but it is hard to be certain; "stock" may be a misspelled "stalk," but one cannot be sure). A slightly different version of half-strength MS should be used to culture cross sections of shoots taken from aseptically grown plants. This medium contains 1 mg NAA l^{-1}, 3 mg kinetin l^{-1}, 10 ml adenine l^{-1}, 800 mg PVP l^{-1}, 15 g sucrose l^{-1}, and 15% CW solidified with 2 g Gellan gum l^{-1} plus 6 g agar l^{-1} (Table Paph-15). There are no recommendations in the patent document for a medium on which to culture plantlets that may be produced on the starting media (Tables Paph-14 and Paph-15). An appropriate culture solution for such plantlets is medium RE (Table Paph-16) which was formulated by Dr. Robert Ernst of the University of California, Irvine (Ernst, 1982; it is actually very similar to the Thomale GD medium).

Procedure. The part of the patent document which describes the procedure is not very clear: "The scape terminal bud of the blooming stock [sic the word probably should be 'stalk' which means stem] and the scape terminal bud isolated from the sterilized floral bud of the stock [sic] before blooming thus obtained were processed while reserving the scales at 0.5 cm from the base of the scape terminal bud." The word "processes" is probably intended to mean "cultured." And "in the cultivation of the cross sectional pieces from an aseptic shoot, a viable plant of a hybrid . . . was employed as . . . material, which was processed, after removal of the root, into 5 sections per seedling, each having a thickness of about 2 mm, by cutting the shoot into round slices from its base. Thus, the cross-sectional pieces thus obtained were embedded on [the] medium . . ."

Developmental Sequence. Shoots formed on both explants after 3 months of culture.

General Comments. The patent document is vague, unclear, and does not contain enough details to make possible the presentation of a workable method. But this does not really matter because the procedure is presented here for information only,

TABLE PAPH-14. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of scape terminal buds of *Paphiopedilum* (Zhou and Tanaka, 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	3.0	300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin-like substance				
12	Adenine	10.0	1 g 100 ml^{-1} 95% ethanol[f]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Adsorbent				
16	Polyvinylpyrrolidone[g]	800.0	No stock	No stock	Weigh
	Complex additive				
17	Coconut water (CW)[h]	150.0 ml	No stock	No stock	Measure
	Sugar				
18	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[i]	To 1000 ml			
	Solidifier				
20	Gellan gum[i,j]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively. Adenine can be solubilized with a few drops of 0.1 N H_2SO_4.

[g]Polyvinylpyrrolidone (PVP), a catalytically produced polymer of purified 1-vinyl-2-pyrrolidone, occurs as a white to tan powder. It is soluble in water, in alcohol, and in chloroform and insoluble in ether. The pH of a 1 : 20 aqueous solution is between 3 and 7. PVP is used as clarifying agent, separation and filtration aid, stabilizer, adsorbent, bodying additive, tableting compound, dispersant, and to coat fresh fruit.

[h]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[i]Add items 1–9 and 16 and 17 to 700 ml of distilled water (item 19), adjust pH to 5.5, add sugar (item 18), and raise volume to 1000 ml with distilled water (item 19). Add the Gellan gum (item 20) in accordance with the instructions in footnote *j*. When the gellan gum is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10–12), and vitamins (items 13–15) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

[j]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE PAPH-15. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of shoot cross sections of *Paphiopedilum* (Zhou and Tanaka, 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Kinetin	3.0	300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin-like substance				
12	Adenine	10.0	1 g 100 ml^{-1} 95% ethanol[f]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Adsorbent				
16	Polyvinylpyrrolidone[g]	800.0	No stock	No stock	Weigh
	Complex additive				
17	Coconut water (CW)[h]	150.0 ml	No stock	No stock	Measure
	Sugar				
18	Sucrose	15.0 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[i]	To 1000 ml			
	Solidifier				
20	Gellan gum[i,j]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively. Adenine can be solubilized with a few drops of 0.1 N H_2SO_4.

[g]Polyvinylpyrrolidone (PVP), a catalytically produced polymer of purified 1-vinyl-2-pyrrolidone, occurs as a white to tan powder. It is soluble in water, in alcohol, and in chloroform and insoluble in ether. The pH of a 1 : 20 aqueous solution is between 3 and 7. PVP is used as clarifying agent, separation and filtration aid, stabilizer, adsorbent, bodying additive, tableting compound, dispersant, and to coat fresh fruit.

[h]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[i]Add items 1–9 and 16 and 17 to 700 ml of distilled water (item 19), adjust pH to 5.5, add sugar (item 18), and raise volume to 1000 ml with distilled water (item 19). Add the gellan gum (item 20) in accordance with the instructions in footnote *j*. When the gellan gum is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10–12), and vitamins (items 13–15) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

[j]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE PAPH-16. **Modified Robert Ernst (RE) medium (Ernst, 1982) for the culture of *Paphiopedilum* plantlets**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	400.0	40.0 g l^{-1}	10	
2	Ammonium sulfate $(NH_4)_2SO_4$[b]	150.0	15.0 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)_2$[b]	150.0	15.0 g l^{-1}	10	
4	Magnesium nitrate, $Mg(NO_3)_2 \cdot 6H_2O$[b]	100.0	10.5 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	300.0	30.0 g l^{-1}	10	
6	Potassium phosphate (monobasic), KH_2PO_4	300.0	30.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Complex additive				
8	Ripe banana pulp homogenate (BN)[d]	100.0 mg	No stock	No stock	Weigh
	Darkening agent				
9	Vegetable charcoal[e]	2.0 g	No stock	No stock	Weigh
	Sugar				
10	Fructose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Agar[f]	16.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. This medium is similar to the Thomale GD solution (Thomale, 1954, 1957) which can be used in its place (Table Paph-7). The RE medium was formulated by Dr. Robert Ernst who named it after himself.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original recipe of the RE medium calls for 25 mg of $FeSO_4 \cdot SO_4$, but chelated iron is preferable. The difference between 25 and 27.8 mg (2.8 mg) $FeSO_4$ is insignificant and can be ignored. The Thomale GD medium contains 20 mg $FeSO_4$ l^{-1}.

[d]Opinions differ regarding the use of banana in culture media. The predominant opinion is that only homogenized pulp of ripe banana should be used. Other views are that: (1) unripe (green) bananas are preferable; (2) the peel rather than the pulp should be used; (3) unpeeled banana homogenate should be incorporated in media; and (4) slices, ripe or unripe, peeled or unpeeled, rather than homogenate are most effective. Opinions also differ regarding which is the most effective banana variety (Gros Michel, Raja, Pisang Susu, and others). Since there is no experimental evidence to support any view the best approach is to follow the recommendations in the original reports and/or use what is suggested there.

[e]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite which are very different in nature should be used only if required by a specific procedure or except under special circumstances.

[f]Dissolve items 1–7 in 600 ml of distilled water (item 11) in a homogenizer, add banana (item 8) and charcoal (item 9), homogenize for 1 min, stop and homogenize again for 1 min, add sugar and homogenize for another minute, bring volume to 1000 ml and homogenize for 30 s and adjust pH to 5.0–5.4. Heat to 80–90°C and add the agar (item 12) slowly while stirring. When the agar has dissolved completely dispense the medium into culture vessels and autoclave. The Thomale GD medium contains 10 g l^{-1} each of fructose and glucose and does not include banana homogenate, charcoal, and calcium nitrate.

not to encourage infringement of the patent. What this patent suggests is that a method for clonal propagation of *Paphiopedilum* can be formulated. However it is also possible that this method may not be practical because as this is being written (February 2003) there are no reports of mass clonal propagation of *Paphiopedilum* by commercial or hobby laboratories.

Paphiopedilum Cloning in Vitro

Freeing explants from contaminants has been the major obstacle in developing micropropagation methods for *Paphiopedilum*. That is why *Paphiopedilum* is the only orchid of major commercial and hobby interest which is being cloned routinely in vitro. Contaminants can be excluded by using very small explants but their survival is low and multiplication is limited (Huang, 1988). Another problem with research on cloning of *Paphiopedilum* is the availability and high value of mature plants. These problems were circumvented by using aseptically grown seedlings as explant sources (Huang et al., 2001).

Plant Material. Seedlings in flasks of *Paphiopedilum* Billy Cardalino (*Paphiopedilum philippinense* × *Paphiopedilum* Susan Booth) obtained from a commercial grower in Taiwan were used as explant sources for initial experiments. Findings with these explants were tested with seedlings of *Paphiopedilum bellatulum* "Big Spot" × *Paphiopedilum* Joanne's Wine and *Paphiopedilum* Taida Glaucothum (*Paphiopedilum micranthum* × *Paphiopedilum glaucophyllum*). Each of the flasks obtained from the commercial grower contained 25 seedlings. The seedlings consisted of one shoot, 1.5–2.5 cm in height, each. To increase their number these plants were cultured on shoot multiplication medium (Table Paph-9) at 26 ± 2°C under 16-h photoperiods of 26 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps. "Clusters of three 4 cm tall plants served as explants for experiments . . ."

Surface Sterilization. Aseptically grown seedlings do not require surface sterilization.

Culture Vessels. Erlenmeyer flasks, 500-ml capacity, containing 125 ml medium should be used. Other containers are also suitable.

Culture Conditions. Explants should be cultured at 26 ± 2°C under 16-h photoperiods of 26 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps.

Culture Media. For proliferation the seedlings should be cultured on a modified MS medium (Murashige and Skoog, 1962) with higher vitamin concentrations (Murashige and Tucker, 1969), 13 μmol BA l^{-1}, 1.6 μmol NAA l^{-1}, 0.15 μmol adenine sulfate dihydrate l^{-1}, 1.28 mmol $NaH_2PO_4 \cdot H_2O$ l^{-1} and 15% (v/v) coconut water *or* 1 g casein hydrolysate l^{-1} *or* 10 g potato tuber sections l^{-1} (Table Paph-17). Plantlets produced on this medium can be grown to a larger size on the Thomale GD (Table Paph-7; Thomale, 1954, 1957) or RE (Table Paph-16; Ernst, 1982) media.

Procedure. When enough seedlings become available clusters of three 4-cm-tall plantlets should be cultured on the first medium (Table Paph-17) for 6 weeks. Plants produced during this period should be subcultured in the same medium once or twice to increase their number. When the new plantlets start crowding the flask they should be separated from each other and cultured on the Hans Thomale GD (Table Paph-7) or Robert Ernst RE (Table Paph-16) medium or moved to community pots.

TABLE PAPH-17. **Modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for cloning of *Paphiopedilum* seedlings (Huang et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	Naphthaleneacetic acid (NAA)	0.3	30 mg 100 ml^{-1} 95% ethanol[e,f]	1	1.6 µmoles l^{-1} (MW is 186.21)
	Cytokinin				
12	N^6-benzyladenine (BA)	2.93	293 mg 100 ml^{-1} 95% ethanol[e,f]	1	444 µmol l^{-1} (MW is 225.26)
	Cytokinin-like substance				
13	Adenine sulfate dihydrate, adenine $SO_4 \cdot 2H_2O$	60.66	No stock	No stock	Weigh, 0.15 mmol l^{-1} (FW is 404.37). These concentrations are higher than usual
	Vitamins				
14	Niacin (nicotinic acid)	5.0	500 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Pyridoxine (vitamin B_6)	10.0	1 g 100 ml^{-1} 95% ethanol[e]	1	
16	Thiamine (vitamin B_1)	10.0	1 g 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
17	Potato tuber sections[g–i]	10.0 g[f]	No stock	No stock	Weigh[f]
	Sugar				
18	Sucrose	61.61 g	No stock	No stock	Weigh, 0.18 mol l^{-1} (MW is 342.30). Amount is higher than in other media
	Solvent				
19	Water, distilled[j]	To 1000 ml			
	Solidifier				
20	Gelrite[j,k]	10.0 g	No stock	No stock	Weigh

Developmental Sequence. The seedlings grow and proliferate on the modified MS medium (Table Paph-17). They only increase in size on the GD (Table Paph-7) and RE (Table Paph-16) media.

General Comments. This procedure can be used to increase the number of seedlings, but not to propagate selected clones. As a rule there are two obstacles in the development of a micropropagation method. One is an effective surface sterilization procedure which does not damage the explants. The second is a suitable culture medium. By formulating an appropriate medium this method can serve as a starting point for the development of more effective protocols for the cloning of mature plants. Given the high cost of selected *Paphiopedilum* clones such a protocol should utilize explants which do not endanger the original plant like leaf or flower-stem sections.

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively.

[g]If coconut water from very young nuts (less than 1.2 kg in weight at harvest) is available 15% (v/v) should be used in preference to potato tuber sections which are listed. Casein hydrolysate, 1 g l^{-1}, can also be used. Potato tuber sections are recommended here because potatoes are less expensive than casein hydrolysate (see footnote *h*) and more readily available than coconut water from very young nuts (see footnote *i*) in many parts of the world. No indication is given if the potatoes were peeled before use, but peeling then is advisable. The paper states that "potato tuber sections were obtained from freshly purchased potatoes. The tubers were diced into small cubes [size not indicated, 0.5–1 cm would be appropriate], weighed and added before incorporating 0.25% Gelrite™ (Kleco) and autoclaving." An interesting speculation is whether homogenizing the potatoes might not be better.

[h]Not all casein hydrolysates are the same. Their compositions and analyses vary depending on the methods by which they were produced. A product sold by Merck (www.merck.de) as casein hydrolysate (CH) is obtained by degrading casein with hydrochloric acid; vitamins and other growth-promoting compounds are largely destroyed in this process. The CH sold by Sigma-Aldrich (www.sigmaaldrich.com) is pancreatic hydrolysate of casein. There is also a CH which is hydrolysed by enzymes. Becton, Dickinson and Company (www.bd.com) sells Difco products including a number of different casamino acid products all of which are casein hydrolysates. Therefore if a method specifies a source for the CH in its culture medium it is best to obtain the additive from the same vendor. If a source is not listed it is best to purchase CH which is described as being suitable as a supplement for culture media. Failing that, CH should be purchased from a source which specializes in tissue culture and micropropagation supplies. One such source is www.caissonlabs.com. They list casein rather than CH as Stock #: SPCA205. Another source for CH is www.duchefa.com (item 1301). Additional sources include www.fishersci.com, www.thomassci.com, and others. The casein hydrolysate used in the original research with *Paphiopedilum* in this case (Huang et al., 2001) was obtained from ICN Biochemicals (www.icinbiomed.com), but there is no indication which of their seven offerings in this area was employed.

[i]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures). In this case coconut water "(liquid endosperm of *Cocos nucifera*) was obtained from green fruits that weighed 1.2 kg or less at harvest. Large volumes were collected at each harvest, filtered through cheese cloth, mixed, divided into convenient aliquots, and frozen until needed."

[j]Add items 1–17 to 8500 ml of distilled water (item 19), adjust pH 5.7, add sugar (item 18), raise volume to 1000 ml with distilled water (item 19), add Gelrite in accordance with the instructions in footnote *k*, and mix well. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave. In the original research the flasks were closed with MEICO gas permeable stoppers obtained from Microwave Enterprises, Taipei, Taiwan.

[k]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear, and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

Plantlet Production from Nodal Explants of *Paphiopedilum*

The importance of *Paphiopedilum* and the difficulties encountered in trying to culture mature flowering plants of species and hybrids of this genus have led to the development of techniques which utilize seedlings as explant sources. One such procedure utilizes nodal explants of stems (Chen et al., 2002*b*).

Plant Material. Nodal segments of stems, 3 mm in length from in vitro grown seedlings of two *Paphiopedilum philippinense* (Fig. Paph-5) hybrids, PH59 and PH60 were cultured.

Surface Sterilization. Seedlings which are growing in vitro do not require surface sterilization.

Culture Vessels. Culture tubes, 20 × 150 mm, containing 10 ml of medium were used in the original research. Other containers are also suitable.

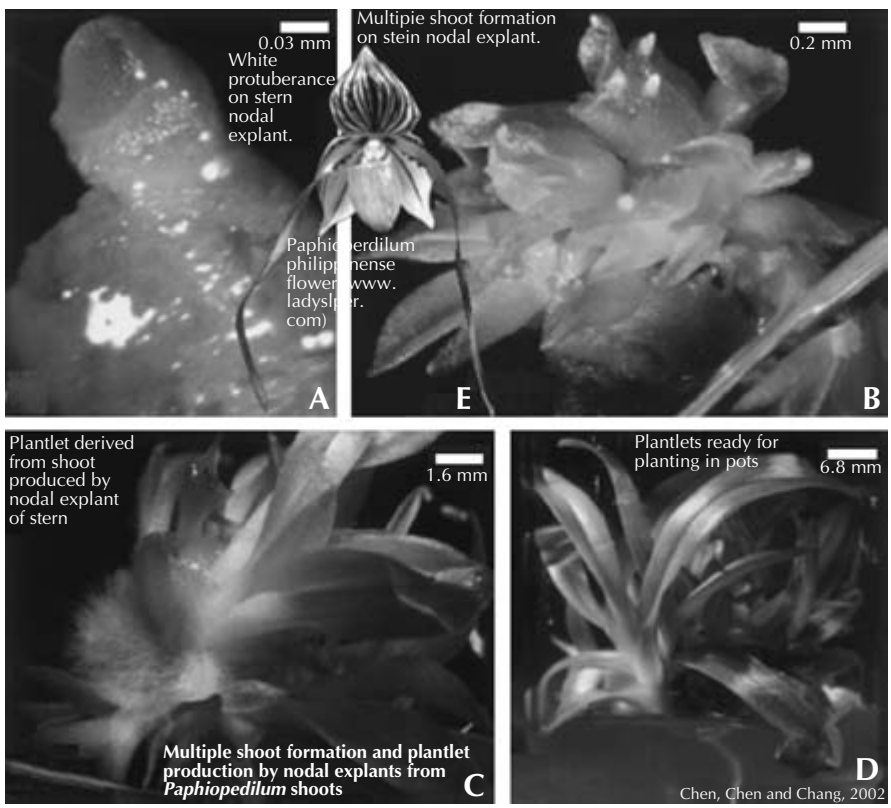


FIG. PAPH-5. **Micropropagation of seedlings of two *Paphiopedilum philippinense* hybrids. (A–D, Chen et al., 2002*b*. E, www.ladyslipper.com.)**

Culture Conditions. Cultures were maintained at 26 ± 1°C under 16-h photoperiods of 28–38 μmol m^{-2} s^{-1} provided by 40-W daylight fluorescent tubes FL-30D/29 (China Electric Co., Taipei, Taiwan). Standard culture room conditions should also prove to be suitable.

Culture Media. The basal medium should be half-strength MS medium (Murashige and Skoog, 1962) containing 170 mg NaH_2PO_4 l^{-1} and 1 g peptone l^{-1} and solidified with 2.2 g Gelrite l^{-1}. When this medium is supplemented with 4.52 μmol 2,4-D l^{-1} and 0.45 μmol TDZ l^{-1} both hybrids will produce the highest percentage of shoots (66.7 and 80%, respectively, for PH59 and PH60). Hybrid PH59 will also produce the highest number of shoots per explant (1.5) on this medium (Table Paph-18). The other hybrid, PH60 will produce the highest number of shoots per explant on a medium with only 4.52 μmol 2,4-D l^{-1} (Table Paph-19). Shoots should be cultured on hormone-free basal medium (Table Paph-20) for rooting.

Procedure. Explants, 3 mm long and each containing one node, of hybrid PH59 should be placed on the surface of the first medium (Table Paph-18) to induce shoot formation. The explants of PH60 should be placed on the second medium (Table Paph-19). When shoots form they should be transferred to the hormone-free medium (Table Paph-20) for plantlet development. Once the plantlets are large enough they should be potted in sphagnum moss and grown in a greenhouse.

Developmental Sequence. Small white protuberances appear on the explants after 2 weeks of culture (Fig. Paph-5A). Multiple shoots (Fig. Paph-5B) form after 22 weeks of culture without subculture. All of these shoots develop into plantlets (Fig. Paph-5C) after 3 months of culture on hormone-free medium. When these plantlets grow to an appropriate size (Fig. Paph-5D) they should be potted in sphagnum moss for further development.

General Comments. Like other protocols which employ explants from seedlings, this procedure cannot be used to propagate selected plants or specific clones. It can be used to increase the number of seedlings and could serve as a starting point for the development of micropropagation methods for mature plants (Fig. Paph-6).

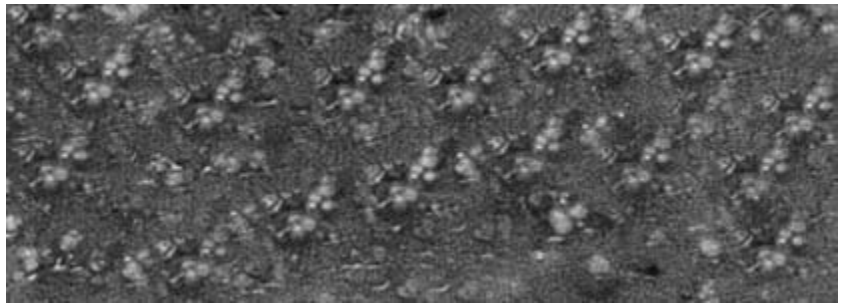


FIG. PAPH-6. **Germinating seeds and young seedlings of *Paphiopedilum* (computer enhanced image taken from http://perso.club-internet.fr/baruk/home/iv.htm).**

TABLE PAPH-18. **Modified half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of *Paphiopedilum philippinense* hybrid PH59 explants (Chen et al., 2002*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	2,4-Dichlorophenoxyacetic acid (2,4-D)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	4.52 µmol, MW is 221.04
	Cytokinin				
12	Thidiazuron (TDZ)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	0.45 µmol, MW is 220.25
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
16	Peptone	1.0 g	No stock	No stock	Weigh
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively.

[g]Add items 1–16 to 900 ml of distilled water (item 18), adjust pH to 5.2, add sugar (item 17), and raise volume to 1000 ml with distilled water (item 18). Add the Gelrite (item 19) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved distribute the medium into culture vessels and autoclave.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite (item 19)] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE PAPH-19. **Modified half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of *Paphiopedilum philippinense* hybrid PH60 explants (Chen et al., 2002*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	2,4-Dichlorophenoxyacetic acid (2,4-D)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	4.52 µmol, MW is 221.04
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
15	Peptone	1.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl respectively.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.2, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Add the Gelrite (item 18) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved distribute the medium into culture vessels and autoclave.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite (item 18)] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE PAPH-20. **Modified half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of shoots of *Paphiopedilum philippinense* (Chen et al., 2002*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
14	Peptone	1.0 g	No stock	No stock	Weigh
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Gelrite[f,g]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

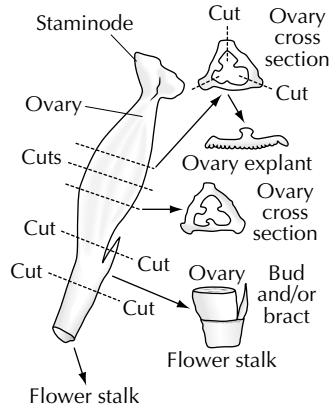
[e]Keep frozen between uses.

[f]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.2, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Add the Gelrite (item 17) in accordance with the instructions in footnote *h*. When the Gelrite is completely dissolved distribute the medium into culture vessels and autoclave.

[g]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite (item 17)] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

Clonal Propagation of *Paphiopedilum* by Tissue Culture

Ovaries, flower stalks, and undeveloped flower buds of flower stalks from *Paphiopedilum* cultivars were cultured in vitro for the purpose of clonal propagation. Bacterial contamination of explants was not experienced to any degree. The rapid growth of the white material was observed on the segments of the ovaries from the open flowers, but most of it turned brown soon after and subsequently died. In the case of the non-open flowers, growth was slow, but the material produced callus-like tissue on which plantlets were then formed. Observation under a light microscope revealed that it was obvious that the white material was formed by the ovules and placenta that increased in size. The undeveloped flower buds proved to grow into a plantlet, although small in number, in the culture from the flower stalks of both open and non-open flowers. No growth was recognized on the segments of the flower stalks. The best response for clonal propagation by using ovaries and undeveloped flower buds was achieved on Vacin and Went medium containing 5 mg/l NAA, 10 mg/l BA, 20 g/l sucrose, and 8 g/l agar. (Kawase, 1990)



Ovary and flower stalk explants (Kawase, 1990).

Plant Material. "Ovaries, flowers stalks and undeveloped flower buds" were used (Kawase, 1990).

Surface Sterilization. Wash plant material with mild detergent, running water and a toothbrush, rinse with distilled water, dip in 70% ethanol (74 ml 95% ethanol diluted to 100 ml with distilled water) for 5–10 s, rinse with sterile distilled water and immerse in 5% household bleach (v/v) for 15 min, and rinse three times with sterile distilled water.

Culture Vessels. Culture tubes, 10.5 × 100 mm, filled with culture medium to 20–30% of their volume, were used in the original research. Other containers are also suitable.

Culture Conditions. In the original research the cultures were maintained at 25°C under 3000 lx illumination. Standard culture room conditions should also prove to be suitable.

Culture Media. Vacin and Went (VW) medium (Vacin and Went, 1949) containing 5 mg NAA l^{-1} and 10 mg BA l^{-1} (Table Paph-21) gave the best results with ovaries and undeveloped flower buds. Plantlets produced on this medium could be cultured to pot size on the RE medium (see Table Paph-16).

Procedure. The plant material is washed and sectioned and the sections should be placed in culture on the first medium (Table Paph-21). Callus and plantlets which form on this medium (Table Paph-21) can be subcultured on it. Plantlets can also be moved to the RE medium (see Table Paph-16).

TABLE PAPH-21. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of *Paphiopedilum* explants (Kawase, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
8	1-Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	6-Benzyladenine (BA)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[g]	To 1000 ml			
	Solidifier				
11	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 9) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]If the auxin and/or cytokinin does not dissolve add a few drops of 0.1 N KOH or HCl respectively.

[f]Keep frozen between uses.

[g]Add items 1, 3–9 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 3), bring volume to 900 ml with distilled water (item 10), adjust pH to 5–5.4, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 10) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary those used as part of the Murashige–Skoog (MS) medium will probably be suitable. Hormones (items 8, 9) are heat labile and are often added after autoclaving to the still hot and liquid solution under sterile conditions with sterilized pipettes, mixed well and the medium is distributed to preautoclaved culture vessels. In this case all components are added before sterilization.

Developmental Sequence. Segments of ovaries from opened flowers produce a "white material" which grows rapidly at first, but eventually turns brown and dies. Ovary segments from unopened flowers produce a "callus-like-tissue" which produced plantlets. Also, a small number of undeveloped flower buds produced plantlets as did flower-stalk explants.

General Comments. By using ovary and stem sections and flower buds as explants this procedure eliminates the two major problems (surface sterilization difficulties and endangering expensive plants) which have hindered the micropropagation of

Paphiopedilum. Despite producing a relatively small number of clones the method seems to be very useful. It has not attracted the attention it deserves because it was not published in English and in a widely distributed journal. It would be interesting to identify the tissue in the ovary explants which gives rise to callus and to count the chromosomes in the plants which are produced by it. Another point to consider is the level of BA used in the medium. It is relatively high and could cause undesirable mutations. Altogether this work is a major advance in the quest for a micropropagation method for *Paphiopedilum*.

Plant Regeneration through Bud Formation from Leaf Explants

Success with *Paphiopedilum* tissue culture protocols has been and, as this was being written, continues to be limited. A procedure using leaf explants was developed in an attempt to remedy the problem (Chen et al., 2004).

Plant Material. Whole leaves, 15 mm long, and leaf sections, 5 mm in length, were taken from 3-year-old in vitro seedlings of two *Paphiopedilum philippinense* hybrids (PH59 and PH60) cultured on MS medium (Murashige and Skoog, 1962) which did not contain growth regulators.

Surface Sterilization. Explants taken from plants growing in vitro do not require surface sterilization. However, they must be washed with sterile distilled water to remove medium residues.

Culture Vessels. Culture tubes, 20×150 mm, were used in the original research. Other containers are also suitable.

Culture Conditions. The research cultures were maintained at $26 \pm 1°C$ under 16-h photoperiods of 28–36 $\mu mol\ m^{-2}\ s^{-1}$ provided by daylight fluorescent tubes (FL-30D/29, 40W, China Electric Co., Taipei). Standard culture room conditions may also be suitable.

Culture Media. Modified MS medium (Murashige and Skoog, 1962) was used and supplemented with 2,4-D and TDZ. Intact leaves, leaf segments, and the two hybrids responded differently to 2,4-D and TDZ levels and combinations. Some of the most effective concentrations are high and may be mutagenic. The concentrations suggested here are lower, less effective, and probably not as likely to cause mutations (Tables Paph-22 to Paph-25). Shoots produced on any of these media should be cultured on hormone-free medium (Table Paph-24) to produce plantlets. The Hans Thomale GD (Table Paph-7) and Robert Ernst RE (Table Paph-16) media can probably also be used for plantlet production and to grow them to potting stage.

Procedure. Whole leaves of hybrid PH59 were placed on a medium containing 0.1 mg (0.44 μmol) l^{-1} TDZ (Table Paph-22). A medium containing 1 mg (4.52 μmol) 2,4-D l^{-1} (Table Paph-23) is suitable for hybrid PH60. Hormone-free medium is best for leaf sections of PH59 (Table Paph-24). Only a medium containing 2,4-D and

TABLE PAPH-22. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of whole leaves of *Paphiopedilum philippinense* hybrid PH59 (Chen et al., 2004)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	70	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
11	Thidiazuron (TDZ)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
15	Peptone	1.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.2, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Add the Gelrite in accordance with instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into a 2-l flask and autoclave. Agar is not added to liquid media.

[h]Gellan gum [(available as such from www.caissonlabs.com) and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require higher concentrations of gellan gum or additional calcium or magnesium salts. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE PAPH-23. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of whole leaves of *Paphiopedilum philippinense* hybrid PH60 (Chen et al., 2004)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	70	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	2,4-Dichlorophenoxyacetic acid (2,4-D)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
15	Peptone	1.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.2, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Add the Gelrite in accordance with instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into a 2-l flask and autoclave. Agar is not added to liquid media.

[h]Gellan gum [(available as such from www.caissonlabs.com) and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require higher concentrations of gellan gum or additional calcium or magnesium salts. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE PAPH-24. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for culture of leaf explants, shoots or plantlets of *Paphiopedilum philippinense* hybrid PH59 (Chen et al., 2004)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	70	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
14	Peptone	1.0 g	No stock	No stock	Weigh
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f,g]	To 1000 ml			
	Solidifier				
17	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]This medium does not contain auxin and cytokinin.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.2, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Add the Gelrite in accordance with instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into a 2-l flask and autoclave. Agar is not added to liquid media.

[h]Gellan gum [(available as such from www.caissonlabs.com) and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require higher concentrations of gellan gum or additional calcium or magnesium salts. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TABLE PAPH-25. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of leaf explants of *Paphiopedilum philippinense* hybrid PH60 (Chen et al., 2004)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	70	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
11	2,4-Dichlorophenoxyacetic acid (2,4-D)[f]	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin				
12	Thidiazuron (TDZ)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
16	Peptone	1.0 g	No stock	No stock	Weigh
	Sugar				
17	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

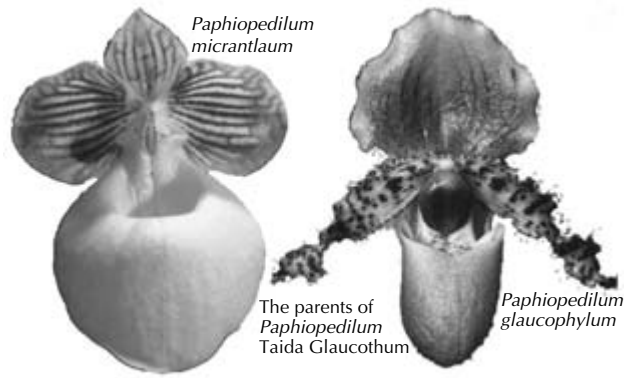
[g]Add items 1–16 to 900 ml of distilled water (item 18), adjust pH to 5.2, add sugar (item 17), and raise volume to 1000 ml with distilled water (item 18). Add the Gelrite in accordance with instructions in footnote *h*. When the Gelrite is completely dissolved pour the solution into a 2-l flask and autoclave. Agar is not added to liquid media.

[h]Gellan gum [(available as such from www.caissonlabs.com) and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require higher concentrations of gellan gum or additional calcium or magnesium salts. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum has dissolved pour the medium into culture vessels and autoclave.

TDZ (Table Paph-25) is suitable for leaf sections of PH60. The different responses of whole leaves, leaf sections, and two hybrids of one species suggest that these media (Tables Paph-22 to Paph-25) may or may not be suitable for other *Paphiopedilum* hybrids. If they are not suitable concentrations of TDZ ranging from 0 to 5 mg (0–22.71 μmol) l^{-1} and 2,4-D levels of 0–10 mg (0–45.25 μmol) l^{-1} should be tested singly and in various combinations.

Developmental Sequence. Buds and later shoots and roots are formed on the initial media (Tables Paph-22 to Paph-25) after 150 (entire leaves) and 170 (leaf sections) days in culture. Plantlets ready for potting develop after 22 months on the hormone-free medium (Table Paph-24). Plantlet formation may require a longer or shorter period on the GD (Table Paph-7) or RE (Table Paph-16) media. It is impossible to predict the developmental sequence of other *Paphiopedilum* species and hybrids.

General Comments. The paper which describes this procedure is excellent, like other papers from this laboratory. Its concluding statement that "a reliable protocol via direct shoot formation for *Paphiopedilum* propagation was established" is accurate as it stands. However, given the different responses of two hybrids of one species' whole leaves and leaf sections, it is not possible to predict if this procedure will also be effective with other *Paphiopedilum* plants.



Papilionanthe

See *Vanda*.

Phaius

Joao (Juan) de Loureiro, a Portuguese missionary in Cochin, China named the genus *Phaius* (sometimes spelled *Phajus* which is not correct) in his *Flora Cochinchinensis* in 1790 and taxonomists have been moving taxa in and out of it ever since. Rudolph Schlechter estimated the number of species in the genus as being around 50, but a more recent estimate by James Comber put the count at 30. The genus is distributed from Africa to India, China, South East Asia, New Guinea, Australia, and the Pacific Islands (Comber, 1990, 2002; Bechtel et al., 1992).

Tissue Culture of *Phaius tankervilleae* Flower-stalk Nodes

There are seven *Phaius* species on Java "all of which occur elsewhere" (Comber, 2002). One of these is *Phaius tankervilleae*, which is distributed from India through South and South East Asia to Australia and some Pacific Islands. This species was named in England where it was grown as far back as the 18th century (Comber, 1990), 1788 to be exact (Bechtel et al., 1992). All plants of this species that were studied by one investigator in Java are reported to be autogamous (Comber, 1990). The ones in North Sumatra are not (Comber 2002). Autogamous flowers in Java are described as lacking a rostellum (Comber, 1990). Their pollinia drop into the stigma before anthesis (Gandawijaja and Arditti, 1982) "or as soon as the flowers open. Flowers then immediately hang their heads down as if in shame" (Comber, 1990) and produce fruits (Gandawijaja and Arditti, 1982). A method for the culture of flower-stalk nodes of an autogamous plant was developed at the Bogor Botanical Garden (Kebun Raya, Bogor, Indonesia) by the late Dr. Djunaedi (Adjun) Gandawijaja (1940–1999). The method was never published due to Dr. Gandawijaja's tragic and untimely death after he was struck by a truck on a street in Bogor.

Plant Material. Explants were taken from the basal, non-flower-bearing parts of inflorescences (Fig. Phaius-1A, B).

Surface Sterilization. Stalks should be washed with a soft toothbrush, mild detergent, and running water before being cut into sections, 6–10 cm long with a node in the middle (Fig. Phaius-1C), and rinsed. The sections should be wiped quickly, but thoroughly, with cotton dipped in 95% ethanol or plunged in 70% ethanol for a second or two. After that they should be soaked in saturated (7.15 g 100 ml^{-1} distilled water) solution (see the section on liquid sterilants in Chapter 2) of calcium hypochlorite (Wilson, 1915) for 10 min and rinsed with sterile distilled water. The bracts should be removed following the rinse (Fig. Phaius-1D) and the sections should be placed in saturated calcium hypochlorite solution diluted 1 : 2 (v/v) with sterile

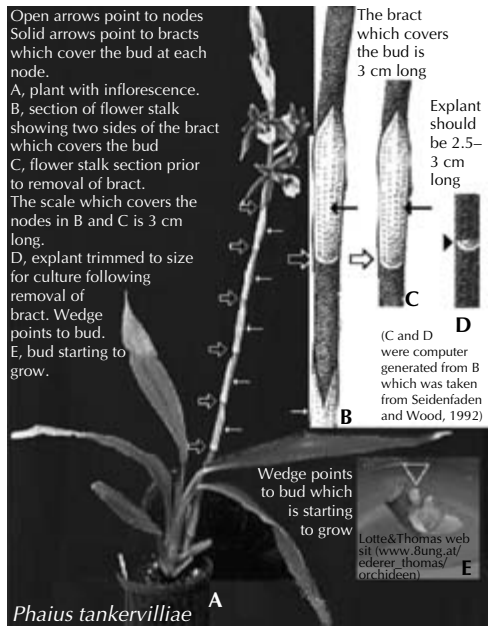


FIG. PHAIUS-1. ***Phaius*** **flower-stalk node culture.**

distilled water for 10 min, rinsed three times with sterile distilled water, and reduced in length to 2.3–3 cm (Fig. Phaius-1D) by cutting sections of the stem on each side of the bud. After this step the bud must be roughly in the center of the section (Fig. Phaius-1D).

Culture Vessels. Erlenmeyer flasks, 250-ml capacity, containing 100 ml of medium were used by Dr. Gandawijaja. Other containers are also suitable.

Culture Conditions. Dr. Gandawijaja maintained his cultures under approximately 12-h photoperiods (Bogor is located at 6° 34′ south and 106° 45′ east, which means that the days are always close to 12 h in length) of normal daylight. Temperature during the day is usually ca. 29°C. The night temperature is 25–26°C. Standard laboratory conditions are also suitable.

Culture Media. Dr. Gandawijaja used solid modified Knop medium (Knop, 1884; Mosich et al., 1973, 1974*a*, 1974*b*) containing 14.8 mg *trans*-cinnamic acid l^{-1} (Table Phaius-1) for the initial culture of the nodes and moved the resulting plantlets to potting medium when they reached a height of 4 cm. It may be wise to subculture the plantlets onto another medium for a while to obtain more vigorous growth. Suitable media are one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (see Table Pln-1), or the Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6,

TABLE PHAIUS-1. **Modified Knop medium for the culture of *Phaius tankevilliae* flower stalk nodes (D. Gandawijaja, unpublished)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	500.0	50.0 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	125.0	12.5 g l^{-1}	10	
3	Potassium nitrate, KNO_3[b]	125.0	12.5 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	125.0	12.5 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
6	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Anti-auxin				
7	*trans*-cinnamic acid (tCA)	14.8	1.48 g 100 ml^{-1} 95% ethanol[e,f]		
	Vitamin[g]				
8	Thiamine (vitamin B_1)	0.4	40 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
9	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[h]	To 1000 ml			
	Solidifier				
11	Agar[h]	13.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. In his unpublished manuscript Dr. Gandawijaja refers only to "solid Knop's medium." He may have used a medium similar to the one he employed for *Dendrobium* nodes (see Table Den-19), but without cytokinins. The assumptions about Dr. Gandawijaja's medium are based on a conversation with him on the subject.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. The original formulation uses 10 mg ferric citrate, $FeC_3H_5O_7 \cdot 3H_2O$ l^{-1}, but chelated iron is preferable.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispenses volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effect. The initial modification of the Knopp medium contains a different combination of microelements than the one used by Dr. Gandawijaja, but the formulation (from the MS solution) suggested here is used very commonly, has proven its suitability for many orchids, and is preferable.

[e]Keep frozen between uses.

[f]If the anti-auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]It is not clear whether Dr. Gandawijaja used this vitamin in his solution. Thiamine is recommended here because it is part of the initial modification, it may be required, and its presence can do no harm.

[h]Add items 1–6 to 900 ml of distilled water (item 10), adjust pH to 5.2–5.5, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the anti-auxin (item 7) and vitamin (item 8) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. The addition of 13 g agar l^{-1} will cause the medium to be more solid than usual. Agar is not added to liquid media.

Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C that does contain this darkening agent (see Table Pln-1, footnote *f*).

Procedure. The stalk is washed following its removal from the plant; it is then surface-sterilized, sectioned, and placed in culture. The plants can be allowed to reach a height of approximately 4 cm on the original medium and then potted. Or, preferably, they can be moved to another medium (Knudson C with banana homogenate for example) on reaching a height of 2 cm where they are grown until they become vigorous, develop a few roots, and reach a height of 4–6 cm before being moved to pots.

Developmental Sequence. Nodes can be expected to produce shoots approximately 20 days after being placed in culture. The first root can be seen following approximately 45 days of culture and the second and third roots, 5 days after that. The plantlets reach a height of 4 cm after 90 days.

General Comments. The advantage of this procedure is that the taking of explants does not endanger the original plant. It also allows the flowers to be enjoyed before the stalk is harvested.

Dr. Djunaidi (Adjun) Gandawijaja was in the process of trying to develop a callus-inducing procedure when fate took him away from the laboratory, his wife Tatin, and daughters Iin and Wulan. He was born in Sumedang, Java, Indonesia on July 12, 1940 and was killed in a traffic accident in Bogor, Java, Indonesia on May 7, 1999. I visited his widow in Bogor several times to offer condolences and support. Adjun received his B.Sc. (1972) and Ph.D. (1992) from the Bogor Agricultural Institute (Institute Pertanian Bogor) in Indonesia and his M.Sc. from the University of Birmingham (1978) in the UK. He accepted employment at the Bogor Botanical Gardens (Kebun Raya, Indonesia) in 1972. I met him that year while teaching a summer course there with the late Professor Charles Lamoureax from the University of Hawaii. Adjun and I collaborated on several research projects after that, and published together. He referred to me once (sarcastically no doubt because I always pushed him to publish more) as his "best loved teacher and also best loved boss." We were actually close friends. His daughters called me "uncle." I am proud to have known Adjun and miss his dry humor.

Propagation of *Phaius tankervilleae*

Sometimes a simple approach will accomplish as much as or more than a high tech method. And the resourcefulness and enthusiasm of a young student can lead to useful findings. This is what happened when a student in the Kew Diploma course "undertook a project on the propagation of *Phaius tankervilleae*" (Danks, 2003).

Plant Material. "Cuttings, 15 cm long . . . from . . . spent inflorescence."

Surface Sterilization. None is required.

Culture Vessels. None are needed.

Culture Conditions. Cuttings are maintained presumably in a greenhouse or a propagation chamber "at a minimum day temperature of 12°C [night temperature is not given] . . . misted daily . . . at 70% humidity, shaded. . . ."

Growing Media. Of the three media tested, the best results were obtained with a medium consisting of one part coir (fiber obtained from coconut husks) and one part perlite. Plantlets obtained by this method should be potted in a mix consisting of equal parts of fine bark, charcoal, and pumice.

Procedure. Cuttings can be inserted into the growing medium or laid on it. Plantlets form on the nodes and should be left in place until their roots reach a length of approximately 15 cm at which point they should be removed and potted in the potting mix.

Developmental Sequence. Plantlets (keikis) form on each node 8 weeks after the cuttings are placed in the growing medium in both horizontal and vertical position. Roots are produced faster on vertically placed cuttings.

General Comments. This is not an in vitro micropropagation procedure, but is included here because it is simple, effective, and rapid.

Phaius tankervilliae plant in flower: The inflorescence is 90 cm tall (Holttum, 1964)

Phalaenopsis

Phalaenopsis is a monopodial genus and as such does not normally form offshoots. Certain of the smaller flowered species in the section *Zebrinae* such as *Phalaenopsis lueddemanniana* usually produce plantlets (keikis) on their flower stalks (Fig. Phal-1A), a phenomenon first described over a century ago (Williams and Williams, 1894). Keikis can also be formed by *Phalaenopsis* species and hybrids of other sections. For example, a watercolor of *Phalaenopsis kunstleri* in Kew Gardens, reproduced in *The genus Phalaenopsis* (Sweet, 1980), clearly shows a well-developed plantlet arising from an inflorescence node. Plantlets also form on flat roots of the Philippine species *Phalaenopsis stuartiana* (a tendency also first noted in the 19th century; Williams

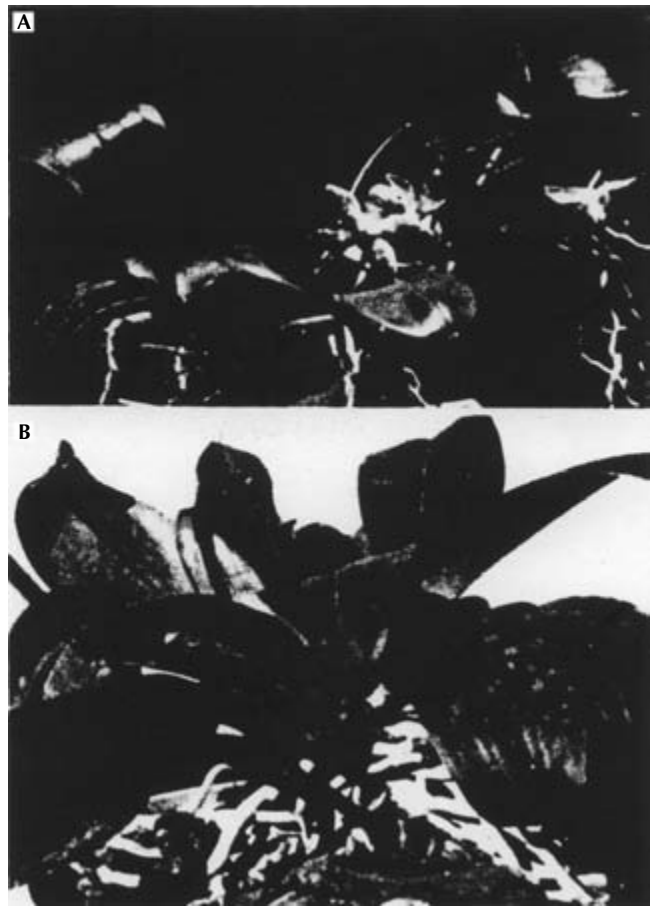


FIG. PHAL-1. **Plantlet production by *Phalaenopsis* plants. A. *Phalaenopsis lueddemannian* producing plantlets (keikis) from flower stalks under cultivation (× 0.4). B. *Phalaenopsis* Irma Anesi producing several shoots from basal buds following injury to the crown (× 0.45). (Ernst, 1986.)**

and Williams, 1894) and *Phalaenopsis schilleriana* (Davis and Steiner, 1952). Keikis developing on roots have likewise been observed in *Phalaenopsis deliciosa* (Reichenbach, 1885), which is presently known as *Kingiella decumbens* P.F. Hunt (Bechtel et al., 1981).

Phalaenopsis plants, under unfavorable culture conditions, frequently produce keikis on their flower stalks, particularly if their tips have been removed.

Clonal Propagation using Whole Shoots

Phalaenopsis plants whose crowns have been injured or severed sometimes give rise to one or several shoots from dormant buds on the base (Fig. Phal-1B). This observation led daring growers to deliberately cut the top of *Phalaenopsis* plants below the aerial roots and cultivate both parts separately in the hope of propagating a desired clone. The process is known as topping. Shoot development from the basal portion is reportedly enhanced by temperatures of 27°C, which has been described as growth promoting (Tran Thanh Van, 1974). A clone may be tripled or quadrupled in this fashion within a 10-month period (Tran Thanh Van, 1974). Vegetative shoots arise from dormant buds on the *Phalaenopsis* plant axis, two of which occur above each other in the leaf axil (Koch, 1974*a*, 1974*b*; Hölters, 1983).

Multiple shoots have also been reported to develop when *Phalaenopsis* plants were treated with morphactins such as the *n*-butyl ester of 9-hydroxyfluorene-(9)-carboxylic acid (Koch, 1974*a*; Hölters, 1983). No details were given regarding this treatment, but it was noted that this morphactin is available from Merck and Company (Koch, 1974*a*). Morphactin has been shown to stimulate carbohydrate synthesis as well as the formation of buds in the dark in lilies (Kato, 1978).

Clonal Propagation through the Production of Plantlets on Flower Stalks

Shoot production on *Phalaenopsis* flower stalks was induced by wrapping sphagnum around the inflorescence node and by keeping it moist (Shara, 1938, 1952). Sphagnum has also been used to produce plantlets from cut flower-stalk sections by a semi-aseptic method (Grimes, 1987).

In recent years, plant hormones have been used to induce the formation of plantlets on flower-stalk nodes in situ. A number of cytokinin-containing pastes are offered by commercial laboratories for this purpose (McFarlane, 1977; Haas, 1977*d*; Brasch and Kocsis, 1980). The method and hormone paste described here were devised at the Technical University of Hannover, Hannover, Germany (Zimmer and Pieper, 1979; Zimmer, 1980).

Plant Material. *Phalaenopsis* plants with fully developed inflorescences, either with buds or open flowers, gave the best results.

Culture Conditions. Greenhouse conditions suitable for *Phalaenopsis* are appropriate.

Hormone Paste. The paste consists of the following (in grams):

Vaseline (petroleum jelly)	44.0
Cetyl/stearyl alcohol	9.0
Paraffin oil (liquid petrolatum)	5.0
Tween 40	2.0
Benzylaminopurine (benzyl adenine)	0.3–0.5
Water	40.0

Vaseline (a paste available in most pharmacies) and cetyl/stearyl alcohol (a solid melting at about 50°C, sold by most chemical supply houses; see Appendix 2) are melted in a beaker placed in a hot water bath at a temperature of about 55°C. Paraffin oil and Tween 40 (both available from chemical or biochemical supply houses) are stirred into the melted mixture and after that the water is added with vigorous stirring. A mechanical stirring device is recommended. BA (available from biochemical supply houses) is dissolved in a minimum volume of ethanol (ethyl alcohol) and stirred into the warm mixture. The paste should be stored in the refrigerator (not freezer) until use.

Procedure. The paste should be applied to buds twice during a 1–3-week period following removal of the bracts. A fully developed inflorescence with buds and/or open flowers can be expected to produce best results.

Developmental Sequence. In some cases a single dormant bud can give rise to more than one shoot. Some of the dormant buds may develop into flower stems. A few can form callus tissue, and others could die (Brasch and Kocsis, 1980; Hölters, 1983). Multiple keikis on a single *Phalaenopsis* flower stem can form following application of this paste, containing 2500–5000 ppm BA (Zimmer, 1978, 1980; Zimmer and Peiper, 1979; Hölters, 1983; Schultz, 1983).

Keikis developing from flower-stem buds of *Phalaenopsis* Inspiration following application of a lanolin-based paste containing 1500 ppm of BA are shown in Fig. Phal-1A.

A gel that can be used for the same purpose (Haas-von Schmude, 1983) reportedly contains less than 50 ppm BA. No formula for the preparation of such a gel is given; however, a widely used gel-forming agent is 1–2% agar dissolved in water by heating the solution to 80–90°C until it becomes clear and allowing it to cool to ambient temperatures.

Antiauxin-containing Hormone Pastes as Plantlet Inducers

A lanolin-based keiki paste containing both the antiauxin *trans*-cinnamic acid (tCA) and the cytokinin BAP was reported to be effective in inducing shoot growth on *Phalaenopsis* inflorescence buds (Griesbach, 1984). Such a combination was used for the induction of vegetative growth of *Phalaenopsis* flower-stalk nodal sections in vitro (Ball et al., 1974/75; Reisinger et al., 1976; Arditti et al., 1977).

Plant Material. In the original research (Griesbach, 1984), inflorescences on flowering plants of *Phalaenopsis* Betty Hausermann were cut back to the third node from the base (the fifth bud usually develops into a flower). The bract covering the bud to be treated was removed prior to the application of the paste.

Culture Conditions. Plants were grown in a greenhouse under a light intensity of 80 $\mu E\ m^{-2}\ s^{-1}$ at 27°C during the day and 21°C at night, and 40% relative humidity. Photoperiods were typical for spring in Beltsville, Maryland, USA.

Hormone Paste. A lanolin paste containing 50 mg tCA and 5 mg BA per milliliter of lanolin is most effective. This paste can be prepared by the method developed at the Technical University of Hannover.

Procedure. Inflorescences of a *Phalaenopsis* hybrid were cut back to the third nodal bud from the base. The bract surrounding the bud was removed before the hormone containing lanolin paste was applied to it.

Developmental Sequence. After 4–8 weeks the buds developed into 1.25 cm inflorescences. One-eighth to one-quarter of the apex of the flower stem was cut off and the remaining bases were retreated with this same hormone/lanolin paste. Several shoots developed from each base. This was followed by root formation.

General Comments. The method of treatment is of interest because it combines the breaking of dormancy with the induction of multiple plantlets.

Vegetative Propagation of *Phalaenopsis* Species and Hybrids in Vitro

The first clonal propagation in vitro of orchids was achieved by Gavino Rotor while he was a graduate student of Lawrence McDaniels at Cornell University (Rotor, 1949). He employed sections of flower stalks each containing a lateral bud. This method is for relatively limited production of plantlets. Rotor's method seems to have been ignored at first but about 10 years later others devised new procedures (Sagawa and Niimoto, 1960; Sagawa, 1961, 1963; Kotomori and Murashige, 1965; Urata and lwanage, 1695; Scully, 1966; Tse et al., 1971; Intuwong et al., 1972*a*, 1972*b*; Ball et al., 1974/75; Koch, 1974*a*; Reisinger et al., 1976; Arditti et al., 1977; Tanaka and Sakanishi, 1977, 1978; Valmayor, 1977; Fast, 1979; Johnson et al., 1982; Ernst, 1984; Gil, 1987). To conserve the spirit of Rotor's pioneering work his method will be presented here as described in his original paper.

Plant Material.

> After the flowers have been picked the stalk is [removed,] washed in tap water and rinsed in distilled water. The bracts at nodes are then removed [Fig. Phal-2A]. These bracts cover the buds. Each bud is removed with a piece of the stem three-fourths of an inch above and below it. Usually, 1 or 2 nodes at the basal portion of the stalk have no bud. These are discarded. The upper portion which bore the flowers was also found to be useless. About 4 or 6 buds are usable [Fig. Phal-2B].

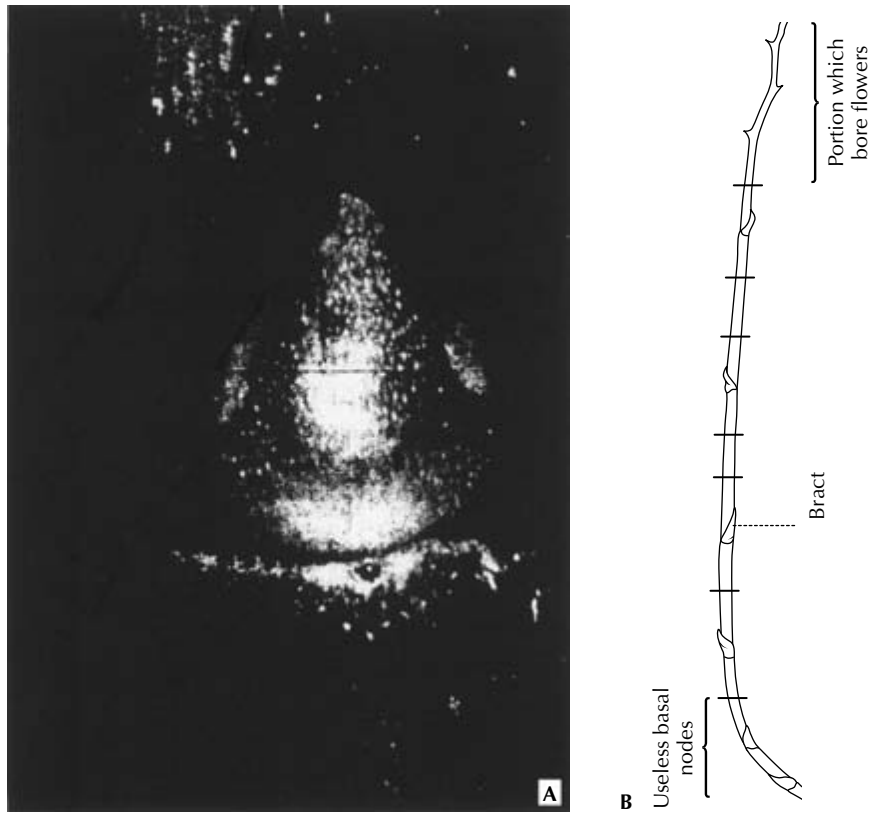


FIG. PHAL-2. **Flower-stem nodes and buds in *Phalaenopsis*. A. Node with bract removed (×16). B. Cuts separating nodes for clonal multiplication. (Sources: A, Ernst, 1986; B, Rotor, 1949.)**

These instructions are still valid at present. However, propagators may cut the flower stalks before the flowers open.

Surface Sterilization.

> The pieces are then freed of microorganisms by immersing them in a solution of calcium hypochlorite. The solution is prepared as for the disinfection of orchid seed. . . . Pieces of shoot are immersed for from two to five minutes. . . . This solution is made by dissolving 7 g calcium hypochlorite in 100 ml distilled water, stirring vigorously at 5- to 10-min intervals and decanting the supernatant or filtering the solution.

An immersion of 2–5 min is probably too short for decontamination; 15–20 min is more appropriate. Household bleach sodium hypochlorite (NaOCl), 20 ml diluted to 100 ml with water, is more commonly used as decontaminant at present. Following removal of the bracts that cover the buds, flower-stalk segments are first wiped with 95% ethanol and then immersed in the sterilant. They are shaken periodically and after about 20 min the NaOCl solution is removed and replaced with

sterile water. The rinse with sterile distilled water may be repeated two to three times. Stem sections may be left in the rinse solution until it is convenient to cut them into smaller sections for planting. These sections should consist of one bud and 1–2 cm of stem on each end.

Culture Vessels. The sections "are then planted in flasks, test tubes or bottles. . . ."

Culture Conditions. "The cultures may be kept in any room that is suitable for germination of orchid seeds." Elevated temperatures (25–30°C) promote growth (Tran Thanh Van, 1974; Tanaka and Sakanishi, 1977; Fu, 1978).

Culture Medium. "Solution [Knudson] C as used for germination of orchid seed" is employed (set Table Aranda-7). Culture media suitable for orchid seed germination are generally suitable.

Procedure. The author (Rotor, 1949) states that "in planting the pieces are simply laid on the surface of the agar." It is actually better to push the sections vertically into the agar with the bud pointing upward.

Developmental Sequence. Rotor obtained active growth (swelling of the bud and appearance of the first leaf) after 2 weeks.

> Sometimes growth may not occur until after two months. In general, conspicuous, well-developed buds grow earlier than small ones and the larger the diameter of the flower stalk, the more vigorous the plants produced. Out of 65 buds that had been cultured only 7 have failed to develop into plants. Roots appear after 2 or 3 leaves have been produced. These plants may then be transplanted to pots. It is probable that such plants will flower in a year or two.

General Comments. This procedure may have failed to receive the early attention it deserved because of low yields due to contamination. Rotor's method must be recognized as the first successful in vitro clonal propagation of orchids and perhaps of plants in general.

Phalaenopsis Flower-stalk Node Propagation

Among the numerous modifications of Rotor's method, the following employs a two-stage sterilization with Clorox (a commercial bleach) and a modified Vacin and Went medium (Table Phal-1; Vacin and Went, 1949; Intuwong et al., 1972*a*, 1972*b*).

Plant Material. Use clean, healthy, vigorous flower stalks with buds in their nodes. Stalks on which only a few flowers have bloomed are best; old stalks should be avoided.

Surface Sterilization. Wipe stalks two to three times using cheesecloth wetted with 95% ethyl alcohol (ethanol) or 10% Clorox (10 ml Clorox diluted to 100 ml with distilled water plus two to three drops of Tween 20, a non-ionic surfactant). After sectioning, place the nodes for 15 min in 10% Clorox. Following removal of the

TABLE PHAL-1. **Vacin and Went medium (Vacin and Went, 1949) modified for propagation of flower-stalk cuttings of *Phalaenopsis* (Intuwong et al., 1972*a*, 1972*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	5	750 mg l^{-1}	10	
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, so it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add other components of medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Add items 1 and 3–7 to the 500 ml distilled water (item 9) that contains item 2, adjust pH to 5.00–5.60, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Omit agar for liquid medium.

bracts, immerse the sections in 5% Clorox (5 ml Clorox diluted to 100 ml with water plus two to three drops of Tween 20) for 10 min and dip in sterile distilled water for 3 min. If Tween 20 is not available, use two to three drops of a mild household detergent.

Culture Vessels. Use 10 × 100-mm vials containing 12 ml medium.

Culture Conditions. Maintain cultures under approximately 200 ft-c of continuous illumination. Cultures may be maintained under illumination usually employed for seedlings, which includes General Electric Power Groove white fluorescent tubes or Sylvania Gro Lux lamps. Temperatures may range from 23 to 30°C with the higher temperature levels being preferable.

Culture Medium. A modified Vacin and Went medium (Table Phal-1) is used.

Procedure. Cut flower stalks into 65-mm sections with a sterile razor blade or scalpel, leaving 25 mm above the node and 40 mm below it. Expose the bud by removing the bract that covers it with sterile forceps. Care must be taken to completely remove

the bract without injuring the bud. Then sterilize with a 5% Clorox solution (10 ml Clorox diluted to 100 ml with water). Place the sections on a sterile Petri dish or glass slide. Cut 12 mm from each end with a sterile razor blade (preferably a Valet-type blade, which has one cutting edge and a blunt side covered with a sleeve) or scalpel. All tools must be dipped frequently in 95% ethanol or 10% Clorox (or flamed using a Bunsen burner or alcohol lamp) to maintain sterility. Transfer the sections from the Petri dish or the glass slide to culture vials, inserting them at a slight angle to just below the bud with the longer portion of the section submerged; in this way the emerging shoot will always point upwards.

Developmental Sequence. Shoots generally appear within a month. Most shoots are well rooted and ready for removal from the vial after 2 months. After that the plantlets develop and should be treated like seedlings.

If inflorescences appear instead of plantlets, allow the cultures to remain undisturbed and aseptically remove the terminal portion of the new flower stalk. As a result of this step plantlets may form from the lowermost node of new inflorescence.

General Comments. The main advantage of this method is that the plant itself is not damaged or endangered. A disadvantage is the lack of callus formation. This limits propagation to the available flower-stalk buds. "In our laboratory, some buds have produced two plantlets on occasion. Also, not all sections survive and some buds fail to grow. A great improvement of this procedure would be the induction of callus, and we are currently working on that" (Intuwong et al., 1972*a*).

Use of Plant Hormones to Improve Shoot Growth of *Phalaenopsis* Stem Nodes

An early study of mass clonal multiplication of *Phalaenopsis* from flower-stem nodes showed that the addition of BA promoted the growth of shoots (Koch, 1974*a*). The weight of the shoots generally increased with increasing amounts of BA within the 0.1–3 ppm range. Formation of multiple shoots per node was not reported. Addition of 2 ppm of the synthetic auxin NAA reduced shoot formation (Tse et al., 1971). The natural auxin IAA or the synthetic auxins potassium α-naphthaleneacetate (KNA) as well as 2,4-D increased initial shoot length but inhibited development subsequently (Koch, 1974*a*, 1974*b*).

Regeneration of Multiple *Phalaenopsis* Plantlets from Flower-stalk Nodes

Our work has shown that much higher levels of cytokinins, 25–125 ppm BAP in Knudson C medium (see Table Aranda-7) or REM medium (Table Phal-2), usually induce multiple shoots (Fig. Phal-3A, B) and even rosettes of plantlets from a single flower-stem node (Fig. Phal-3C; Ernst, 1986).

Plant Material. Flower stalks are removed from *Phalaenopsis* preferably before the first flower opens or at least as soon as the flowers begin to wilt.

TABLE PHAL-2. **REM medium for the propagation of *Phalaenopsis* through the culture of flower-stalk cuttings (Ernst, 1986)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[c]	150	15 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[c]	300	30 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	100	10 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	300	30 g l^{-1}	10	
5	Dipotassium phosphate, K_2HPO_4	50	5 g l^{-1}	10	
6	Ammonium nitrate, NH_4NO_4[a]	400	No stock	No stock	Weigh
	Iron[d]				
7	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
	Microelements				
8	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	3	300 mg l^{-1}	10	
9	Boric acid, H_3BO_3	0.1	10 mg l^{-1}	10	
10	Cupric sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}	10	
11	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}	10	
12	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50 mg l^{-1}	10	
	Cytokinin				
13	Benzylaminopurine (BAP)[e]	25	250 mg 10 ml^{-1} 95% ethanol		
	Sugar				
14	Sucrose	25 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[f]	To 1000 ml			
	Solidifier				
16	Agar[f]	2 g	No stock	No stock	Weigh

[a]Except for the omission of BAP (item 13) and addition of 10% (w/v) banana homogenate, this is the same medium employed for transflasking shoots in order to develop roots.

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]It is preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]BAP is heat-stable and can be added to medium prior to autoclaving. It can also be added after autoclaving under sterile conditions.

[f]Add items 1–12 to 900 ml distilled water (item 15), adjust pH to 5.0–5.6, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add cytokinin (item 13) to hot solution under sterile conditions with sterilized pipette, mix well, and distribute medium to preautoclaved culture vesels. Omit agar for liquid medium.

Surface Sterilization. The flower stalk is cut into sections with about 2 cm of stalk on each side of the bud, wiped with 95% ethanol, and immersed for about 20 min in a 1% solution of sodium hypochlorite. It is convenient to dilute 20 ml of commercial household bleach with 80 ml water in a 100-ml volumetric cylinder, place the nodal sections into this sodium hypochlorite solution, and shake the cylinder intermittently several times to assure wetting of the sections. After 20 min the liquid in the cylinder is drained and sterile distilled water is poured in to rinse the explants. The nodal segments are then placed in a sterile Petri dish, and the bracts are carefully removed with sterilized forceps. These sections are then reimmersed in a 0.5% sodium hypochlorite solution (10 ml commercial bleach diluted with 90 ml distilled

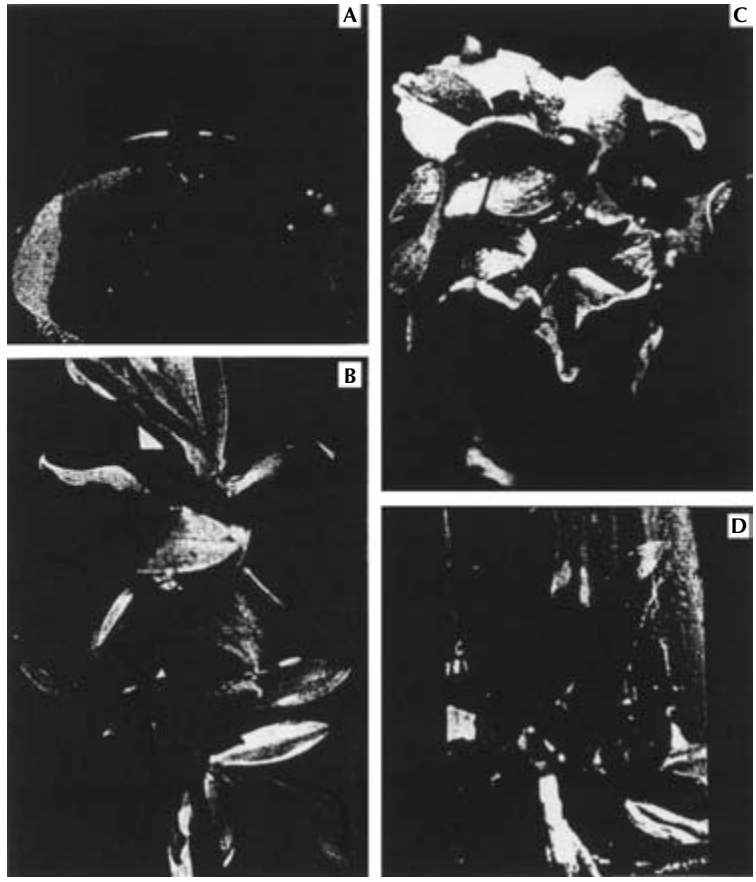


FIG. PHAL-3. **In vitro cultures of *Phalaenopsis* flower-stem nodes. A. Multiple shoots and PLBs developing from a node grown on REM medium containing 25 ppm BA (× 2). B. Multiple shoots but no roots growing from two flower-stalk nodes of a hybrid on REM medium containing 25 ppm BA (× 1.8). C. Rosette of shoots growing from one bud of a hybrid on REM medium containing 50 ppm BAP (× 3). D. Root development on multiple shoots from a single flower-stem bud, grown initially on REM medium containing 25 ppm BA and moved after 3 months to REM medium containing only 10% (w/v) ripe banana homogenate as additive (× 1.25). (Ernst, 1986.)**

water) and shaken occasionally for 10 min. After this period the bleach solution is drained once more and the cylinder is filled again with autoclaved distilled water for rinsing. Sections are then placed on a sterile Petri dish again and recut so that the stalk on each end is about 1–1.5 cm. The section can now be inserted into the nutrient medium such that the bud points upward and the section is partially inside the agar.

Culture Media. To stimulate multiple shoot formation, REM medium, which contains 25 mg l^{-1} BA (Table Phal-2), is employed. After shoots have developed, the

shoot-bearing sections are transferred without rinsing to REM medium, containing no hormone but 10% (w/v) banana homogenate (Table Phal-2) to stimulate root formation (Fig. Phal-3D).

Culture Vessels. Test tubes or small (125-ml) Erlenmeyer flasks are suitable for this procedure. Aluminum foil may serve as cover.

Developmental Sequence. Multiple shoots usually develop within 2–3 months. Roots form 1–2 months following transfer to a hormone-free medium that contains banana homogenate (Table Phal-2). The clones can then be moved to the greenhouse into a customary seedling mixture.

Shoot Growth of *Phalaenopsis* Flower-stalk Nodes in the Presence of Anti-auxin

Phalaenopsis flower-stem nodes were grown in the presence of the anti-auxin *trans*-cinnamic acid (tCA) to overcome possible dormancy of the buds (Ball et al., 1974/75; Reisinger et al., 1976; Arditti et al., 1977).

Plant Material. Use mature flower stalks. Best results are obtained with buds from nodes on the upper sections of the stalk (Fig. Phal-2B).

Surface Sterilization. Remove the bract (scale) that covers each bud. Wash the stalk section with distilled water containing 2–3 drops of a mild detergent per 100 ml. Rinse with distilled water and immerse in 50% Clorox (or any 5–5.5% sodium hypochlorite household bleach diluted with an equal volume of distilled water) for 4–6 min. Dip momentarily in sterile distilled water to remove bleach residues, and insert the sections into the culture medium.

Culture Vessels. A variety of containers can be used, including Erlenmeyer flasks (125–250-ml capacity), test tubes (150 or 250 mm × 25 mm), and prescription bottles (100 ml). Fill culture vessels with medium to approximately 25% of the containers' volume, and autoclave for 30 min at about 1 atm (15 pounds per square inch, psi).

Culture Conditions. Maintain cultures under approximately 150 ft-c and 16-h photoperiods. Sylvania Gro Lux fluorescent tubes were used in the original experiments, but other suitable lighting sources could be employed. The temperature should be 22–25°C.

Culture Medium. A modified Knop's medium (Table Phal-3) was used. The medium should contain isoleucine (13 mg l^{-1}) and tCA at a concentration that depends on the source of the stalk section:

- 1.48 mg l^{-1} tCA for upper and base section nodes;
- 14.8 mg l^{-1} tCA for mid section nodes.

TABLE PHAL-3. **Modified Knop's solution (Knop, 1884) for the culture of *Phalaenopsis* node sections (Ball et al., 1974/75)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b,c]	500	50 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$[c]	125	12.5 g l^{-1}	10	
3	Monopotassium phosphate, KH_2PO_4	125	12.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	125	12.5 g l^{-1}	10	
	Iron				
5	Ferric citrate, $FeC_6H_5O_7 \cdot 5H_2O$[d]	10	No stock	No stock	Weigh
	Minor elements				
6	Boric acid, H_3BO_3	56 µg	56 mg l^{-1}	0.25	
7	Molybdenum trioxide, MoO_3	16 µg	16 mg l^{-1}	0.25	
8	Cupric sulfate, $CuSO_4$	40 µg	40 mg l^{-1}	0.25	
9	Zinc sulfate, $ZnSO_4$	33.1 µg	33.1 mg l^{-1}	0.25	
	Polyol				
10	Inositol	100	No stock	No stock	Weigh
	Amino acid				
11	L-isoleucine	13	650 mg 25 $m1^{-1}$ 95% ethanol[e]	0.5	
	Vitamin				
12	Thiamine·HCl	0.4	100 mg 100 ml^{-1} 95% ethanol[e]	0.4	
	Anti-auxin				
13	*trans*-cinnamic acid (tCA)	148	14.8 g 100 ml^{-1} 95% ethanol[e]	0.1	
	Cytokinin				
14	Benzylaminopurine (BAP)	20	100 mg 100 ml^{-1} 95% ethanol[e]	20	
	Sugar				
15	Sucrose[f]	20 g	No stock	No stock	Weigh
	Solvent				
16	Distilled water[g]	To 1000 ml			
	Solidifier				
17	Agar[f,g]	130 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The number of waters of hydration is not given in the original paper.

[d]Knop's solution does not contain iron which should be added, preferably in chelated form. To prepare a stock solution add 3.73 g l^{-1} chelating agent (Na_2EDTA) and 2.78 g l^{-1} ferrous sulfate ($FeSO_4 \cdot 7H_2O$). Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Keep refrigerated between uses.

[f]As originally formulated, the solution does not contain sugar or agar. These components should be added as 20 g l^{-1} sucrose (item 15) and 12–16 g l^{-1} agar (item 17).

[g]Add items 1–10 and 15 to 900 ml distilled water (item 16), adjust pH to 5.0–5.6, and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add remaining organics (items 11–14) after autoclaving to the hot solution under sterile conditions with sterilized pipettes. Mix well, and distribute medium to preautoclaved culture vessels.

BA and inositol are also included because of their generally beneficial effects on tissue cultures (Murashige, 1974).

Developmental Sequence. A swelling of the buds is the first sign that a culture might be successful. This is followed by leaf development and finally by root formation. Subsequent growth resembles that of seedlings and leads to plants that should be potted in fir bark.

Clonal Propagation of *Phalaenopsis* through Injured Nodal Flower-stalk Buds

Some flower-stem buds of *Phalaenopsis* do not develop into shoots but form masses of callus (Tse et al., 1971; Hackett et al., 1973). Adventitious buds form on callus surfaces and develop into shoots. Twelve plantlets can form within 6 months on one node, and additional ones may be produced. This observation led these authors to develop methods for callus induction.

Plant Material. Inflorescences of a number of *Phalaenopsis* hybrids were used in the original research.

Surface Sterilization. Use the method employed in the section *Phalaenopsis* Flower-stalk Node Propagation (see p. 911, Vol. II).

Culture Media. Knudson C (see Table Aranda-7) or MS (Murashige and Skoog, 1962; see Table Cym-11) media, each solidified with 1% agar, are used. In some cases 2 mg l^{-1} NAA is added.

Culture Vessels. Glass vials of 10-ml capacity were used in the original research. Other culture vessels may also be used.

Procedure. Before inserting the node section into one of the media described above, injure the buds by one of the following methods:

1 Remove two-thirds of each bud by cuts that are oblique to its axis and parallel to the stem axis.
2 Bisect the buds by cutting longitudinally through their axes.
3 Puncture the buds longitudinally from apex to base with a sharp sterile needle.

Methods 1 and 3 yielded the highest callus formation (Table Phal-4). The addition of auxin (NAA) proved detrimental with Knudson C medium (see Table Aranda-7) but resulted in some improvement with MS medium (see Table Cym-11), as shown in method 1 in Table Phal-4.

Phalaenopsis comes from the Greek phalaina (moth) and opsis (moths), alluding to the resemblance of the flowers of type species to some tropical moths (Schultes and Pease, 1963).

TABLE PHAL-4. **Callus and/or shoot formation by flower-stalk node explants of *Phalaenopsis* (Tse et al., 1971)**

Injury technique	Medium[a]	NAA,[a] mg l^{-1}	Percentage of nodes forming	
			Callus and shoots	Single shoots
1. Removing 2/3 of each bud	KC	0	50	0
	KC	2	20	0
	MS	0	30	0
	MS	2	60	0
2. Longitudinal cut	KC	0	10	0
	KC	2	0	0
	MS	0	0	30
	MS	2	0	48
3. Puncturing	KC	0	20	20
	KC	2	0	0
	MS	0	60	0
	MS	2	50	0
4. Control (intact bud)	KC	0	0	60
	KC	2	0	40
	MS	0	0	60
	MS	2	0	50

[a]KC, Knudson C (see Table Aranda-7); MS, Murashige–Skoog (see Table Cym-11 without item 13); NAA, naphthaleneacetic acid.

Mass Clonal Propagation of *Phalaenopsis* through Tissue Culture

Considerable difficulties have been encountered in the development of tissue-culture methods for *Phalaenopsis*. A common problem encountered is the high level of phenolics released by tissues. The resulting oxidation products of these substances, which diffuse into the culture media, appear to be toxic and cause the explants to turn brown or black and die (Morel, 1974; Flamee and Boesman, 1977; Fast, 1979). Numerous attempts have been made to eliminate these exudates and especially their oxidation products with antioxidants, polyphenoloxidase inhibitors, polyvinylpyrrolidone, activated charcoal, and many other adsorbents. Most effective has been the frequent transfer of explants (every 2–3 weeks) to fresh medium (Flamee and Boesman, 1977).

Clonal Propagation of *Phalaenopsis* by Shoot-tip Culture

An early, successful method for shoot-tip culture of *Phalaenopsis* was reported from the University of Hawaii (Intuwong and Sagawa, 1974).

Plant Material. *Phalaenopsis amabilis*, *Phalaenopsis* × Star of Santa Cruz, *Phalaenopsis* × Surfrider, *Phalaenopsis* × Ruby Lips, *Phalaenopsis* × Arcadia, and *Phalaenopsis cochlearis* were used. Explants were taken from vegetative shoots of adult plants with six to seven leaves or from aseptically grown plantlets obtained from flower-stalk nodes; the latter method does not sacrifice the mother plant.

Surface Sterilization. Leaves are removed and the shoot axis is then sterilized with 10% Clorox (10 ml Clorox diluted to 100 ml with water; about 0.5% NaOCl) for

15 min. The leaf bases are removed next, and the tissue is again sterilized in 5% Clorox (5 ml Clorox diluted to 100 ml with sterile distilled water) for 10 min. Explants that are 2–3 mm^3 in size and consist of the apical meristem, two to four leaf primordia, and two to three axillary buds covered with scale-like leaves are removed and rinsed in sterile distilled water before culturing. There is no need to sterilize explants taken from aseptically grown plantlets.

Culture Vessels. Use 50-ml Erlenmeyer flasks containing 20 ml medium.

Culture Conditions. Cultures are maintained under continuous illumination of 200 ft-c (General Electric Power Groove white fluorescent lamps were used in the original work) at 26 ± 2°C. Liquid cultures should be agitated at 160 rpm (a New Brunswick Model V shaker was used for the initial experiments).

Culture Medium. Solid or liquid Vacin and Went medium modified as for the culture of flower-stalk sections (Intuwong et al., 1972*a*, 1972*b*) and enriched with 15% (v/v) coconut liquid endosperm (Table Phal-5) is employed. Sucrose is omitted from

TABLE PHAL-5. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of *Phalaenopsis* flower-stalk cuttings (Intuwong and Sagawa, 1974)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	Or weigh
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	20 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	Or weigh
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	Weigh
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	5.75	5.7 g l^{-1}	1	
	Complex additives				
8	Coconut water	150 ml			
	Sugar				
9	Sucrose[e]	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, so it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Then add other components of medium.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Do not add sugar for a medium used to culture PCBs.

[f]Add items 1 and 3–8 to the 500 ml distilled water (item 10) that contains tem 2 and adjust pH to 5.0–5.6. Add sugar (item 9) for liquid modified Vacin and Went medium. Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When the agar is completely dissolved, dispense the medium into culture vessels and autoclave. Omit agar for liquid medium.

the solid medium onto which PLBs are transferred after being in liquid for a month. The solid medium used to induce further proliferation is also free of sucrose.

Procedure. Culture explants in agitated liquid medium (Table Phal-5), change every 10 days for a month, and then transfer onto a solid sucrose-free medium. Frequent subcultures to new medium without sucrose results in the production of numerous pseudobulb-like bodies.

Developmental Sequence. Yellowish PLBs are produced on axillary and terminal bud explants within a month of culture. The same happens on inflorescence nodes. On transfer to solid sucrose-free medium, the PLBs turn green. If left undisturbed on solid medium, these bodies form plantlets within 3–5 months. However, if they are frequently subcultured onto new, solid sucrose-free medium, numerous new PLBs will be produced. At first the PLBs are less than 0.5 mm in diameter, rough, and globular. On reaching a diameter of 0.5–1 mm they become shiny and develop numerous trichomes on their lower half. When they reach a diameter of 1–3 mm, a dark green spot becomes apparent (from which the first leaf later appears), and many trichomes are still present. An expanded leaf and a root near its base appear when the PLBs are 4 mm long. They are still 4 mm in diameter when the leaf enlarges, the root elongates, and velamen develops. Three months are required for this developmental process.

General Comments. Numerous plants were produced by this technique at the University of Hawaii. It should therefore prove useful to orchid propagators everywhere.

Mass Clonal Propagation of *Phalaenopsis* from Leaf Tissue

Production and proliferation of PLBs on the bases of thickened leaf sections (rather than on meristems) arising from clonal propagation of flower-stalk nodes was demonstrated in a doctoral dissertation (Koch, 1974*a*) by a student of K. Zimmer. A liquid modified Knudson C medium containing BA and potassium naphthaleneacetate (KNA) induced the formation of PLBs. Further proliferation of PLBs took place on different modifications of the Knudson C medium.

This method was extended further by Zimmer and his collaborators at the Technical University of Hannover, Germany (Zimmer and Pieper, 1976, 1978, 1979; Zimmer, 1980).

Plant Material. Flower-stalk node sections of pink *Phalaenopsis* hybrids Zada × Zada, Lipperose, and Zauberrose were used.

Surface Sterilization. Node sections, 4 cm in length, of the flower stalks are washed with a household detergent, following removal of the bracts and immersed for 5–6 min in a solution of calcium hypochlorite ($CaCl_2O$, 20 g in 140 ml distilled water, shaken a few times, and decanted). This is followed by a rinse with sterile distilled water before the bud is removed.

Buds further away from the base of the stalk tend to become less contaminated (Koch, 1974*a*).

Culture Media. Owing to the difficulty and expense of obtaining coconut endosperm in Europe, 10% (v/v) of bleeding sap of birch trees (Pieper and Zimmer, 1974, 1976*b*; Zimmer and Pieper, 1976) was added to a modified Knudson C medium (Table Phal-6; Zimmer and Pieper, 1978) for the development of PLBs.

TABLE PHAL-6. **Knudson C medium (Knudson, 1946) for the clonal propagation of *Phalaenopsis* (Koch, 1974*a*; Zimmer and Pieper, 1978)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	500 g	50 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Ammonium nitrate, NH_4NO_3[b]	500	50 g l^{-1}	10	
	Iron				
6	Chelated iron, Fe_2EDTA	30	No stock	No stock	Weigh
	Microelements[c]				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7	7 g l^{-1}	1	
8	Zinc sulfate, $ZnSO_4$	1	1 g l^{-1}	1	
9	Boric acid, H_3BO_3	1	1 g l^{-1}	1	
10	Cupric sulfate, $CuSO_4$	0.03	30 mg l^{-1}	1	
11	Aluminum chloride, $AlCl_3$	0.03	30 mg l^{-1}	1	
12	Nickel chloride, $NiCl_2$	0.03	30 mg l^{-1}	1	
13	Potassium iodide, KI	1	1 g l^{-1}	1	
	Auxin				
14	Potassium naphthylacetate (KNA)	0.3	30 mg 100 ml^{-1} 95% ethanol[d]	1	
	Cytokinin				
15	Benzylaminopurine (BAP)[e]	2	200 mg 100 ml^{-1} 95% ethanol[d]	1	
	Complex additive				
16	Bleeding sap of birch trees[f]	100 g	No stock	No stock	Weigh
	Sugar				
17	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Agar[g]	5 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The microelements (items 7–13) can be dissolved together in 1 l of distilled water and 1 ml of this solution may be used for each 1000 ml medium.

[d]Keep refrigerated between uses.

[e]Add BAP to the medium only when it will be used to culture parts of broken plantlets.

[f]This can be replaced with an equal volume of filtered coconut water.

[g]Add items 1–13 to 800 ml distilled water (item 18), adjust pH to 5.0–5.6, add sugar (item 17), and bring volume to 1000 ml with distilled water (item 18). While stirring, slowly add agar (item 19) and heat the medium until it turns clear (about 80–90°C). The hormones (items 14 and 15) may be added to the final solution before or after sterilization in the autoclave, since they are sufficiently heat-stable. If added after autoclaving, incorporate them while the solution is still hot, under sterile conditions, using sterile pipettes. Mix well and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

Culture Conditions. The buds are cultivated in the dark at 26°C for 2 weeks. Then necrotic stalk tissue is removed and the node sections are transferred to fresh medium. Temperature should be lowered to 22°C and the cultures illuminated with (per square meter) two 40-W warm white fluorescent tubes and two 40-W "Fluora" tubes (similar to Sylvania Gro Lux) at a distance of 60 cm for the culture period.

Procedure. The flower stalk buds are excised following removal of the bracts by making a semicircular cut. They should be cultured as indicated above.

Developmental Sequence. After about 2 months the buds develop into plantlets. These can be divided into leaves, leaf sections, roots, and stem tissues by breaking them; this seems to be more effective than cutting with a razor blade or a scalpel. These fragments are cultured at 26°C in the dark on a modified Knudson C medium (Table Phal-6). After 2 weeks the temperature should be lowered to 22°C and the cultures must be illuminated. Leaves, leaf sections, stem tissue, and, in some cases roots, form numerous PLBs within 8 weeks (Figs Phal-4 and Phal-5). Newly grown plantlets can be broken again and cultured in the dark and the entire cycle repeated to form more PLBs and plantlets.

General Comments. About 500–1000 plants can be produced by this method from a single flower-stem bud (Zimmer and Pieper, 1978; Hölters, 1983). However, bleeding sap of birch trees may not be easy to obtain.

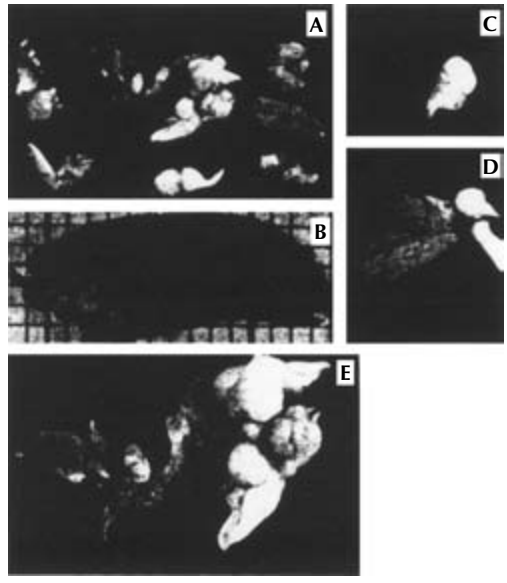


FIG. PHAL-4. **A. Development of PLBs on broken pieces of leaf, shoot, and root tissues of *Phalaenopsis*. B–E. PLBs developing on broken leaf tissue of *Phalaenopsis*. (Zimmer and Peiper, 1978; Hölters, 1983.)**

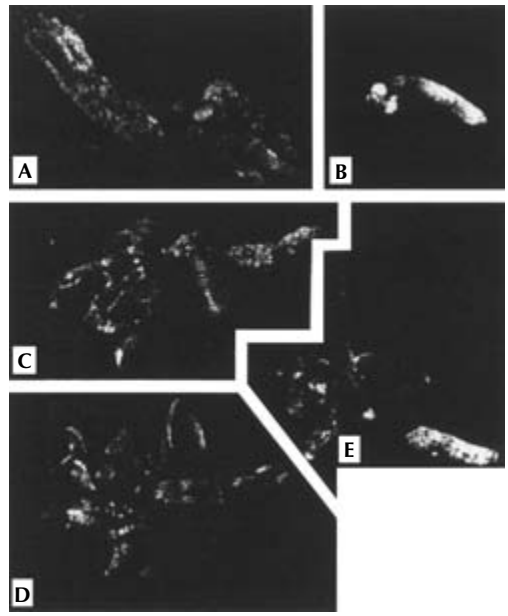


FIG. PHAL-5. **A–E. PLBs and plantlets developing on broken root parts of *Phalaenopsis* hybrid clones derived from flower-stem nodes. Proliferation on leaf, stem, and root explants. (Zimmer and Pieper, 1978; Hölters, 1983.)**

Mass Clonal Propagation of *Phalaenopsis* from Leaf Tissues

Investigations on the clonal propagation of *Phalaenopsis* from leaf tissues were carried out by M. Tanaka and his collaborators at the University of Osaka, Japan (Tanaka et al., 1974; Tanaka and Sakanishi, 1977, 1980, 1985). Initial experiments were carried out with emerging leaves on mature plants and those taken from seedlings. Leaves of mature plants did not produce PLBs, but they developed on leaf explants taken from very young seedlings. Production of PLBs was reduced with increased age of the seedlings (Tanaka et al., 1974). The method outlined here was developed with leaf tissue from nodal shoots of *Phalaenopsis* flower-stalk sections (Tanaka and Sakanishi, 1977, 1980).

Plant Material. Flower stalks were obtained from a 5–6-year-old *Phalaenopsis amabilis* hybrid.

Surface Sterilization. The flower stalks are surface-sterilized by wiping them three times with gauze wetted with 70% ethanol. They are then sliced into sections consisting of one lateral bud and 3 cm of flower stalk on each end. The sections are then treated for 10 min in a filtered aqueous solution of 7% calcium hypochlorite containing 0.1% wetting agent (Tween 20). This is followed by rinsing them three times with sterile water and removing the bracts with sterile forceps. The flower-stalk sections are then trimmed so that only 0.5–1.0 cm of the stalk on each

TABLE PHAL-7. **Vacin and Went medium (Vacin and Went, 1949) modified for the propagation of *Phalaenopsis* from leaf tissues (Tanaka and Sakanishi, 1977, 1980)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	Weigh
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	Weigh
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water[e]	200 ml			
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[g]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, so it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add other components of medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]If coconut water is not available, substitute 1–1.5 mg l^{-1} benzylaminopurine.

[f]Add items 1 and 3–8 to the 500 ml distilled water (item 10) that contains item 2, adjust pH to 5.0–5.6, add sugar (item 9), and adjust solution with distilled water (item 10) to 1000 ml. Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added while stirring to the cold solution, which is then brought to a boil. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar for liquid medium.

side of the node remains; the trimmed sections are inserted in a modified Vacin and Went medium (Table Phal-7) with the base of the node submerged.

Culture Vessels. Test tubes (25 × 200 mm) containing 16 ml medium were used in the original research. After planting the node sections the tubes were covered with aluminum foil.

Culture Conditions. Cultures should be maintained at 28°C for maximum vegetative shoot production (Fig. Phal-6; Table Phal-8; Tanaka and Sakanishi, 1978) under 16-h photoperiods and an intensity of approximately 500 lx provided by fluorescent lamps.

Culture Media. A modified Vacin and Went medium (Table Phal-7) with the addition of 2.5 ppm BAP is used. Propagation of leaf sections excised from shoots

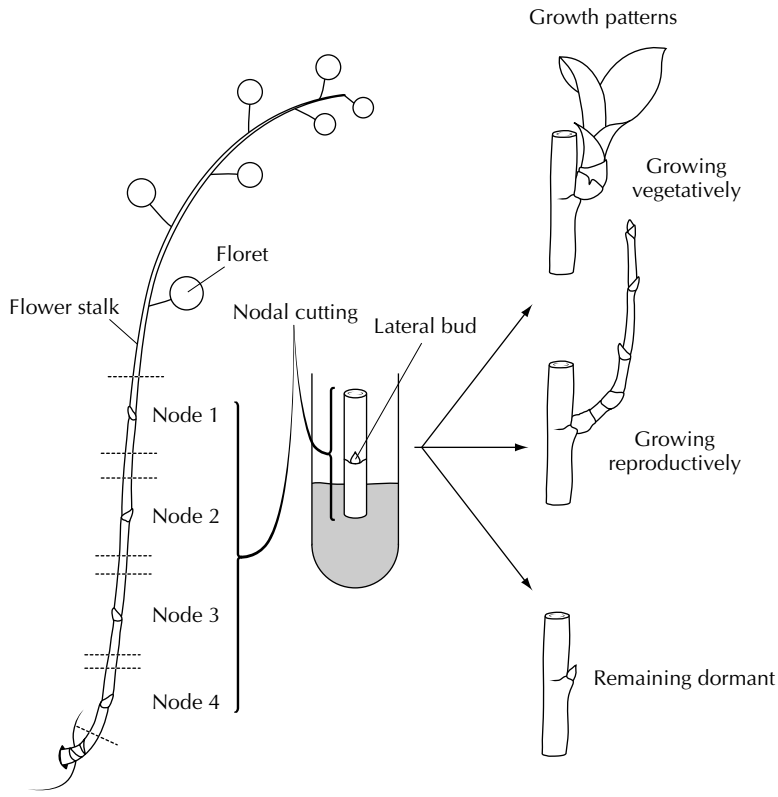


FIG. PHAL-6. ***Phalaenopsis*** **flower stalk showing node positions and different growth patterns of lateral buds from nodal cuttings (Tanaka and Sakanishi, 1977, 1978, 1980).**

TABLE PHAL-8. **Growth of *Phalaenopsis* flower-stalk buds taken from inflorescences that elongated under different temperatures (Tanaka and Sakanishi, 1978)**[a]

Temperature, °C		Number of buds		Percentage of buds growing into shoots		
During flower-stalk elongation	During bud culture	Cultured	Surviving	Vegetative	Reproductive	Dormant
18	28	24	20	65	0	35
18	20	24	21	5	52	43
28	28	20	14	57	0	43
28	20	20	15	6	47	47

[a]Observation after 15 weeks of culture.

produced in vitro is carried out on a modified MS medium (Table Phal-9). A liquid Vacin and Went medium containing 20% coconut water (v/v) is employed for further proliferation of the PLBs (Table Phal-10; Tanaka and Sakanishi, 1978).

Developmental Sequence. Flower-stalk sections cultured at 28°C from nodes 4 and 3 (Fig. Phal-6) showed the highest ratio of vegetative growth and no or few

TABLE PHAL-9. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for the formation of protocorm-like bodies of *Phalaenopsis* in clonal propagation (Tanaka and Sakanishi, 1977, 1980)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Cupric sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid	1	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	10	100 mg 100 ml^{-1} 95% ethanol[e]	10	
	Vitamins				
12	Niacin (nicotinic acid)	l	100 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Purine				
15	Adenine	10	100 mg 100 ml^{-1} 95% ethanol[e]	10	
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[f]	To 1000 ml			
	Solidifier				
18	Agar[f]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep refrigerated between uses.

[f]Add items 1–9 to 900 ml distilled water (item 17), adjust pH to 5.0–5.6, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to cold solution while stirring, and then brought to a boil. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid, hormones, vitamins, and nucleotide (items 8 and 10–15) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium into preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE PHAL-10. **Liquid Vacin and Went medium (Vacin and Went, 1949) modified for proliferation of leaf-tissue-derived protocorm-like bodies of *Phalaenopsis* (Tanaka and Sakanishi, 1980)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	Weigh
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water[e]	200 ml			
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, so it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add other components of medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Filter before using.

[f]Add items 1 and 3–7 to the 500 ml distilled water (item 10) that contains item 2, adjust pH to 5.0–5.6, add sugar (item 9) and coconut water (item 8), and adjust solution to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, dispense into culture vessels, and autoclave.

dormant buds, whereas many buds of nodes 2 and 1 remained dormant (Fig. Phal-6). When the culture temperature was lowered to 20 or 25°C, a substantial number of sections from nodes 1–4 grew reproductively (Fig. Phal-6). After the first leaf on the shoots expands, about 2 months after the start of culture on the modified Vacin and Went medium (Table Phal-7), leaf segments are excised aseptically and transplanted to modified MS medium (Table Phal-9). PLBs ranging from one to seven per segment and averaging 3.8, form on the adaxial surfaces of the explants (Figs Phal-7 and Phal-8A–C, E). These PLBs can be further proliferated in liquid Vacin and Went medium, supplemented with 20% (v/v) of coconut water (Table Phal-10) on a rotary shaker (160 rpm; Figs Phal-7 and Phal-8F; Tanaka, 1978). A solid Vacin and Went medium supplemented with 20% (v/v) coconut water (Fig. Phal-8G) can also be used. The PLBs can be differentiated on media used for seed germination of *Phalaenopsis* (Fig. Phal-9) and later grown under greenhouse conditions to the flowering stage (Fig. Phal-9B, C).

General Comments. Elevated temperatures (28–30°C) during cultivation of lateral buds may be an important factor in preventing their dormancy (Fu, 1978). Since the quality of flowers that seedlings would produce is an unknown factor, their clonal

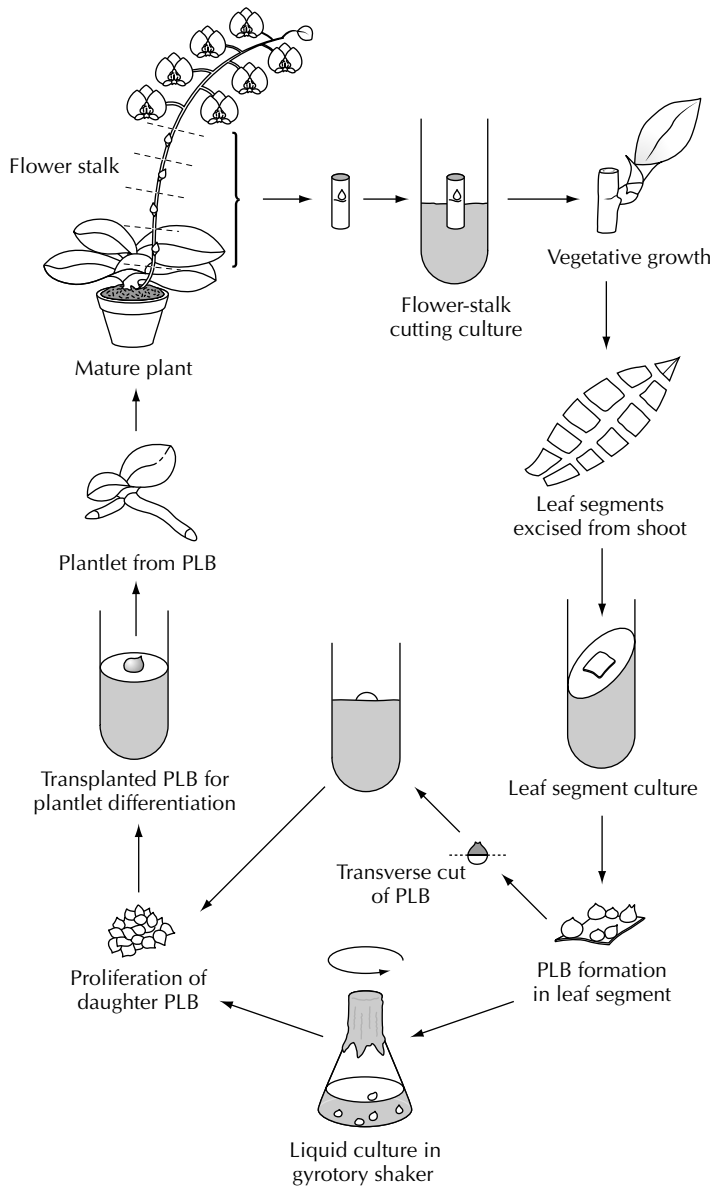


FIG. PHAL-7. **Sequence of mass clonal propagation of *Phalaenopsis* through flower-stalk cuttings followed by proliferation of PLBs cultured from their excised leaf segments (Tanaka and Sakanishi, 1978, 1980; Tanaka, 1987).**

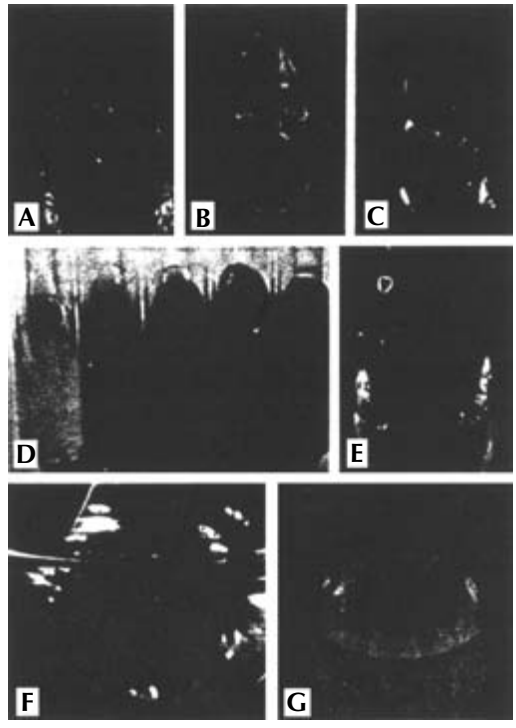


FIG. PHAL-8. **PLBs formed on leaf sections taken from shoots of flower-stalk cuttings. A, B. *Phalaenopsis* Lavender Lady × [Lavender Lady × (Clarelen × Zada)]. C. *Phalaenopsis stuartiana*. D. Medium blackening through release of phenolics from leaf explants. E. *Phalaenopsis amboinensis*, proliferation of secondary PLBs. F. In liquid medium (Table Phal-9) on reciprocating rotary shaker, after 165 days of culture. G. Sectioned transversally and placed on solid medium (Table Phal-11), after 55 days of culture. (Tanaka and Sakanishi, 1980; Tanaka, 1987.)**

multiplication may be of little practical use. However, clonal propagation of leaves or other tissue from aseptic plantlets obtained from flower-stalk nodes makes possible the multiplication of desirable plants. This procedure is reported to regenerate plants that produce uniform flowers (Fig. Phal-9D, E).

A problem previously discussed is the release of phenolics from *Phalaenopsis* tissues; oxidates of these phenolics gradually darken the media and prove toxic to the explants (Fig. Phal-9D).

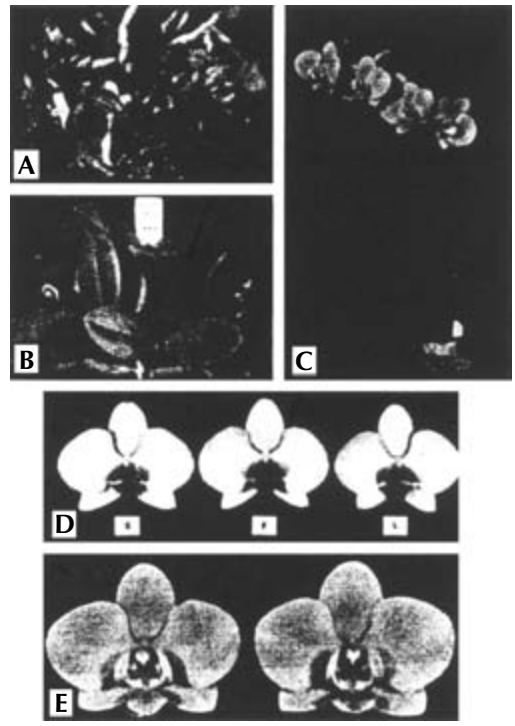


FIG. PHAL-9. **Plantlet regeneration. A. Plantlets derived from PLBs produced by explants of *Phalaenopsis* [(White Falcon × Persistent) × Jimmy Hall] after 8 months on a seedling medium. B. *Phalaenopsis* Callie Flynn plantlets growing in a community pot. C. First flowering of a *Phalaenopsis* Callie Flynn clone produced from leaf tissues, 3 years after transfer to a community pot. D. Flowers, reported to be from a stock plant (s) and clones (F and L). E. Flowers from a stock plant [Callie Flynn (left) and from a clone (right)]. (Tanaka, 1987.)**

Induction of Protocorm-like Bodies on Mature *Phalaenopsis* Leaf Explants

Additional studies by M. Tanaka and his associates showed that hormones used in the MS medium employed for leaf explants from flower-stalk buds can induce PLBs on emerging leaves of mature plants (Tanaka and Sakanishi, 1985).

Plant Material. The last emerging (uppermost) leaf of a *Phalaenopsis amabilis* hybrid (average 5 cm in length) is removed (Fig. Phal-10A).

Surface Sterilization. Leaf explants are soaked in 70% ethanol for 2–3 sec and immersed in a filtered 7% calcium hypochlorite solution containing 1 drop of Tween 20 per 100 ml bleach. After sterilization for 10 min the leaf is rinsed with sterile water. Segments, 8–10 mm^2, are excised from surface-sterilized leaves under sterile conditions with a razor blade (Fig. Phal-10B).

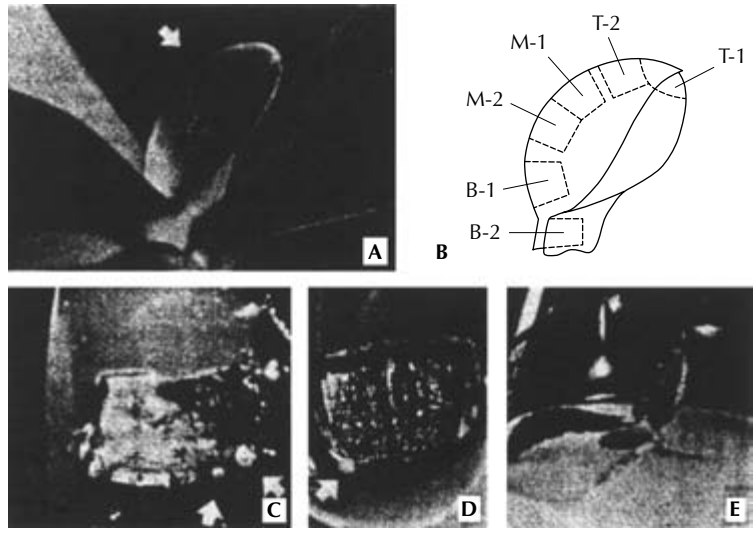


FIG. PHAL-10. **Clonal propagation of mature *Phalaenopsis* from leaf explants. A. Last emerging (uppermost) leaf (arrow), ca. 5 cm in length, which is used as a source of explants for clonal propagation of a *Phalaenopsis amabilis* hybrid. B. Explants, 8–10 mm^3, are cut from the leaf; section B-2 gave explants that produced PLBs, but only from an etiolated, proximal region. C, D. Two sections with PLBs after 173 days (C) and 239 days (D) of culture (arrows). E. A plantlet reported to have differentiated from this mature hybrid 10 months after transplanting to a Knudson C medium. (Tanaka and Sakanishi, 1985.)**

Culture Vessels. Test tubes (24 × 200 mm) containing 16 ml of nutrients were used in the original research, but other containers can be employed. The sealed tubes were sterilized at 121°C for 15 min.

Culture Conditions. In the original work, cultures were maintained at 25°C under 16-h photoperiods of approximately 500 lx provided by plant-growth fluorescent lamps.

Culture Medium. A modified MS medium (Table Phal-11) was used. PLBs were transplanted onto a modified Knudson C medium (Table Phal-12) in Erlenmeyer flasks to develop into plantlets.

Developmental Sequence. Some leaf explants turned black and died; others remained green for as much as 7 months, but failed to produce callus or PLBs. Only two segments of etiolated, proximal leaf sections from region B-2 (Fig. Phal-10B) developed one or two protuberance(s), which formed PLBs after 173 and 239 days, respectively (Fig. Phal-10C, D). Plantlet(s) were obtained from these PLBs (Fig. Phal-10E) upon culture on modified Knudson C medium (Table Phal-12).

General Comments. This method shows that emerging leaves of adult plants can be utilized in the clonal propagation of *Phalaenopsis*; however the yield is low. Success may depend largely on the plant material used.

TABLE PHAL-11. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for clonal propagation of leaf explants from adult *Phalaenopsis* plants (Tanaka and Sakanishi, 1985)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1	100 mg 100 ml^{-1} 95% ethanol[f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	10	1 g 100 ml^{-1} 95% ethanol[f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	l	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Purine				
15	Adenine	10	1 g 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar[i]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin do not dissolve, add a few drops of dilute KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 17), adjust pH to 5.0–5.6, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid, hormones, vitamins, and purine (items 8, 10–15) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar when preparing liquid medium.

TABLE PHAL-12. **Knudson C medium (Knudson, 1946) modified for differentiation of protocorm-like bodies from adult leaf explants of *Phalaenopsis* (Tanaka and Sakanishi, 1985)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
	Iron[c]				
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Polyol				
7	Inositol	100 g	No stock	No stock	Weigh
	Vitamins				
8	Nicotinic acid (niacin)[d]	1	100 mg 100 ml^{-1} 5% ethanol[e]	1	
9	Thiamine·HCl[d]	1	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
10	Peptone	2 g	No stock	No stock	Weigh
	Sugar				
11	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[f]	To 1000 ml			
	Solidifier				
13	Agar[f]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[d]Add vitamins (items 8 and 9) to medium after autoclaving and while solution is still hot, under sterile conditions with sterilized pipettes. Then dispense medium into preautoclaved culture vessels.

[e]Keep frozen between uses.

[f]Add items 1–7 to 900 ml distilled water (item 12), adjust pH to 5.0–5.6, add peptone (item 10) and sugar (item 11), and adjust volume to 1000 ml with distilled water (item 12). Bring solution to a gentle boil, and add agar (item 13) while constantly stirring. Agar can also be added while constantly stirring, to the cold water which is then brought to a gentle boil. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Omit agar for liquid medium.

Mass Clonal Propagation of *Phalaenopsis* from Flower-stalk Nodes and their Leaves

A method that produced PLBs from flower-stalk node cuttings of *Phalaenopsis* as well as from leaf sections generated from them was developed by Dr. Norbert Haas-von Schmude (1983, 1985).

Plant Material. Flower-stem sections, 1–1.5 cm long and each having one bud, were taken from *Phalaenopsis* hybrids Münsterland Stern "Alpha," Babette "Symphony," Windspiel "Düsseldorf," and Barbara Moler "Firecracker."

Surface Sterilization. Node sections are decontaminated by soaking them for about 15 min in 0.6% sodium hypochlorite (12 ml household bleach diluted to 100 ml with distilled water). The sections are then rinsed with sterile distilled water.

Culture Conditions. The cultures were kept under artificial light for 18-h photoperiods at 27°C.

Culture Media. Two modifications of MS medium (Murashige and Skoog, 1962) are used, one for proliferation (Table Phal-13) and the second for differentiation (Table Phal-14).

Procedure. Place half of the node sections in liquid modified MS medium (Table Phal-13) and shake at 100 rpm for several weeks. Culture the other half on the same (but solidified) medium. Buds in liquid culture develop PLBs while those on solid medium produce plantlets.

After cutting the leaves produced by the plantlets cultured on the solid modified MS medium, culture these explants again on the same substrate (Table Phal-13). PLBs appear along the cut basal part, the tip, and the edges of the leaf explants. Small cells with large nuclei were seen in the embryonic tissue that forms. This tissue may be cut and shaken in liquid modified MS medium (Table Phal-13) as described above.

Developmental Sequence. The PLBs developing in liquid solution are transferred to the solid differentiation medium (Table Phal-14). The PLBs form no chlorophyll in the dark but turn green and develop normally when illuminated.

General Comments. With this method it was possible to produce more than 30,000 plantlets from one flower stalk of the *Phalaenopsis* Babette "Symphony" in 3 years. *Phalaenopsis* Münsterland Stern "Alpha" yielded 10,000 plants in 2 years, and *Phalaenopsis* Barbara Moler "Firecracker" gave 3000 plantlets in 18 months (Haas-von Schmude, 1983). All three of these hybrids have *Phalaenopsis lueddemanniana* and *Phalaenopsis fasciata* in their background. These species have a natural tendency to form keikis and may well be easier to proliferate than hybrids of *Phalaenopsis amabilis* and other large-flowered types.

The fact that not all genotypes of *Phalaenopsis* behave in the same manner under identical culture conditions has been pointed out (Reuter, 1983).

TABLE PHAL-13. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) used for proliferation in the clonal propagation of *Phalaenopsis* (Haas-von Schmude, 1983)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot H_2O$	40	4 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	9	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
11	Pyridoxine·HCl (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
12	Thiamine·HCl (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[f]	1	
	Complex additives				
13	Casein hydrolysate	1 g	No stock	No stock	Weigh
14	Coconut water[g]	580 ml			
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Keep refrigerated between uses.

[g]Filter before use.

[h]Add items 1–7 and 9 to 350 ml distilled water (item 16); then add sugar (item 15) and casein hydrolysate (item 13). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added while stirring, to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, add coconut water (item 14), adjust volume to 1000 ml and the pH to 5.0–5.6 with dilute KOH or HCl, as needed. Pour into a 2-l flask and autoclave. Add amino acid (item 8) and vitamins (items 10–12) to hot and still liquid solution under sterile conditions, using sterile pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE PHAL-14. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962), used for differentiation in the clonal propagation of *Phalaenopsis* (Haas-von Schmude, 1983)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
11	Pyridoxine·HCl (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
12	Thiamine·HCl (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[f]	1	
	Complex additives				
13	Casein hydrolysate	1 g	No stock	No stock	Weigh
14	Coconut water[g]	200 ml			
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Keep refrigerated between uses.

[g]Filter before use.

[h]Add items 1–7 and 9 to 700 ml distilled water (item 16). Add sugar (item 15) and casein hydrolysate (item 13). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be stirred into the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, add coconut water (item 14) and adjust volume to 1000 ml with distilled water (item 16). Bring pH to 5.0–5.6 with dilute KOH or HCl, as needed. Pour into a 2-l flask and autoclave. Add amino acid (item 8) and vitamins (items 10–12) to the hot, autoclaved solution under sterile conditions, using sterile pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

Indoleacetyl–Amino Acid Conjugates in Mass Clonal Propagation of *Phalaenopsis*

Conjugates of IAA and certain amino acids, such as indoleacetyl alanine (IAA-ala) or indoleacetyl glycine (IAA-gly), substitute well as proliferation-inducing hormones (Griesbach, 1983) in place of the combination of the auxin IAA and the cytokinin BA used by previous investigators. These conjugates have been found to increase the proliferation and differentiation of tomato and tobacco tissue cultures (Hangarter et al., 1980).

Plant Material. Two-centimeter-long sections of a flower stalk, having one dormant bud, were cut from the *Phalaenopsis* Berry Hausermann, and their bracts were removed.

Surface Sterilization. The sections are sterilized for 30 min in a 25% Clorox solution (25 ml Clorox diluted to 100 ml with water) containing 0.05% Tween surfactant (type not given). After that the sections are rinsed in sterile distilled water, dipped in 70% aq ethanol for 30 sec, and rinsed again in sterile distilled water.

Culture Conditions. All cultures are grown at 28°C under 16-h photoperiods at 65 μEM^{-2} sec^{-1}. Liquid cultures are agitated at 2–3 rpm.

Culture Media. Half-strength MS medium supplemented with 2 g l^{-1} yeast extract, 13 mg l^{-1} isoleucine, and 100 mg l^{-1} *trans*-cinnamic acid (Table Phal-15; Reisinger et al., 1976) is used. A liquid MS medium supplemented with 2 mg l^{-1} yeast, 0.5 mg l^{-1} NAA, and 2 mg l^{-1} BA (Table Phal-16) is employed. Another liquid modified MS medium, containing 2 g l^{-1} yeast extract and one of four different IAA–amino acid conjugates is used for further proliferation (Table Phal-17). Differentiation is induced on solid modified MS medium (Table Phal-18).

Procedure. Once the second leaf develops from the buds of the flower-stalk sections on solid modified MS medium (Table Phal-15), remove the basal portions of the shoots, place in liquid modified MS solution (Table Phal-16), and rotate at a speed of 2–3 rpm. PLBs develop from the basal sections of the shoots. Place these in liquid MS medium that contains not NAA and BAP, but rather one of the IAA–amino acid conjugates (Table Phal-17). Other hormone combinations (Table Phal-19; Koch, 1974*a*, 1974*b*; Zimmer and Pieper, 1978) also give good results.

Differentiation is then induced by transferring the PLBs to solid MS medium supplemented with 2 g l^{-1} Bacto-Difco peptone (Table Phal-18).

General Comments. The medium with IAA-ala induced more PLBs than did the one with IAA-gly, which brought about initiation of more shoots. It appears that IAA-ala retards differentiation when compared with IAA-gly. However, absence of statistical analysis makes it impossible to determine the significance of the small differences between the use of conventionally employed hormones and the indoleacetyl amino acids (Table Phal-19). Question: Why use uncommon chemicals when simpler ones work as well or better?

TABLE PHAL-15. **Murashige–Skoog medium (Murashige and Skoog, 1962), modified for clonal propagation of *Phalaenopsis* through the culture of flower-stalk nodes (Griesbach, 1983)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot H_2O$	220	22 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950	95 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	18.7	1.87 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	13.9	1.39 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.0125	1.25 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.0125	1.25 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.15	1.115 g l^{-1}		
(e)	Potassium iodide, KI	0.415	415 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430 mg l^{-1}		
	Amino acid				
8	Glycine	1	100 mg 100 ml^{-1} 95% ethanol[e]	1	
9	L-isoleucine	13	1.3 g 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	50	No stock	No stock	Weigh
	Auxin				
11	Indoleacetic acid (IAA)	1	100 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
12	Kinetin	1	10 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.25	25 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Pyridoxine·HCl (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
15	Thiamine·HCl (vitamin B_1)	0.5	5 mg 100 ml^{-1} 95% ethanol[g]	1	
	Anti-auxin				
16	*trans*-cinnamic acid (tCA)	100	No stock	No stock	Weigh
	Complex additives				
17	Yeast extract	2 g	No stock	No stock	Weigh
	Sugar				
18	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[h]	To 1000 ml			
	Solidifier				
20	Agar[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin and cytokinin do not dissolve, add a few drops of dilute KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–7, 10, 16, and 17 to 900 ml distilled water (item 19); adjust pH to 5.0–5.6, add sugar (item 18), and adjust volume to 1000 ml with distilled water (item 19). Bring solution to a gentle boil, and add agar (item 20) slowly while stirring. Agar can also be added while stirring to cold solution, which is then brought to a boil. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acids, hormones, and vitamins (items 8, 9, and 11–15) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE PHAL-16. **Liquid Murashige–Skoog medium (Murashige and Skoog, 1962) modified for clonal propagation of *Phalaenopsis* through the culture of basal shoot sections (Griesbach, 1983)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	2	200 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
15	Yeast extract	2	No stock	No stock	Weigh
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin do not dissolve, add a few drops of dilute KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 17), adjust pH to 5.0–5.6, add sugar (item 16) and yeast extract (item 15), and adjust volume to 1000 ml with distilled water (item 17). Warm solution and stir until all salts and additives are completely dissolved. Pour into a 2-l flask and autoclave. Add amino acid, hormones, and vitamins (items 8 and 10–14) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

TABLE PHAL-17. **Liquid Murashige–Skoog medium (Murashige and Skoog, 1962) modified for the proliferation of protocorm-like bodies of *Phalaenopsis* (Griesbach, 1983)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Indoleacetyl amino acid				
10	Indoleacetyl glycine (IAA-gly)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[f]	1	
	Complex additive				
14	Yeast extract	2	No stock	No stock	Weigh
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Keep refrigerated between uses.

[g]Add items 1–7 and 9 to 900 ml distilled water (item 16), adjust pH to 5.0–5.6, add yeast extract (item 14) and sugar (item 15), adjust volume to 1000 ml with distilled water (item 16). Warm solution and stir until all salts and additives are completely dissolved. Pour into a 2-l flask and autoclave. Add the amino acid (item 8), the indolacetyl amino acid (item 10) and the vitamins (items 11–13) to the autoclaved solution while hot under sterile conditions, using sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

TABLE PHAL-18. **Murashige-Skoog medium (Murashige and Skoog, 1962) modified for the differentiation of protocorm-like bodies of *Phalaenopsis* (Griesbach, 1983)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[f]	1	
	Complex additive				
13	Peptone, Bacto-Difco	2 g	No stock	No stock	Weigh
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Keep refrigerated between uses.

[g]Add items 1–7 and 9 to 900 ml distilled water (item 15), adjust pH to 5.0–5.6, add sugar (item 14) and peptone (item 13), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil while stirring, and slowly add agar (item 16). Agar can also be added with constant stirring to the cold solution, which is then brought to a gentle boil. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid and vitamins (items 8 and 10–12) to hot solution under sterile conditions with sterile pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

TABLE PHAL-19. **Effect of indoleacetic acid, indoleacetyl amino acids, and an auxin–cytokinin combination on the proliferation of *Phalaenopsis* protocorm-like bodies (Griesbach, 1983)**

Treatment	No. of shoots produced by 100 protocorms
Control, no hormones	152
0.5 mg NAA[a] l^{-1} + 2 mg BA[a] l^{-1}	250
Indoleacetic acid (IAA)	
0.5 mg l^{-1}	0
5 mg l^{-1}	0
Indoleacetyl-alanine	
0.05 mg l^{-1}	257
0.5 mg l^{-1}	259
5 mg l^{-1}	240
Indoleacetyl-glycine	
0.05 mg l^{-1}	256
0.5 mg l^{-1}	400
5 mg l^{-1}	325

[a]NAA, naphthaleneacetic acid; BA, benzylaminopurine.

Mass Clonal Multiplication of *Phalaenopsis* from Internodal Sections of Young Flower Stalks

Several procedures, in which the shoot tissue derived from nodal flower-stem sections serves as explant material in the clonal multiplication of *Phalaenopsis*, have been published (Fu, 1979*a*; Kushnir and Budak, 1980; Yoneda et al., 1983). Clonal multiplication of *Phalaenopsis* obtained by culturing thin disks of young internodal sections of flower stalks was reported by Lin (1986).

Plant Material. Flower-stalk internodes of *Phalaenopsis* hybrids grown for 3 years under greenhouse conditions are used as explants. Highest PLB yields are obtained from newly emerging, 35–45-day-old flower stalks. At this stage only two to three internodes generally develop. The production of PLBs is usually about double that obtained with 60–75-day-old stalks. No PLBs are obtained from stalks 180 or more days old. Older flower stalks yield PLBs only from internodal sections near the tips. Nodes are removed from sterilized stalks, and the internodal sections are cut into 1–1.5-mm disks under sterile conditions (Fig. Phal-11). These disks are the explants to be cultured.

Surface Sterilization. Excised flower stalks are wiped with 70% aqueous ethanol and then sterilized in 1% sodium hypochlorite solution (20 ml household bleach diluted to 100 ml with water) containing 0.05% Tween 20. The sterilized stalks are then rinsed three times with sterile distilled water.

Culture Conditions. Cultures are maintained under about 500 lx of fluorescent light (FL 40D lamps, Taiwan), 16-h photoperiods, and temperatures of 26 ± 2°C.

Culture Medium. A modified solid Vacin and Went medium is used (Table Phal-20).

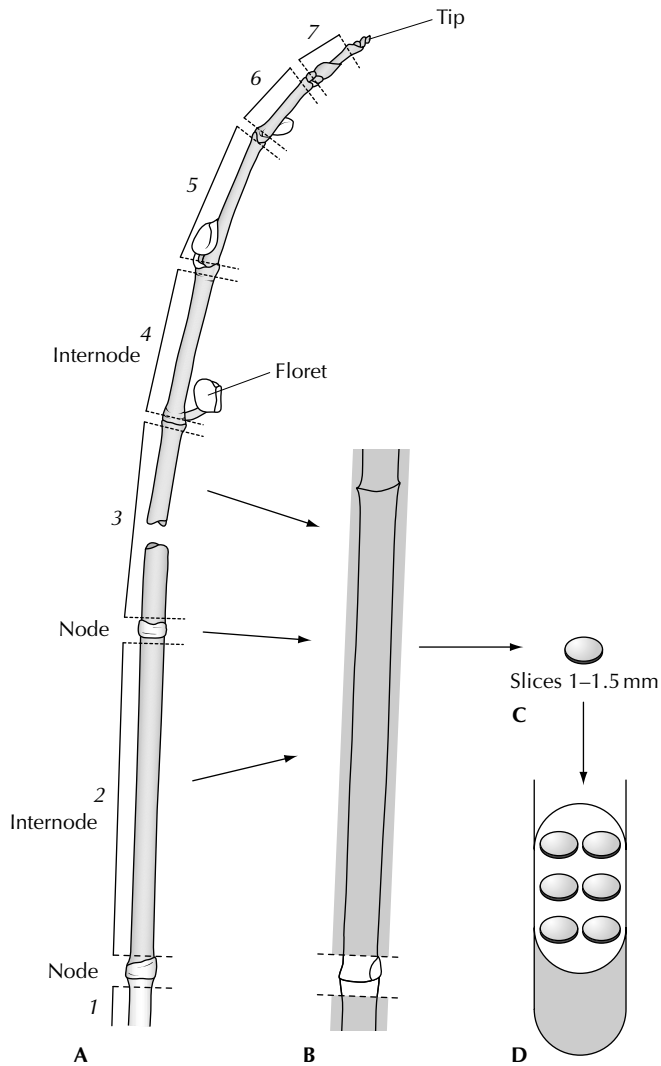


FIG. PHAL-11. **Clonal propagation of *Phalaenopsis* from a flower stalk. A. Internodal segments of young flower stalks. B–D. Slices of 1–1.5 mm, cut from the plant shown in A, are used as explants for mass clonal propagation (D). (Lin, 1986.)**

Developmental Sequence. Most sections cultured on solid medium enlarged within 15 days and produced PLBs after 30 days of culture. Within 45 days the PLBs elongated and after 2 months produced a few adventitious buds. Yellowish callus formed on some stalk sections. The frequency of callus induction was 26% on medium with 1 mg BA l^{-1} and 53% on medium with 5 mg BA l^{-1}. PLBs formed on the epidermis and cut surfaces of the sections that were cultured. After the first flower became visible, sections from the base stalks did not produce PLBs; only sections near the tip of these stalks produced such bodies (Fig. Phal-11) with a frequency of

TABLE PHAL-20. **Vacin and Went medium (Vacin and Went, 1949) modified for clonal propagation of *Phalaenopsis* from flower-stalk internode sections (Lin, 1986)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	600	60 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	240	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	300	30 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	300	30 g l^{-1}	10	
5	Potassium nitrate, KNO_3	630	63 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	33.6	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	6	750 mg l^{-1}	10	
	Polyol				
8	*myo*-inositol	100	No stock	No stock	Weigh
	Vitamins				
9	Thiamine·HCl	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
10	Pyridoxine·HCl	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Nicotinic acid (niacin)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin				
12	Benzylaminopurine (BAP)[f]	5	50 mg 100 ml^{-1} 95% ethanol[e]	10	
	Sugar				
13	Sucrose	20 g	No stock	No stock	Weigh
	Darkening agent				
14	Activated charcoal	2 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	8 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, so it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, stir and/or heat until it dissolves. Add other components of medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution, dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Keep vitamin and hormone solutions refrigerated between uses.

[f]For differentiation into plantlets divide protocorm-like bodies and subculture them on the same medium, except that the BAP concentration is reduced to 1 mg l^{-1}. The cytokinin can be solubilized with dilute HCl.

[g]Add items 1 and 3–8 to the 500 ml distilled water (item 15) that contains item 2, and adjust pH to 5.0–5.6. Add sugar (item 13), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. When agar is completely dissolved, add powdered charcoal (item 14) with rapid agitation (use a blendor or similar device), pour solution into a 2-l flask and autoclave. After autoclaving add to the still hot solution the vitamins (items 9–11) and the cytokinin (item 12) under sterile conditions using sterilized pipettes, mix well, and distribute medium to preautoclaved vessels. Omit agar for liquid medium.

27.5–44.7%. The highest frequency of PLB induction (62.9–77.1%) occurred on sections taken from flower stalks before the first flower became visible. *Phalaenopsis* stalks could still produce some PLBs (4.8–6.5%) near the tip following opening of the last flower.

The PLBs were subcultured on the same solid medium, containing 1 mg BA l^{-1} but without auxin to develop into plantlets.

TABLE PHAL-21. **Effect of growth regulators and stage of inflorescence development on the formation of protocorm-like bodies (PLBs) from young flower-stalk internode sections of *Phalaenopsis* (Lin, 1986), showing the ratio of the number of segments that formed PLBs to the total number of segments**

	Internode, number[a] from bottom (days after stalk forms)								
Growth regulators[b]	1 (30–45 days)		2 (60–75 days)		3 (90–105 days)		4 (120–135 days)		Percent PLBs formed
Control (none)	0/15	0/15	0/15	0/15	0/15	0/17	0/21	0/15	0
1 mg BAP l^{-1}	2/2	3/3	4/6	13/16	52/70	21/27	8/20	8/10	73.4
5 mg BAP l^{-1}	16/21	13/25	28/48	15/35	11/15	20/33	16/45	18/31	46.8
1 mg BAP l^{-1} + 1 mg NAA l^{-1}	0/2	0/5	0/4	2/15	5/18	3/24	11/6	2/6	12.6
5 mg BAP l^{-1} + 1 mg NAA l^{-1}	0/2	0/5	0/4	0/15	2/18	1/24	2/6	1/6	5.9
1 mg KIN l^{-1}	0/21	0/25	0/20	0/25	0/15	0/33	0/45	0/31	0

[a]See Fig. Phal-11 for intemode positions. Internode segments higher then 5 (180 days or older) produced no PLBs.
[b]BAP, benzylaminopurine; KIN, kinetin; NAA, naphthaleneacetic acid.

General Comments. The highest yield of PLBs was obtained with 1 mg BA l^{-1} and no auxin, followed by 5 mg BA l^{-1} and no auxin. Some PLBs formed when the auxin NAA (1 mg l^{-1}) was added to the cytokinin BA, but this combination reduced PLB production sharply. No PLB formation occurred with the cytokinin kinetin or with the control (Table Phal-21).

Clonal Multiplication of Stem-propagated *Phalaenopsis* Root Tissue

A procedure for root-tip culture developed by Tanaka et al. (1976) is as follows.

Plant Material. Seedlings of *Phalaenopsis amabilis* hybrids, 194 and 349 days old, were taken from aseptic culture. After removing them from the flask, root tips (3 mm long) were removed with a sterile razor blade and cultured on two solid media.

Culture Vessels. In most cases 18×180 mm test tubes plugged with cotton bungs were used as culture containers in the original research. Other vessels can also be used.

Culture Conditions. All cultures are maintained at 25°C under 16-h photoperiods provided by white fluorescent lamps (NEC Company Vita Lux or equivalent).

Culture Medium. Modified MS medium is used in which the inorganic nutrients are reduced to half their original concentration and auxins and cytokinins are omitted (Table Phal-22).

Developmental Sequence. Only four of 10 explants from the 349-day-old seedlings produced PLBs during culture periods of 120–272 days on the modified MS medium (Table Phal-22). A single culture on medium O (Ojima and Fujiwara, 1962) produced PLBs after 260 days. About 3 months later an increase in cellular

TABLE PHAL-22. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for the clonal propagation of *Phalaenopsis* from root tips (Tanaka et al., 1976)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot H_2O$	220	22 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950	95 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	18.7	1.87 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	13.9	1.39 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.0125	1.25 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.0125	1.25 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.15	1.115 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.415	415 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430 mg l^{-1}		
	Amino acid				
8	Glycine	2	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
11	Pyridoxine·HCl (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[f]	1	
12	Thiamine·HCl (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[f]	1	
	Sugar				
13	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[g]	To 1000 ml			
	Solidifier				
15	Agar[g]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration, a difference probably without much effect.

[e]Keep frozen between uses.

[f]Keep refrigerated between uses.

[g]Add items 1–7 and 9 to 900 ml distilled water (item 14), adjust pH to 5.0–5.6, add sugar (item 13), and adjust volume to 1000 ml with distilled water (item 14). Bring solution to a gentle boil, and add agar (item 15) slowly while stirring. Agar can also be added while stirring to the cold solution, which is then brought to a boil. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid and vitamins (items 8 and 10–12) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

activity was observed in these cases. Small bumps formed at the tip or lateral sides of the root-tip explant. These bumps differentiated into a callus from which PLBs developed gradually.

Only one culture from 10 explants of 194-day-old seedlings formed a small mass of cream-colored callus on modified MS medium (Table Phal-22). When this callus was excised and transplanted on the same medium, 12 PLBs were obtained. The PLBs can then be grown into plantlets on media suitable for seedling culture. Some of the root tips that failed to produce PLBs elongated or developed a shoot.

General Comments. Root explants from seedlings have little practical value, since their use does not allow for selection prior to multiplication. However, PLBs and plantlets have also been obtained from broken root explants of young plantlets derived from flower-stalk nodes (Zimmer and Pieper, 1978). Since the flower quality of such plants is known, this process would be useful (Zimmer and Pieper, 1978).

Isolation and Fusion of *Phalaenopsis* Protoplasts

The isolation of protoplasts from leaves of *Phalaenopsis* and subsequent intraspecific cell fusion employing polyethylene glycol, was reported by Teo and Neumann (1978*a*, 1978*b*). Also shown in these papers is a photomicrograph of interspecific cell fusion of the bigeneric hybrid *Renantanda* (*Renanthera* × *Vanda*) with *Phalaenopsis*, brought about by the use of polyethylene glycol. However, mitotic divisions or callus formation were not observed.

Plant Material. Plant material for this study was grown aseptically and therefore required no sterilization. Protoplasts were isolated from young leaves of *Phalaenopsis* and from protocorms of *Renantanda*.

Isolation of Protoplasts. Protoplasts were obtained as shown by a previous method (Teo and Neumann, 1978*c*; see also *Renantanda* entry).

Protoplast Fusion. Protoplast fusion was carried out in the presence of 28% polyethylene glycol (Elmheuser, 1977).

Protoplast Staining. To separate *Renantanda* protoplasts from those of *Phalaenopsis*, protoplasts from *Renantanda* Rosalind Cheok were stained with 0.001% bisbenzimide (available from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178) for 1 min and then promptly washed off with sterile nutrient. The stained nucleus emitted bright fluorescence under 490-nm light.

Histological Observations of *Phalaenopsis* Protocorm-like Bodies from Flower-stalk Internodes

Phalaenopsis flower-stalk internode sections were cultured for histological observations of PLBs (Lin, 1987).

Plant Material. Three internodes from near the tips of young flower stalks of *Phalaenopsis* (species or hybrid not given) were used as explants. Young internodes, 4–10 cm long taken from under the flower-stalk tip, were used as control explants.

Surface Sterilization. Explants to be cultured were surface-sterilized for 30 min in a 1% sodium hypochlorite solution containing 0.05% Tween 20 and then rinsed three times with sterile distilled water. They were then cut into sections 1–1.5 mm thick (see Fig. Phal-11) for culture.

Culture Vessels. The type of culture containers was not given in the original research, but test tubes were probably used (see Fig. Phal-11).

Culture Conditions. Cultures were maintained under 500 lx provided by FL-40D lamps and 16-h photoperiods at 26 ± 2°C.

Culture Medium. A modified Vacin and Went solid medium (see Table Phal-20) was used.

Developmental Sequence and Cytological Observations. After 3 days of culture (in the presence of 1 mg BA l^{-1}) no visible changes in the cultured tissues were observed. Cell division occurred in the epidermis only, after 7 days. After 11 days, cell division extended to the first layer of the cortex and to immature vascular bundles under cut surfaces. Fifteen days following the start of culture small protuberances consisting of cells that formed as a result of divisions were visible on the epidermis and cut surfaces. PLBs formed first on the epidermis and vascular bundles and became visible after 30 days of culture. PLBs formed on the epidermis and cortex, immature vascular bundles, and ground parenchyma following 45–60 days.

General Comments. These histological observations based on light microscopic examinations should be useful to others working on the micropropagation *of Phalaenopsis*.

Micropropagation of *Phalaenopsis* through the Culture of Leaf Segments

Dr. Michio Tanaka of the Laboratory of Floriculture, Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa 761-07, Japan started to work on the culture of *Phalaenopsis* leaf segments in 1972. By 1990 he perfected a simple yet effective micropropagation procedure (Tanaka, 1990, 1992).

Plant Material. Leaf segments, 6–8 × 6–8 mm in size (Fig. Phal-12E1, F) were taken from the apical, median, and basal parts of the uppermost leaves (about 2 cm in length) on shoots derived from flower-stalk nodes in vitro (Fig. Phal-12A–D). Segments should be placed on the medium adaxial side up (Fig. Phal-12G). To increase production of PLBs from leaf segments, the shoots or plantlets from which the explants are taken should be cultured at 25°C under photoperiods of more than 8 h, a light

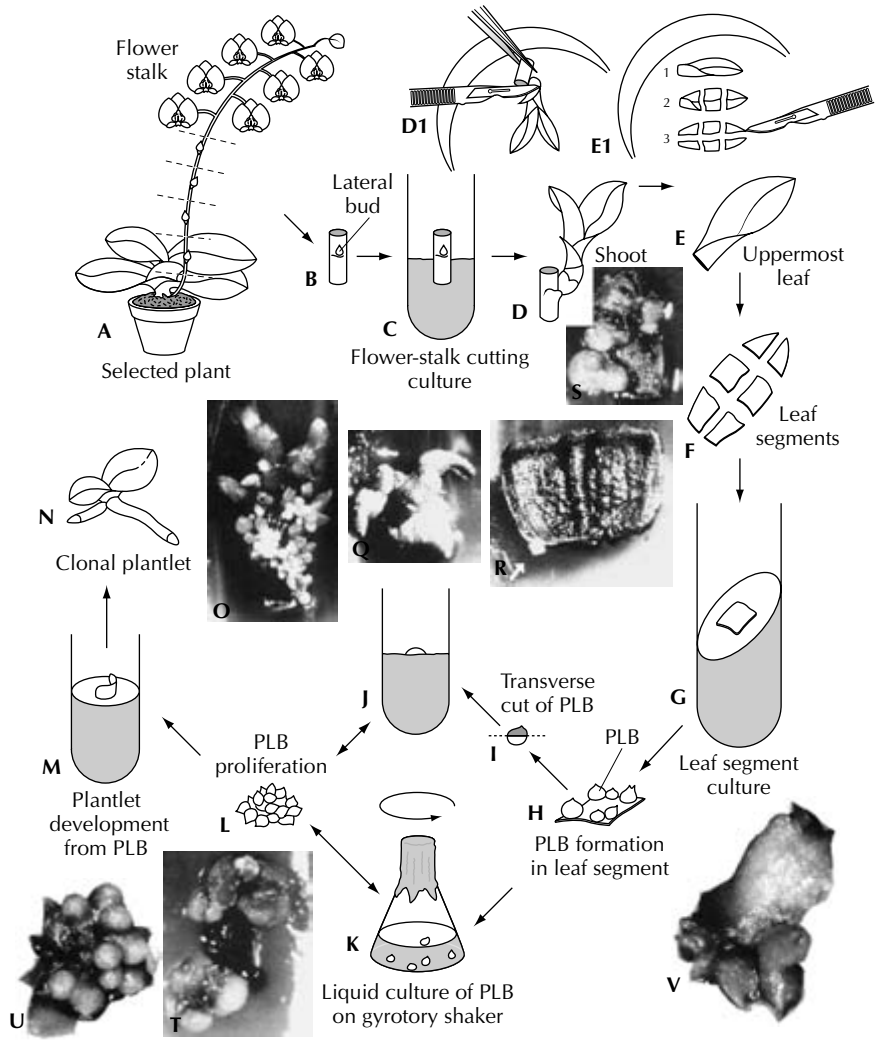


FIG. PHAL-12. **Micropropagation of *Phalaenopsis*. A. Source of explants. B. Explant. C. Explant in culture. D. Explant with shoot. D1. Excision of leaf. E. Uppermost leaf. E1. Sectioning of leaf. F. Leaf segments. G. Leaf segment in culture. H. PLBs produced by a leaf segment. I. Sectioning a PLB transversely. J. A PLB section in culture. K. PLBs in liquid culture. L. Proliferated PLBs. M. Plant formation from a PLB. N. Plantlet. O–R. PLBs on the adaxial surface of a leaf segment. S, T. PLBs on leaf segments after 120 days in culture. U. Proliferation on the basal part of a PLB. V. Proliferation on the apical half of a PLB. (NB there is no part P; Tanaka, 1990, 1992.)**

intensity of 180 lx, and on a medium that contains 1 or 10 mg BA l^{-1} or 20% (v/v) coconut water.

Surface Sterilization. Explants from plants in vitro do not require surface sterilization.

Culture Vessels. Test tubes, 18 × 180 mm containing 8 ml of medium, were used in the original research. Other containers can also be used.

Culture Conditions. Leaf segments should be cultured at 25°C in darkness for the initial 2 weeks and then under 16-h photoperiods of 900 lx.

Culture Media. Modified Kyoto medium (Tsukamoto et al., 1963) which employs Hyponex (N6.5:P6:K19; www.hyponex.co.jp) as a source of minerals (Table Phal-23) is used to culture leaf segments. To further enhance PLB formation and to absorb

TABLE PHAL-23. **Modified Hyponex/Kyoto medium (Tsukamoto et al., 1963) for the culture of *Phalaenopsis* leaf explants (Tanaka, 1990, 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Hyponex[b]	3.5 g	No stock	No stock	Weigh
	Polyol				
2	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
3	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[c,d]	1	
	Cytokinin				
4	Benzyladenine (BA)	10.0	1000 mg 100 ml^{-1} 95% ethanol[c,d]	1	
	Purine (cytokinin-like substance)				
5	Adenine	10.0	1000 mg 200 ml^{-1} 70% ethanol[e]	2	
	Vitamins				
6	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[c]	1	
7	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[c]	1	
	Sugar				
8	Sucrose	35.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[f]	To 1000 ml			
	Solidifier				
10	Agar[f,g]	15.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]From www.hyponex.co.jp

[c]Keep frozen between uses.

[d]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively. This stock solution may be at the limit of solubility of BA. If a precipitate forms shake well before dispensing.

[e]Suspend the adenine in 55 ml of distilled water, add 0.6 ml of 1 N sulfuric acid (H_2SO_4), and bring up volume to 200 ml with 95% ethanol. This stock solution may be at the limit of solubility of adenine. If a precipitate forms shake well before dispensing.

[f]Add items 1–7 to 900 ml of distilled water (item 9), adjust pH to 5.0 (Tsukamoto et al., 1963), add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring the solution to a gentle boil and add the agar (item 10) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

[g]This is the amount of agar listed in the original formulation (Tsukamoto et al., 1963). With this much agar the medium will be fairly hard.

phenolics, which are produced by the explants, and to darken the medium it is advisable to add 800 mg polyvinylpyrrolidone (PVP) l^{-1} to the medium. PLBs produced by leaf segments (Fig. Phal-12H) can be proliferated (Fig. Phal-12J–L, U, V) by cutting them transversely and placing the halves upside down on Vacin and Went medium (Vacin and Went, 1949) supplemented with 20% (v/v) coconut water (Table Phal-24). Proliferation can also be brought about by culturing the PLBs in liquid medium (Table Phal-24 without agar) with agitation. To induce plantlet development (Fig. Phal-12M, N), PLBs should be cultured on a different modification of the Hyponex/Kyoto medium (Table Phal-25).

Procedure. When shoots produced by flower-stalk nodes (Fig. Phal-12A-C) produce growths (Fig. Phal-12D) with large enough leaves, the uppermost leaf should be sectioned (Fig. Phal-12D1–F) and the sections should be placed in culture (Fig. 12G) on the first medium (Table Phal-23). After PLBs are produced (Fig. Phal-12H, O–T) they should be sectioned transversely (Fig. Phal-12I) and proliferated (Fig. Phal-12L) on solid (Fig. Phal-12J, U, V) or in liquid (Phal-12K) medium (Table Phal-25). To produce plantlets (Fig. Phal-12M, N), the PLBs should be placed on the third medium (Table Phal-25).

Developmental Sequence. Explants produce PLBs on the first medium (Table Phal-23). The PLBs proliferate when they are sectioned (Fig. Phal-12I) and the halves are placed on the second medium (Table Phal-24) in solid form. Uncut PLBs proliferate when cultured on the second medium in liquid form (Table Phal-24 without agar). Basal halves produce more PLBs than apical ones. PLBs produce plantlets on the third medium (Table Phal-25).

General Comments. This is an efficient and effective procedure for the micropropagation of *Phalaenopsis*. It can produce a large number of plants without endangering the original plants. However, excessive proliferation must be avoided because it can cause undesirable mutations. The proportion of leaf segments that produce PLBs, and the number of PLBs per segment, can vary with the hybrid being cultured. PLBs generated through this method were used to produce transgenic *Phalaenopsis* (Anzai and Tanaka, 2001).

The formation of protocorm-like bodies (PLBs) and shoot development from basal and apical sections of leaf segment-derived PLBs (Tanaka, 1992, table 1).

		Developed organ on culture				
Section	No. of cultures	PLB and shoots, %	No. of PLBs culture	PLBs, %	No. of PLBs culture	Shoot, %
Upper	16	31	1.0	38	5.3	31
Lower	16	0	–	100	13.7	0

Hybrid used: Lavender Lady × [Lavender Lady × (Clarelen × Zada)].
PLBs were transversally bisected, and cultured on Vacin and Went + coconut milk 20% (v/v) medium, at 25°C with a 16-h light (900 lx).
Data were recorded 55 days after culture.

TABLE PHAL-24. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the proliferation of leaf-segment-derived *Phalaenopsis* protocorm-like bodies (Tanaka, 1990, 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water (CW)[e]	200.0 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 10), adjust pH to 5–5.4, add sugar (item 9), and raise volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar should not be added to liquid medium. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

TABLE PHAL-25. **Modified Hyponex/Kyoto medium (Tsukamoto et al., 1963) for plantlet formation from protocorm-like bodies derived from *Phalaenopsis* leaf explants (Tanaka, 1990, 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelement				
1	Hyponex[b]	3.5 g	No stock	No stock	Weigh
2	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	0.037	7.45 mg l^{-1}	5	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	0.028	5.57 mg l^{-1}		
3	**Microelements**[c]				
(a)	Boric acid, H_3BO_3	10.0	1.0 g	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	0.25	25.0 g		
(e)	Sodium molybdate, $NaMoO_4 \cdot 2H_2O$	0.25	25.0 mg		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	10.0	1.0 g		
	Polyol				
4	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamin				
5	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[d]	1	
	Darkening agent				
6	Activated charcoal[e]	2.0	No stock	No stock	Weigh
	Sugar				
7	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
8	Water, distilled[f]	To 1000 ml			
	Solidifier				
9	Agar[f]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]From www.hyponex.co.jp.

[c]These are the microelements used by Nitsch and Nitsch (1967).

[d]Keep frozen between uses.

[e]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances.

[f]Add items 1–5 to 900 ml of distilled water (item 8), adjust pH to 5.0 (Tsukamoto et al., 1963), add sugar (item 7), and adjust volume to 1000 ml with distilled water (item 8). Bring the solution to a gentle boil and add the agar (item 9) slowly while stirring. The agar can also be added to the cold water, which is brought to a boil and stirred. When the agar is completely dissolved, add the charcoal (item 6) with vigorous stirring, distribute the medium to culture vessels, and autoclave. Agar is not added to liquid media.

Callus Induction and Somatic Embryogenesis in *Phalaenopsis*

A callus that can be sectioned and cultured on a medium or media capable of inducing plant formation and/or somatic embryos that can form plantlets facilitate and accelerate micropropagation. Therefore a method for callus induction, somatic embryogenesis, and plantlet production was developed at Kagawa University in Japan (Ishii et al., 1998).

Plant Material. PLBs of *Phalaenopsis* Richard Shaffer 'Santa Cruz' obtained through the culture of leaf segments (see Micropropagation of *Phalaenopsis* through the Culture of Leaf Segments) were cut transversely (as shown here: ⊖) and the

segments were used as one type of explants. The leaf segments were obtained by cutting 2-cm-long leaves into six sections (see Fig. Phal-12E, F) taken from a shoot produced by flower-stalk node culture [see *Phalaenopsis* Flower-stalk Node Propagation (p. 911, Vol. II), Use of Plant Hormones to Improve Shoot Growth of *Phalaenopsis* Stem Nodes (p. 913, Vol. II), Shoot Growth of *Phalaenopsis* Flower-stalk Nodes in the Presence of Anti-auxin (p. 916, Vol. II), and Clonal Propagation of *Phalaenopsis* through Injured Nodal Flower-stalk Buds (p. 918, Vol. II)]. Very few leaf segments produced calli.

Surface Sterilization. Explants taken from PLBs growing in vitro do not require sterilization.

Culture Vessels. Erlenmeyer flasks, 250-ml capacity containing 40–60 ml medium, are suitable as culture vessels. Other containers can also be used.

Culture Conditions. All explants should be cultured at 25°C. PLB sections should be cultured under 16-h photoperiods (no intensity given) provided by Homolux lamps (Matsushita Electric Industrial Co.) for 8 weeks. Leaf explants must be kept in the dark for 2 weeks, moved to the same illumination as PLB sections for another 14 days, and cultured for 3 months.

Culture Media. PLB sections should be cultured on Vacin and Went medium (Vacin and Went, 1949) containing 40 g sucrose l^{-1} and 200 ml coconut water (CW) l^{-1} solidified with 2 g gellan gum l^{-1} (Table Phal-26). Leaf segments should be cultured on Vacin and Went medium solidified with gellan gum, but without CW, and containing 40 g sucrose l^{-1}, 1 mg 2,4-D l^{-1}, and 0.1 mg BA l^{-1} (Table Phal-27). Callus masses produce PLBs after being transferred to Vacin and Went medium without sucrose, supplemented with 200 ml CW l^{-1}, and solidified with 8 g agar l^{-1} (Table Phal-28). PLBs produced on the sugar-free medium (Table Phal-28) develop plantlets on a modified Hyponex medium (Table Phal-29).

Procedure. PLB sections and leaf segments should be cultured on the first medium (Table Phal-26) for at least 8 weeks under illumination. When enough callus becomes available, portions should be moved to the sugar-free medium (Table Phal-28) for PLB production. Leaf segments should be placed on the hormone-containing medium (Table Phal-27) first in the dark for 2 weeks, and then in the light for 14 weeks to produce callus. To generate PLBs from this callus, portions of it should also be moved to the sugar-free substrate (Table Phal-28). Once PLBs form on the sugar-free medium, they should be moved to the Hyponex solution (Table Phal-29) for plantlet production.

Developmental Sequence. PLB sections and leaf segments produce callus (Fig. Phal-13A) on the first medium (Tables Phal-26 and Phal-27, respectively). Regardless of its origin, the callus produces PLBs (Fig. Phal-13A) on the second medium (Table Phal-28). Histological observations suggest that the PLBs originate from somatic embryos (Fig. Phal-13B, C). Plantlets (Fig. Phal-13D) are formed on the Hyponex medium (Table Phal-29).

TABLE PHAL-26. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for callus production from sections of protocorm-like bodies of *Phalaenopsis* (Ishii et al., 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water (CW)[e]	200.0 ml	No stock	No stock	Weigh
	Sugar				
9	Sucrose	40.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Gellan gum[f,g]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 2), if necessary bring volume to 900 ml with distilled water (item 10), adjust pH to 5.3, add sugar (item 9), and raise volume to 1000 ml with distilled water (item 10). Add the gellan gum (item 11) in accordance with the instruction in footnote *g*. When the gellan gum (item 11) is completely dissolved, pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

[g]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Phytagel (gellan gum, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

TABLE PHAL-27. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for callus production from segments of young *Phalaenopsis* leaves (Ishii et al., 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
8	2,4-Dichlorophenoxyacetic acid (2,4-D)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	Benzyladenine (BA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
10	Sucrose	40.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Gellan gum[g,h]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 11) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[f]Keep frozen between uses.

[g]Add items 1 and 3–9 to the 500–700 ml of distilled water (item 11) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 11), adjust pH to 5.3, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Add the gellan gum (item 12) in accordance with the instruction in footnote *h*. When the Gellan gum (item 12) is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable. As a rule hormones should be added after autoclaving but in this case all components seem to have been incorporated in the medium before it was autoclaved.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear, and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Phytagel (Gellan gum, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

TABLE PHAL-28. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the induction of protocorm-like bodies from *Phalaenopsis* callus (Ishii et al., 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water (CW)[e]	200.0 ml	No stock	No stock	Weigh
	Sugar				
9	Sucrose	0 or 20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f,g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 2), if necessary bring volume to 900 ml with distilled water (item 10), adjust pH to 5.3, add sugar (item 9) or not (this medium can also be prepared without sugar), and raise volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar (item 11) is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

TABLE PHAL-29. **Modified Hyponex/Kyoto medium (Tsukamoto et al., 1963) for plantlet formation from protocorm-like bodies derived from *Phalaenopsis* callus (Tanaka, 1990, 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Hyponex[b]	3.0 g	No stock	No stock	Weigh
2	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
3	**Microelements**[c]				
(a)	Boric acid, H_3BO_3	10.0	1.0 g	5	One solution
(b)	Cobalt chloride, $CoCl_2{\cdot}6H_2O$	0.025	2.5 mg		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.025	2.5 mg		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	0.25	25.0 mg		
(e)	Sodium molybdate, $NaMoO_4{\cdot}2H_2O$	0.25	25.0 mg		
(f)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	10.0	1.0 g		
	Polyol				
4	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamin				
5	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[d]	1	
	Complex additive				
6	Potato extract[e]				
	Darkening agent				
7	Activated charcoal[f]	1.0	No stock	No stock	Weigh
	Sugar				
8	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[g]	To 1000 ml			
	Solidifier				
10	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]From www.hyponex.co.jp.

[c]These are the microelements used by Nitsch and Nitsch (1967).

[d]Keep frozen between uses.

[e]No information is given on how to prepare potato extract or where to purchase any. The usual sources of tissue culture supplies do not carry potato extract. One possible method of preparing the extract may be to cut a peeled potato, weigh 30 g of the cubes, boil them for 10–15 min in 100 ml of distilled water, filter the "soup" and add the liquid to the medium. Another possibility is to homogenize 30 g of cubes and add the homogenate to the medium.

[f]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances.

[g]Add items 1–6 to 900 ml of distilled water (item 9), adjust pH to 5.3, add sugar (item 8), and raise volume to 1000 ml with distilled water (item 9). Bring the solution to a gentle boil and add the agar (item 10) slowly while stirring. The agar (item 10) can also be added to the cold water which is brought to a boil and stirred. When the agar (item 10) is completely dissolved, add the charcoal (item 7) with vigorous stirring, distribute the medium to culture vessels, and autoclave. Agar is not added to liquid media.

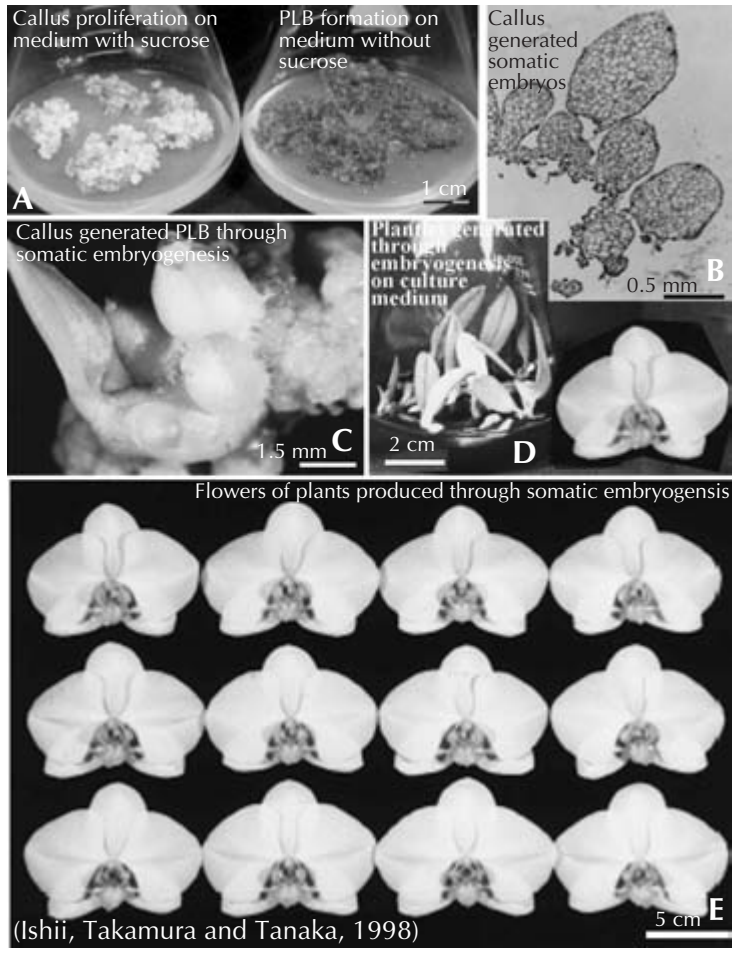


FIG. PHAL-13. **Callus induction and somatic embryogenesis in *Phalaenopsis*.**

General Comments. This method can accelerate propagation in vitro without the danger of undesirable mutations because "no variation was observed in . . . flowering plants" produced through this procedure (Fig. Phal-13E).

Micropropagation of *Phalaenopsis* by Culturing Shoot Tips of Flower-stalk Buds

The methods described in "Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds" (Tokuhara and Mii, 1993) are also suitable for *Phalaenopsis*. When using this method it is important to keep in mind that proliferation, especially if it is excessive, can lead to somaclonal variations and flowers which are not attractive (see Fig. Dtps-4; Tokuhara and Mii, 1998).

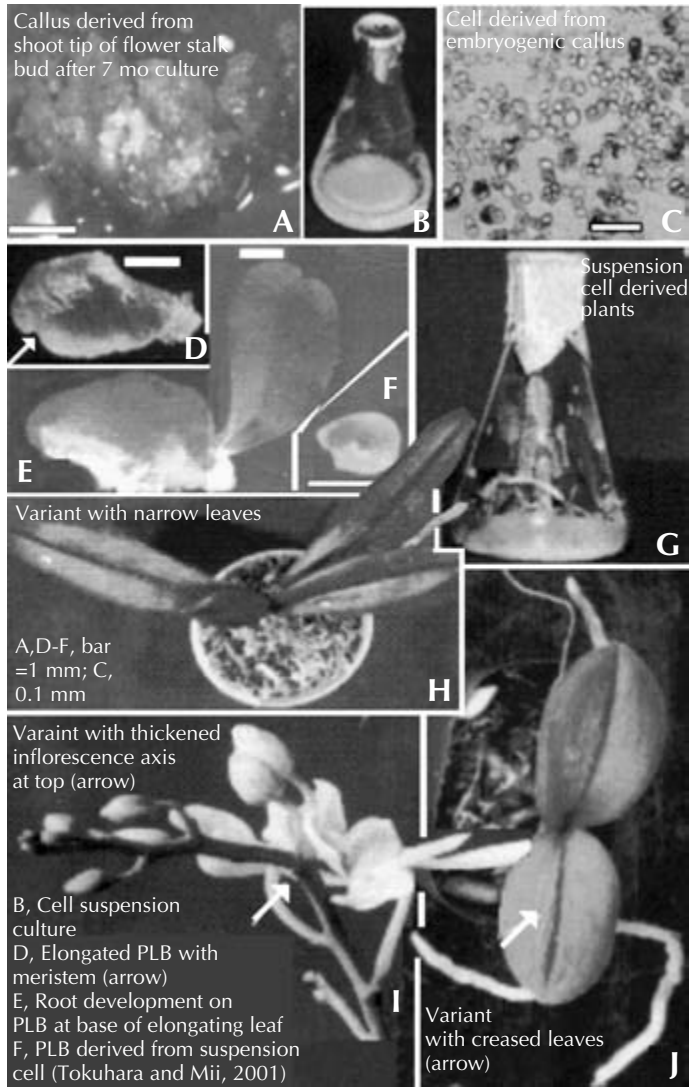


FIG. PHAL-14. **Induction of embryogenic callus and cell suspension cultures from shoot tips excised from flower-stalk buds of *Phalaenopsis* (Tokuhara and Mii, 2001).**

Induction of *Phalaenopsis* Callus and Cell Suspension Cultures from Flower-stalk Bud Tips

A method for the induction of embryogenic callus (Fig. Phal-14A) and/or totipotent cells (Fig. Phal-14B, C), and producing plants (Fig. Phal-14F–J) from them, was developed at the Dogashima Orchid Sanctuary and the Faculty of Agriculture, Chiba University in Japan (Tokuhara and Mii, 2001).

Plant Material. Shoot-tip explants should be obtained by the method used by Tokuhara and Mii (1993).

Surface Sterilization. The procedure is the same as the one used to surface-sterilize explants by Tokuhara and Mii (1993).

Culture Vessels. For callus production, explants should be cultured singly in 24×100 mm culture tubes containing 15 ml medium. To establish cell suspension cultures (Fig. Phal-14C), about 200 mg of callus should be cultured in 100-ml Erlenmeyer flasks (Fig. Phal-14B) containing 40 ml liquid medium. Petri dishes, 9 cm in diameter, containing 25 ml medium should be used for callus production from cells.

Culture Conditions. Both solid and liquid cultures should be maintained at $23 \pm 1°C$ under 14-h photoperiods of 33 μmol m^{-2} s^{-1}. Liquid cultures should be placed on a reciprocal shaker at 80 oscillations per minute (the original paper uses "rpm" or revolutions per minute, which is incorrect in this case because reciprocal shakers do not revolve, they oscillate or move back and forth or to and fro ⇄).

Culture Media. To induce callus, the explants should be cultured on the new Dogashima (ND) medium (Tokuhara and Mii, 1993) containing 0.5 μmol NAA l^{-1}, 4.4 μmol BAP l^{-1}, and 29.2 mmol sucrose l^{-1} (Table Phal-30). Callus should be moved to the same medium, but with 58.4 mmol sucrose l^{-1} (Table Phal-31), after 4 months of culture. To obtain a cell suspension, sections of callus must be cultured in liquid ND medium with 5.4 μmol NAA l^{-1} and 58.4 mmol sucrose l^{-1} (Table Phal-32). Calli and cells from cell suspension cultures produce PLBs (Fig. Phal-14D, E) on ND medium with 29.2 mmol sucrose l^{-1} and probably (the original paper is not very clear on this point) also 5.4 μmol NAA l^{-1} (Table Phal-33). To produce plantlets (Fig. Phal-14G) these PLBs should be moved to a medium free of plant growth regulators and containing potato granules (Basic American Foods, Walnut Creek, CA, USA, www.baf.com; the granules are fortified with vitamin C which probably makes no difference because this vitamin is heat-labile and probably most of it is destroyed during autoclaving), apple juice (the source for the original research was Kyoei Co. Ltd., Kuroishi, Aomori, Japan, but any apple juice completely free of additives, preservatives, and coloring substances and with additional sugar will probably be suitable) and 58.4 mmol sucrose l^{-1} (Table Phal-34).

Procedure. Shoot tips of buds from flower-stalk nodes are cultured for 7 months in test tubes on ND medium to produce embryogenic callus on a medium with 29.2 mmol sucrose (Table Phal-30). The callus is moved to a solution which contains 58.4 mmol sucrose (Table Phal-31), but is otherwise the same as the previous substrate. The callus should be subcultured monthly. To produce cell suspension cultures, the callus is cultured in agitated Erlenmeyer flasks containing liquid ND medium with 58.4 mmol sucrose (Table Phal-32). Cells should be subcultured at 1-month intervals. To induce PLBs, cells should be collected by centrifuging the suspension at $200\times g$ for 5 min and cultured on solid ND medium with 29.2 mmol glucose for 5 months. The embryogenic cells and callus on this medium should be subcultured

TABLE PHAL-30. **New Dogashima (ND) medium (Tokuhara and Mii, 1993) for callus induction from shoot-tip explants from buds of *Phalaenopsis* flower-stalk nodes (Tokuhara and Mii, 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	480.0	48.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	470.0	47.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	550.0	55.0 g l^{-1}	10	
5	Potassium chloride, KCl	150.0	15.9 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	200.0	20.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2{\cdot}6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Sulfuric acid, concentrated	0.5 µl	0.5 ml		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	0.5	50.0 mg l^{-1}		
	Amino acid				
9	*l*-cysteine	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Purine				
11	Adenine	1.0	100 mg 100 ml^{-1} acidified 70% ethanol[e,f]	1	
	Auxin				
12	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,g]	1	
	Cytokinin				
13	6-Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,g]	1	
	Vitamins				
14	Biotin	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Calcium pantothenate	1.0	100 mg 100 ml^{-1} 70% ethanol[e,h]	1	
16	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
17	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
18	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
19	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
20	Water, distilled[f]	To 1000 ml			
	Solidifier				
21	Gelrite[i,j]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Dissolve the adenine in up to 30 ml 0.1 N hydrochloric acid and make up the solution to 100 ml with 95% ethanol.

[g]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively. The actual amount of NAA (MW 186.2) used is 0.5 µmol which comes to (0.5 × 186.21 =) 93.11 µg or 0.093 mg, which is rounded here to 0.1 mg. BA (MW 225.2) was used at 4.4 µmol which is (4.4 × 225.26 =) 991.14 µg. That is rounded to 1 mg here.

[h]Dissolve the calcium pantothenate in 30 ml distilled water and make up the solution to 100 ml with 95% ethanol.

[i]Add items 1–18 to 900 ml of distilled water (item 20), adjust pH to 5.4, add sugar (item 19), and raise volume to 1000 ml with distilled water (item 20). Add the Gelrite (Phytagel, gellan gum) slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps (see also footnote *j*). If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave. The amino acid (item 9), hormones (items 12 and 13) and vitamins (items 14–18) are not heat-stable and are often added to autoclaved and still warm and liquid media with sterilized pipettes and mixed well before distribution to preautoclaved culture vessels. In this case all components of the medium are autoclaved. Sucrose (MW 342.30) at 29.2 mmol is 9995.16 mg, which is rounded to 10 g; 58.4 mmol is 19,990.32 mg or 20 g (rounded).

[j]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

TABLE PHAL-31. **New Dogashima (ND) medium (Tokuhara and Mii, 1993) for the maintenance of callus produced by shoot-tip explants from buds of *Phalaenopsis* flower-stalk nodes (Tokuhara and Mii, 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	480.0	48.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	470.0	47.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	550.0	55.0 g l^{-1}	10	
5	Potassium chloride, KCl	150.0	15.9 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	200.0	20.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Sulfuric acid, concentrated	0.5 µl	0.5 ml		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50.0 mg l^{-1}		
	Amino acid				
9	*l*-cysteine	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Purine				
11	Adenine	1.0	100 mg in 100 ml acidified 70% ethanol[e,f]	1	
	Auxin				
12	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,g]	1	
	Cytokinin				
13	6-Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,g]	1	
	Vitamins				
14	Biotin	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Calcium pantothenate	1.0	100 mg 100 ml^{-1} 70% ethanol[e,h]	1	
16	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
17	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
18	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
19	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
20	Water, distilled[i]	To 1000 ml			
	Solidifier				
21	Gelrite[i,j]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Dissolve the adenine in up to 30 ml 0.1 N hydrochloric acid and make up the solution to 100 ml with 95% ethanol.

[g]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively. The actual amount of NAA (MW 186.21) used is 0.5 µmol which comes to (0.5 × 186.21 =) 93.11 µg or 0.093 mg, that is rounded here to 0.1 mg. BA (MW 225.2) was used at 4.4 µmol which is (4.4 × 225.26 =) 991.14 µg. That is rounded to 1 mg here.

[h]Dissolve the calcium pantothenate in 30 ml distilled water and make up the solution to 100 ml with 95% ethanol.

[i]Add items 1–18 to 900 ml of distilled water (item 20), adjust pH to 5.4, add sugar (item 19), and raise volume to 1000 ml with distilled water (item 20). Add the Gelrite (Phytagel, gellan gum) slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps; see also footnote *j*). If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave. The amino acid (item 9), hormones (items 12 and 13), and vitamins (items 14–18) are not heat-stable and are often added to autoclaved and still warm and liquid media with sterilized pipettes and mixed well before distribution to preautoclaved culture vessels. In this case all components of the medium are autoclaved. Sucrose (MW 342.30) at 29.2 mmol is 9995.16 mg which is rounded to 10 g; 58.4 mmol is 19,990.32 mg or 20 g (rounded).

[j]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

TABLE PHAL-32. **Liquid new Dogashima (ND) medium (Tokuhara and Mii, 1993) for the culture of cell suspensions derived from callus produced by shoot-tip explants from buds of *Phalaenopsis* flower-stalk nodes (Tokuhara and Mii, 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	480.0	48.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	470.0	47.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	550.0	55.0 g l^{-1}	10	
5	Potassium chloride, KCl	150.0	15.9 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	200.0	20.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2{\cdot}6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Sulfuric acid, concentrated	0.5 µl	0.5 ml		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	0.5	50.0 mg l^{-1}		
	Amino acid				
9	*l*-cysteine	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Purine				
11	Adenine	1.0	100 mg in 100 ml acidified 70% ethanol[e,f]	1	
	Auxin				
12	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,g]	1	
	Vitamins				
13	Biotin	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Calcium pantothenate	1.0	100 mg 100 ml^{-1} 70% ethanol[e,h]	1	
15	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
16	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
17	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
18	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[i]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Dissolve the adenine in up to 30 ml 0.1 N hydrochloric acid and make up the solution to 100 ml with 95% ethanol.

[g]If the auxin does not dissolve, add a few drops of 0.1 N KOH. The actual amount of NAA (MW 186.21) used is 5.4 µmol which comes to (5.4 × 186.21=) 1005.53 µg or 1.006 mg, that is rounded here to 1 mg.

[h]Dissolve the calcium pantothenate in 30 ml distilled water and make up the solution to 100 ml with 95% ethanol.

[i]Add items 1–17 to 900 ml of distilled water (item 19), adjust pH to 5.4, add sugar (item 18), and raise volume to 1000 ml with distilled water (item 19), pour the solution into culture vessels, and autoclave. The amino acid (item 9), hormone (item 12) and vitamins (items 13–17) are not heat-stable and are often added to autoclaved and still warm and liquid media with sterilized pipettes and mixed well before distribution to preautoclaved culture vessels. In this case all components of the medium are autoclaved. Sucrose (MW 342.30) at 29.2 mmol is 9995.16 mg, which is rounded to 10 g; 58.4 mmol is 19,990.32 mg or 20 g (rounded).

TABLE PHAL-33. **New Dogashima (ND) medium (Tokuhara and Mii, 1993) modified for protocorm-like body (PLB) and plantlet production from cell suspensions derived from callus produced by shoot-tip explants from buds of *Phalaenopsis* flower-stalk nodes (Tokuhara and Mii, 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	480.0	48.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	470.0	47.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	550.0	55.0 g l^{-1}	10	
5	Potassium chloride, KCl	150.0	15.9 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	200.0	20.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Sulfuric acid, concentrated	0.5 µl	0.5 ml		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50.0 mg l^{-1}		
	Amino acid				
9	*l*-cysteine	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Purine				
11	Adenine	1.0	100 mg in 100 ml acidified 70% ethanol[e,f]	1	
	Auxin				
12	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,g]	1	
	Vitamins				
13	Biotin	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Calcium pantothenate	1.0	100 mg 100 ml^{-1} 70% ethanol[e,h]	1	
15	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
16	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
17	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
18	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[i]	To 1000 ml			
	Solidifier				
20	Gelrite[i,j]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Dissolve the adenine in up to 30 ml 0.1 N hydrochloric acid and make up the solution to 100 ml with 95% ethanol.

[g]If the auxin does not dissolve, add a few drops of 0.1 N KOH. The actual amount of NAA (MW 186.21) used is 5.4 µmol which comes to (5.4 × 186.21 =) 1005.53 µg or 1.006 mg, that is rounded here to 1 mg.

[h]Dissolve the calcium pantothenate in 30 ml distilled water and make up the solution to 100 ml with 95% ethanol.

[i]Add items 1–17 to 900 ml of distilled water (item 19), adjust pH to 5.4, add sugar (item 18), and raise volume to 1000 ml with distilled water (item 19). Add the Gelrite (Phytagel, gellan gum) slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps (see also footnore *j*). If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. When the Gelrite is completely

in Petri dishes twice with 1-month intervals on a suitable medium (Table Phal-33). After 10 months, PLBs that form on this medium are transferred to a fifth solution (Table Phal-34) to bring about plantlet formation.

General Comments. This procedure can produce a large number of plants with somaclonal variations occurring in "less that 10% in six out of eight genotypes examined." The procedure is complex and made more difficult to follow by unclear writing in some parts of the paper, as for example on p. 458: "After proliferation in liquid medium, embryogenic cells of eight genotypes (one genotype of . . .) induced from shoot-tip culture of vegetative buds on flower stalks, on medium consisting of 0.5 μ*M* [this is incorrect usage of terminology because *M* stands for molar, the correct term here is μmoles] NAA, 4.4 μ*M* [see comment above] BA, 58.4 m*M* [see comment above] sucrose and 2 g l^{-1} gellan gum, were subcultured twice at 1-mo intervals. For each phenotype, 50–100 mg of the calluses were transferred . . . and cultured for PLB formation." Is this section dealing with embryogenic cells or with calluses? This quote above is followed by a description of a medium for plantlet induction from the PLBs. But the abstract tells a different story: "These calluses were successfully cultured as cell suspension cultures in liquid NDM supplemented with 5.4 μ*M* [see comment above] NAA and 58.4 m*M* [see comment above] sucrose. By simply reducing the sucrose concentration to 29.2 m*M* [see comment above] the cells grew into plantlets. . . ." So, what to do? Which medium to use? Should the outline in the abstract be followed? Or, is the procedure on p. 458 correct? Dr. Mii was kind enough to clarify the paper and as a result the procedure here (see above) is the one to follow. Those who wish to use this procedure will do well to read the original article and experiment with the procedures. The e-mail address of the first author as given on the paper is tokuhara@dogashima.com.

The new Dogashima culture medium derives its name from the Dogashima Orchid Sanctuary with which the first author of this paper, Ken Tokuhara, is associated. The exact name and address is the Orchid Sanctuary Dogashima, 2848-1 Nishina, Nishizu-cho, Kamo-gun, Shizuoka 410-3514, Japan. The other author, Masahiro Mii, is with the Faculty of Horticulture, Chiba University, Matsudo, Chiba 271-0092, Japan.

dissolved pour the solution into culture vessels and autoclave. The amino acid (item 8), hormone (item 12), and vitamins (items 13–17) are not heat-stable and are often added to autoclaved and still warm and liquid media with sterilized pipettes and mixed well before distribution to preautoclaved culture vessels. In this case all components of the medium are autoclaved. Sucrose (MW 342.30) at 29.2 mmol is 9995.16 mg, which is rounded to 10 g; 58.4 mmol is 19,990.32 mg or 20 g (rounded).

[f]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

TABLE PHAL-34. **New Dogashima (ND) medium (Tokuhara and Mii, 1993) modified for plantlet production from protocorm-like bodies (PLB) produced by suspension cultures derived from callus produced by shoot-tip explants from buds of *Phalaenopsis* flower-stalk nodes (Tokuhara and Mii, 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	480.0	48.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	470.0	47.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	550.0	55.0 g l^{-1}	10	
5	Potassium chloride, KCl	150.0	15.9 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	200.0	20.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Sulfuric acid, concentrated	0.5 µl	0.5 ml		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50.0 mg l^{-1}		
	Amino acid				
9	*l*-cysteine	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
11	Biotin	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Calcium pantothenate	1.0	100 mg 100 ml^{-1} 70% ethanol[e,f]	1	
13	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additives				
16	Potato granules[g]	10.0 g	No stock	No stock	Weigh
17	Apple juice[h]	10.0 g	No stock	No stock	Weigh
	Sugar				
18	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[i]	To 1000 ml			
	Solidifier				
20	Gelrite[i,j]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Dissolve the calcium pantothenate in 30 ml distilled water and make up the solution to 100 ml with 95% ethanol.

[g]Basic American Foods, Walnut Creek, CA, USA, www.baf.com.

[h]The brand used in the original research was Kyoei Co., Ltd., Kuroishi, Aomori, Japan, but any apple juice completely free of additives or additional sugar can be used. In the original paper the amount used is given in grams which is unusual for a liquid.

[i]Add items 1–17 to 700 ml of distilled water (item 19), adjust pH to 5.4, add sugar (item 18), and raise volume to 1000 ml with distilled water (item 19). Add the Gelrite (Phytagel, gellan gum) slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps (see also footnore *j*). If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave. The amino acid (item 9) and vitamins (items 11–15) are not heat-stable and are often added to autoclaved and still warm and liquid media with sterilized pipettes and mixed well before distribution to preautoclaved culture vessels. In this case all components of the medium are autoclaved. Sucrose (MW 342.30) at 29.2 mmol is 9995.16 mg which is rounded to 10 g; 58.4 mmol is 19,990.32 mg or 20 g (rounded).

[j]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

Propagation of *Phalaenopsis* through the Culture of Flower-stalk Sections on Thidiazuron-containing Medium

An often repeated erroneous assertion in the literature is that shoot tips (Morel, 1960) were the first orchid explants to be cultured, when in fact it was *Phalaenopsis* flower-stalk sections (Rotor, 1949). The culture of inflorescence sections is still an important means of micropropagation for *Phalaenopsis*, but most of the available methods produce only a few plants per explants. Approximately 10 years after the very high cytokinin acitivity of TDZ became known (see text box on p. 670, Vol. I in the *Doritaenopsis* section), Dr. Robert Ernst at the Universisty of California, Irvine used it to propagate *Phalaenopsis* stem sections (Ernst, 1994).

Plant Material. Explants should be taken from flower stalks which have finished blooming. The upper flower-bud-bearing portions and the lowermost node stalks should be removed. After that the stalks should be cut into 3-cm sections, each with a bud in the middle.

Surface Sterilization. In the original research, the ends of the sections were sealed with paraffin wax to prevent entry of sterilant into the tissues. This was most probably a superfluous precaution because the amount of sterilant that can enter the sections during the sterilization process is very small and will probably have no effects of any kind. Also, cutting about 0.25–0.5 cm off the ends of sections after the sterilization will probably remove tissue that contains sterilant. Numerous *Phalaenopsis* stem sections which are surface-sterilized and cultured successfully in many laboratories without paraffin sealant are excellent proof that the paraffin wax seal is not necessary. Still, it should not be eliminated without a test. The sealed node sections should be wiped with 95% ethanol and then stirred for 20 min in a 1% solution of sodium hypochlorite (20 ml of household bleach containing 5–5.25% sodium hypochlorite) partially neutralized to pH 9.0 ± 0.2 with 2N hydrochloric acid [17.2 ml of concentrated hydrochloric acid diluted to 100 ml with distilled water; to prevent splattering and possible injury to the operator the acid must be poured into the water (never the water into the acid) slowly, with stirring, while wearing a face guard and gloves]. Bracts that cover the buds should be removed before submerging the sections in the sodium hydroxide sterilant for an additional 10 min. The sections should be placed in culture after rinsing them three times with sterile distilled water and cutting the paraffin-sealed ends.

Culture Vessels. Erlenmeyer flasks, 250-ml capacity containing about 50 ml medium, closed with perforated rubber stoppers that were plugged with cotton. Other containers and/or different plugs can also be used.

Culture Conditions. Research cultures were maintained at 23–24°C under 12-h photoperiods of approximately 25 μmol m^{-2} s^{-1} provided by Sylvania F 30TB 30-W Gro-Lux lamps (Sylvania GTE, Versailles, KY). Standard culture room conditions are probably also suitable.

Culture Media. Explants should be cultured initially in a medium designated XER by its author (X stands for "experimental" and ER are the initials, last name first, of Dr. Robert Ernst who formulated the medium and named it for himself) containing 0.45 μmol TDZ (Table Phal-35). PLBs produced on the first medium should be cultured on XER medium containing 30–45% coconut water from ripe nuts (Table Phal-36) to induce further proliferation. To enhance vegetative growth, PLBs can also be cultured (before or after proliferation) on XER containing 0.2% activated charcoal (www.sigmaaldrich.com) and 5% (w/v) ripe banana pulp homogenate (Table Phal-37).

Procedure. Surface-sterilized sections are placed in culture and maintained on the first medium (Table Phal-35) until PLB or plantlet formation. PLBs can be cultured on coconut-water-containing XER (Table Phal-36) for proliferation. Plantlets or PLBs are cultured on medium supplemented with banana pulp (Table Phal-37) for vegetative growth.

General Comments. This method is the first one to use TDZ for orchids. Its main advantage is that it generates several plantlets per node explant. The method is the same as the one used for *Doritaenopsis* (Ernst, 1994).


Carl Ludwig Blume (1796–1862)

C. L. Blume established the genus *Phalaenopsis* as it is known as present.

TABLE PHAL-35. **XER medium for the culture of *Phalaenopsis* flower-stalk sections (Ernst, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	500.0	50.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	122.0	12.2 g l^{-1}	10	
4	Dibasic potassium phosphate, K_2HPO_4	30.0	3.0 g l^{-1}	10	
5	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
6	Potassium nitrate, KNO_3	500.0	50.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 2H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $NaMoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50.0 mg l^{-1}		
	Cytokinin				
9	Thidizuron (TDZ)	1.0	100 mg 100 ml 1N NaOH in 95% ethanol[e]	1	
	Sugar				
10	Fructose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Gelrite[f,g]	5.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]The original recipe does not include EDTA as a chelating agent. It is included here because iron salts tend not to stay in solution. Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. The original recipe for this medium calls for 28 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$ l^{-1}. The difference (0.2 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored. Sticklers for accuracy can add 28 mg l^{-1}.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]To prepare the solvent (1N NaOH in ethanol) dissolve 4 g NaOH in 100 ml 95% ethanol. Use this as a sterilizing solvent for thidizuron if the cytokinin is to be added to the medium after autoclaving. According to several reports thidiazuron can be added to the medium before autoclaving. If so it can be dissolved in aqueous 1N NaOH. In either case the stock solution should be kept frozen between uses. Thidiazuron can also be dissolved in dimethyl sulfoxide (DMSO). Both thidiazuron and DMSO can be obtained from www.sigmaaldrich.com and other sources. For more information about incorporation of thidiazuron in culture media see Badzian et al. (1989); Bates et al. (1992); Fellman et al. (1987); Huetterman and Preece (1993; excellent source of information); Mok and Mok (1985); Mok et al. (1982); and Neuman et al. (1993).

[f]Add items 1–9 to 900 ml of distilled water (item 11), adjust pH to 5.5, add sugar (item 10), and bring volume to 1000 ml with distilled water (item 11). Phytagel (gellan gum, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave.

[g]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels.

TABLE PHAL-36. **Coconut-water-containing XER medium for the proliferation of protocorm-like bodies produced by flower-stalk explants of *Phalaenopsis* (Ernst, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$[b]	500.0	50.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	122.0	12.2 g l^{-1}	10	
4	Dibasic potassium phosphate, K_2HPO_4	30.0	3.0 g l^{-1}	10	
5	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
6	Potassium nitrate, KNO_3	500.0	50.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}		
(b)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}2H_2O$	3.0	300.0 mg l^{-1}	10	One solution
(e)	Sodium molybdate, $NaMoO_4{\cdot}2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	0.5	50.0 mg l^{-1}		
	Complex additive				
9	Coconut water from ripe nuts[e]	400.0 ml	No stock	No stock	Measure
	Sugar				
10	Fructose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Gelrite[f,g]	5.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]The original recipe does not include EDTA as a chelating agent. It is included here because iron salts tend not to stay in solution. Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. The original recipe for this medium calls for 28 mg ferrous sulfate, $FeSO_4{\cdot}7H_2O$ l^{-1}. The difference (0.2 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored. Sticklers for accuracy can add 28 mg l^{-1}.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts except when indicated otherwise as in this case. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe nuts are not available, water from ripe (brown) ones can be used even for media which require water from green fruits. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Add items 1–9 to 500 ml of distilled water (item 11), adjust pH to 5.5, add sugar (item 10), and bring volume to 1000 ml with distilled water (item 11). Phytagel (gellan gum, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave.

[g]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels.

TABLE PHAL-37. **XER medium containing Banana pulp homogenate and activated charcoal for the enhancement of vegetative growth of plantlets produced by flower-stalk explants of *Phalaenopsis* (Ernst, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	500.0	50.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	122.0	12.2 g l^{-1}	10	
4	Dibasic potassium phosphate, K_2HPO_4	30.0	3.0 g l^{-1}	10	
5	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
6	Potassium nitrate, KNO_3	500.0	50.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	10.0	1.0 g l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	25.0	2.5 g l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	10.0	1.0 g l^{-1}		
	Complex additive				
9	Ripe banana pulp homogenate[e]	50.0 g	No stock	No stock	Weigh
	Sugar				
10	Fructose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f,g]	To 1000 ml			
	Solidifier				
12	Gelrite[f,g]	5.0 g	No stock	No stock	Weigh
	Darkening agent				
13	Activated charcoal[h]	2.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]The original recipe does not include EDTA as a chelating agent. It is included here because iron salts tend not to stay in solution. Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. The original recipe for this medium calls for 28 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$ l^{-1}. The difference (0.2 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored. Sticklers for accuracy can add 28 mg l^{-1}.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Opinions differ regarding the use of banana in culture media. The predominant opinion is that only homogenized pulp of ripe banana should be used. Other views are that: (1) unripe (green) bananas are preferable, (2) the peel rather than the pulp should be used, (3) unpeeled banana homogenate should be incorporated in media, and (4) slices, ripe or unripe, peeled or unpeeled, rather than homogenate are most effective. Opinions also differ regarding which is the most effective variety (Gros Michel, Raja, Pisang Susu, and others). Since there is no experimental evidence to support any view the best approach is to follow the recommendations in the original reports and use what is suggested there.

[f]Pour 900 distilled water (item 11) into a blender, add items 1–9, homogenize thoroughly, adjust pH to 5.5, add sugar (item 10), homogenize again and bring volume to 1000 ml with distilled water (item 11). Phytagel (gellan gum, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave.

[g]Gellan gum [available as such rom www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels.

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite should not be used except in special circumstances.

Effect of Bract Removal Time on the Decontamination and Growth of Flower-stalk Nodes

Research at the Department of Life Science, Aichi University of Education (Ichihashi et al., 2000) has shown that:

1 Fewer cultures became contaminated (Fig. Phal-15) when the bracts were removed (Fig. Phal-16) after sterilization. These findings are not in agreement with conventional wisdom. They may prove to be applicable in all cases or be limited to the conditions at Aichi. Therefore preliminary tests are advisable. Or, the nodes should be surface-sterilized before and after the bract is removed.

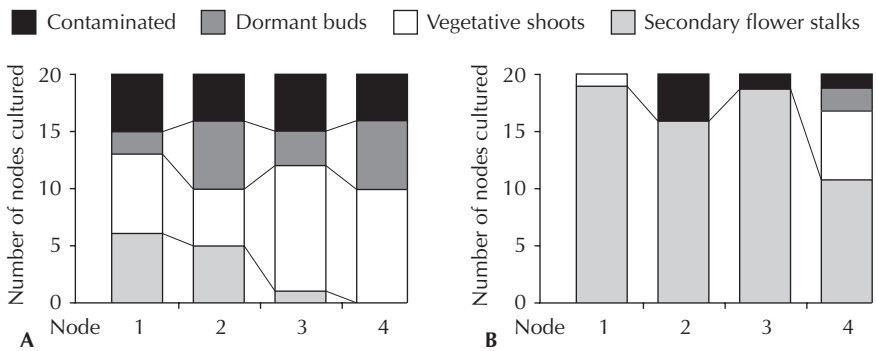


FIG. PHAL-15. **Effects of the time of bract removal on *Phalaenopsis* flower-stalk node cultures. A. Bract removed before sterilization. B. Bract removed after sterilization. (Ichihashi et al., 2000.)**

FIG. PHAL-16. ***Phalaenopsis* flower-stalk nodes before (left) and after (right) removal of the bract. In the right-hand stalk, the bud is visible. (From Lotte and Thomas website, http://www.orchideenvermehrung.at/english/index.htm.)**

TABLE PHAL-38. **Hyponex/Kyoto medium (Tsukamoto et al., 1963) modified for the culture of *Phalaenopsis* flower-stalk nodes (Ichihashi et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Hyponex[b]	3.5 g	No stock	No stock	Weigh
	Polyol				
2	*myo*-inositol	100.0	No stock	No stock	Weigh
	Amino acid				
3	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[c]	1	
	Cytokinin				
4	Benzyladenine (BA)	5.0	500 mg 100 ml^{-1} 95% ethanol[c,d]	1	
	Vitamins				
5	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[c]	1	
6	Pyridoxine·HCl	0.5	50 mg 100 ml^{-1} 95% ethanol[c]	1	
7	Thiamine·HCl (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[c]	1	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]From www.hyponex.co.jp.

[c]Keep frozen between uses.

[d]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[e]Add items 1–7 to 900 ml of distilled water (item 9), adjust pH to 5.6, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring the solution to a gentle boil and add the agar (item 10) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

2 Most buds developed secondary flower stalks when the bract was removed after surface sterilization. If the bract was removed before surface sterilization, most buds formed vegetative shoots. Since the intent of node culture is to produce vegetative shoots and plantlets, removing the bract before sterilization is preferable even if more nodes will become contaminated.

Nodes were cultured on a modified Hyponex medium (Table Phal-38) at 20–23°C under continuous light of 300–500 lx provided by Toshiba Plantlux FL40S tubes.

Phalaenopsis Propagation through the Culture of Sections from Cloned Plantlets Growing in Vitro

Slices, 2.5 or 5 mm thick from the basal parts, 5–10 mm in length, of plantlets 1.5 cm tall, cultured on new *Phalaenopsis* (NP) medium with coconut water and BA (Table Phal-39) at 20–23°C under 300–500 lx produce callus and shoots (Ichihashi et al., 2000).

TABLE PHAL-39. **New *Phalaenopsis* (NP) medium (Ichihashi, 1992*a*, 1992*b*) modified for the culture of plantlet sections (Ichihashi et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	32.0	3.2 g l^{-1}	10	
2	Ammonium sulfate, $(NH_4)_2SO_4$	303.9	30.39 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)_2$	637.6	63.76 g l^{-1}	10	
4	Magnesium nitrate, $Mg(NO_3)_2 \cdot 6H_2O$	256.4	25.64 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	424.0	42.4 g l^{-1}	10	
6	Monopotassium phosphate, KH_2PO_4	462.7	46.27 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.415	41.5 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinin				
14	Benzyladenine (BA)	5.0	100 mg 100 ml 95% ethanol[e,f]	1	
	Complex additive				
15	Coconut water[g]	150.0	No stock	No stock	Measure
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar[h]	3.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the cytokinin fails to dissolve add a few drops of 0.1 N HCl.

[g]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[h]Add items 1–15 to 750 ml of distilled water (item 17), adjust pH to 5.6, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

Light Intensity and Light Quality Effects on Callus and on Callus-derived Plantlets of *Phalaenopsis*

Research with *Phalaenopsis* plantlets grown on new *Phalaenopsis* (NP) medium has shown that both light intensity and quality (i.e., color) can affect the growth and development of both callus and callus-derived plantlets (Islam et al., 2001). Cultures were grown at 25°C under 16-h photoperiods provided by Plant Lux fluorescent lamps (National, Japan). Culture flasks were wrapped with blue (8.79 μmol m^{-2} s^{-1} at plant level inside the vessel), green (22.61 μmol m^{-2} s^{-1}), red (52.17 μmol m^{-2} s^{-1}), and yellow (78.26 μmol m^{-2} s^{-1}) cellophane (Toyo, Japan) to produce the required light colors. The controls were unwrapped flasks (100 μmol m^{-2} s^{-1}). Specifically:

1. Callus grown in the dark was pale.
2. The growth of callus in sucrose-containing medium under 25 μmol m^{-2} s^{-1} illumination was significantly better than in the dark.
3. The growth of callus on sucrose-containing medium was better under red and yellow light than that of callus illuminated with other colors.
4. PLB production on maltose- or sorbitol-containing media illuminated with red or yellow light was better than the control. Green and blue illumination inhibited PLB production.
5. Fresh weight (FW), dry weight (DW), and shoot length as well as the leaf length, width, and thickness of plantlets in vitro increased significantly under illumination of 50 μmol m^{-2} s^{-1} in comparison to 25 and 100 μmol m^{-2} s^{-1}.
6. The FW and DW of plantlets under red light were significantly higher than the weights of plants under other colors and the control despite the fact that the red light intensity was about half that of the controls.

In considering light quality effects it is important to keep in mind that: (1) light transmission levels and spectra of cellophane can vary with manufacturer, and (2) cellophane is not a narrow band light transmission filter.

Micropropagation of *Phalaenopsis* through the Culture of Lateral Buds from Young Flower Stalks

By 1992 several methods were developed for micropropagation of *Phalaenopsis*, each with its shortcomings and advantages. A method using buds from young flower stalks was formulated in an attempt to overcome some of the shortcomings. Formulation of a new medium, especially suitable for *Phalaenopsis*, was another purpose of research by Professor Syoichi Ichihashi, Department of Life Science, Aichi University of Education, Hirosawa, Igaya-cho, Kariya-shi, Aichi-ken, Japan (Ichihashi, 1992*a*, 1992*b*).

Plant Material. Soft flower stalks, less than 15 cm in length, should be used before the appearance of the first flower bud (Fig. Phal-17A, O). Buds (Fig. Phal-17C) should be removed after surface sterilization by making two cuts horizontally and a pair of incisions vertically (Fig. Phal-17B, P), followed by a vertical cut. The excised buds should be rinsed several times with sterile distilled water.

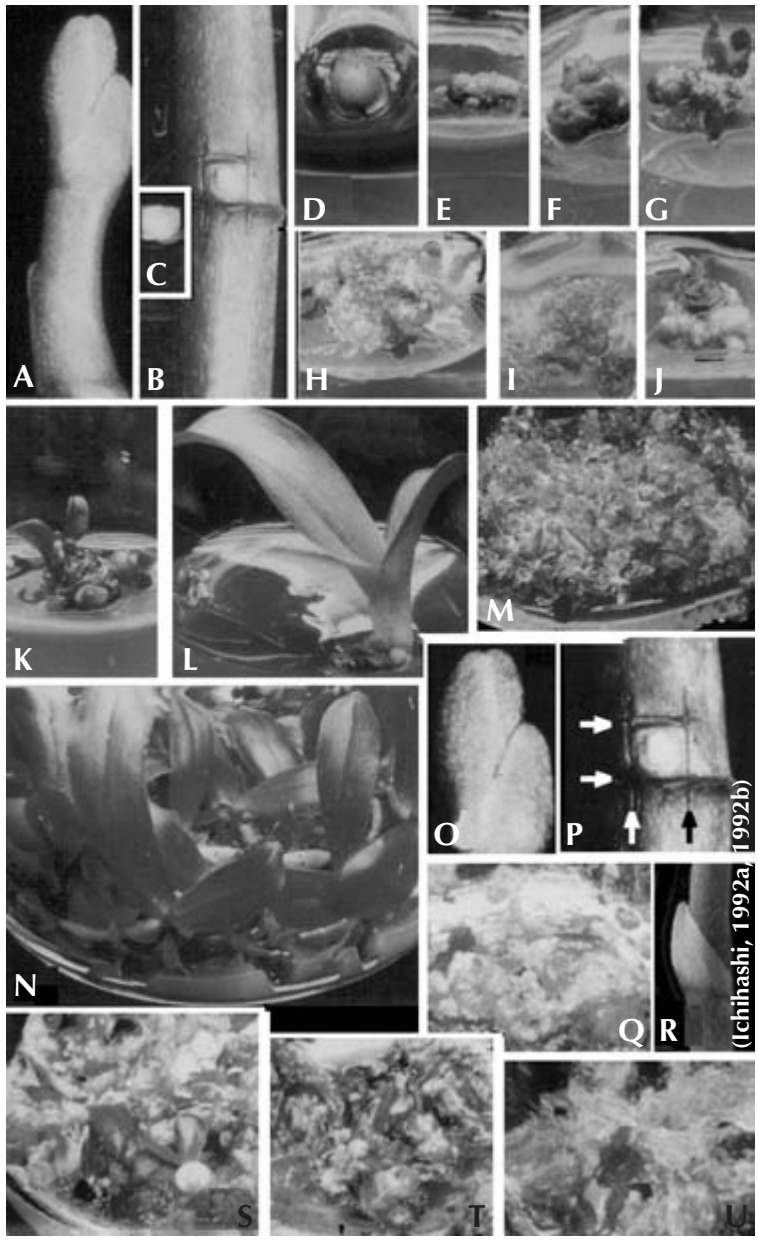


FIG. PHAL-17. **Culture of lateral buds from young flower stalks. A. Upper part of a young stalk. B. Horizontal and vertical incisions above, below, and on the side of a bud. C. Excised bud. D. Growth is starting. E. Proliferating green explant. F. PLBs being formed. G. Callus-like body (CLB). H. Proliferating CLBs. I. CLB. J. PLB. K. PLB and a small plantlet. L. Plantlet. M. CLBs. N. Plantlets formed by CLBs. O. Upper portion of stalk. P. Horizontal (white) and vertical (white and black) incisions. Q. Proliferating PLBs and CLBs. R. Bud covered with a scale. S–U. Proliferating PLBs and CLBs. (Sources: A–Q, S–U, Ichihashi, 1992*a*, 1992*b*; R, Lotte and Thomas website, http://www.orchideenvermehrung.at/english/index.htm.)**

Surface Sterilization. After removing the scales, the second to fourth nodes from the base, all with dormant buds, should be cut into sections approximately 3 cm in length (Fig. Phal-17B). These sections should be surface-sterilized using the following sequence: (1) 30-min immersion in 0.1% benzalkonium chloride, (2) three rinses with sterile distilled water, (3) 3-min immersion in 70% ethyl alcohol (73–74 ml 95% ethyl alcohol diluted to 100 ml with distilled water), (4) three rinses with distilled water, (5) 10-min immersion in 1% sodium hypochlorite (17 or 19 ml of household bleach containing 6 or 5.25% sodium hypochlorite, respectively, diluted to 100 ml with distilled water), and (6) three rinses with sterile distilled water.

Culture Vessels. Culture tubes, 25 × 150 mm or 18 × 180 mm containing 10 ml of medium were used in the original research. Other containers can also be used.

Culture Conditions. Cultures should be maintained at 25 ± 1°C under constant illumination of 1.2 mW cm^{-2} provided by Plantlux (Toshiba) fluorescent tubes. Standard culture room conditions may also be suitable.

Culture Media. The optimal balance of cations for these explants is $NH_4^+(25)$: $K^+(38)$: $Mg^{2+}(10)$. The optimal anion balance is NO_3^- (60) : $H_2PO_4^-$ (17) : SO_4^{2-} (23). A suitable medium was formulated to contain these optimal ratios (Table Phal-40). PLBs formed on this medium can be proliferated by subculturing them on the same solution (Table Phal-40) or a different one (Table Phal-36). Plantlets can be cultured on the first medium (Table Phal-40) or another substrate (Table Phal-37).

Procedure. Young flower stalks, 15 cm long or less (Fig. Phal-17A, O), should be cut from the plants, sectioned (Fig. Phal-17B) into 3-cm-long sections, and surface-sterilized before the buds are excised. The excised buds (Fig. Phal 17C) should be cultured in the first medium (Table Phal-40). PLBs (Fig. Phal-17F) or callus-like bodies (CLBs; Fig. Phal-17G) that form on this medium can be subcultured on it or proliferated on another solution (Table Phal-36). Plantlets (Fig. Phal-17L, N) can also be grown on the first medium (Table Phal-40) or another substrate (Table Phal-37).

Developmental Sequence. Buds start to grow (Fig. Phal-17D), turn green, and begin to proliferate (Fig. Phal-17E), and then form PLBs (Phal-17F, J, K) or CLBs (Fig. Phal-17G, I) which also proliferate (Fig. Phal-17H, M, Q, S–U). Plantlets are formed by PLBs (Fig. Phal-17L) and CLBs (Fig. Phal-17N).

General Comments. One of the original papers states that coconut water (CW) "eliminated growth," "inhibited or eliminated growth," "inhibited growth to none," "CW was inhibitory," but also that "addition of CW [to medium solidified with Gelrite] enhanced growth." Those who may use this method should test media with and without CW. Except for the uncertainty about CW, this procedure which was developed by a leading Japanese orchid scientist seems to be efficient, technically simple, very productive, and suitable even for a small laboratory.

TABLE PHAL-40. **Medium for the culture of excised buds from young flower stalks (Ichihashi, 1992*a*, 1992*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	32.0	3.2 g l^{-1}	10	
2	Ammonium sulfate, $(NH_4)_2SO_4$	303.9	30.39 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)_2$	637.6	63.76 g l^{-1}	10	
4	Magnesium nitrate, $Mg(NO_3)_2 \cdot 6H_2O$	256.4	25.64 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	424.0	42.4 g l^{-1}	10	
6	Monopotassium phosphate, KH_2PO_4	462.7	46.27 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.62	62.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.0025	0.25 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.0025	0.25 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	2.23	223.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.083	0.83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.86	86.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Coconut water				
14	Coconut water (CW), optional[f]	150.0 ml	No stock	No stock	Measure
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Gelrite[g,h]	4.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[g]Add items 1–13 to 900 ml of distilled water (item 16), adjust pH to 5.6 ± 0.1, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Add Gelrite as described in footnote *h* below. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Phytagel (gellan gum, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved, pour the medium into culture vessels and autoclave.

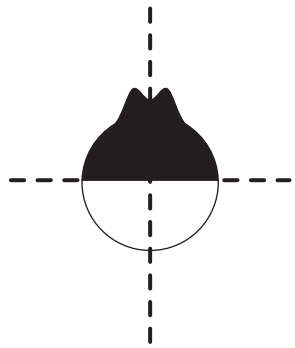


FIG. PHAL-18. **Sectioning a protocorm or a PLB into quarters (Yam et al., 1991 after Tanaka, 1987).**

Coconut Water Effects on the Proliferation of *Phalaenopsis* Protocorms

PLBs are produced by explants or callus in several *Phalaenopsis* micropropagation procedures. The plantlet yields of these procedures can be increased by proliferating the PLBs through a method that keeps undesirable mutations to a minimum. Such a method was developed for seedling protocorms of *Phalaenopsis* [Yam (mispelled as Lam) et al., 1991]. This method is presented here because PLBs and protocorms generally respond in a similar fashion to culture media and their components.

Plant Material. Protocorms or PLBs at the leaf point stage should be sectioned into quarters "by one vertical (tip to base) and one horizontal cut" (Fig. Phal-18).

Surface Sterilization. Protocorms or PLBs taken from in vitro cultures do not require surface sterilization. However, they should be washed with sterile distilled water to remove medium residues.

Culture Vessels. Culture tubes, Erlenmeyer flasks, and other containers filled with medium to 25–30% of their capacity are suitable.

Culture Conditions. Cultures should be maintained at 22 ± 3°C under 16-h photoperiods of 2.5 mW cm^{-2} provided by Gro-Lux fluorescent lamps. Standard culture room conditions will probably also be suitable.

Culture Media. PLB quarter sections should be cultured on XE medium ("X" is for "experimental and "E" stands for Ernst, the last name of Dr. Robert Ernst who formulated the medium and named it for himself) supplemented with 100 ml coconut water (Table Phal-41). New PLBs that form on this medium can be subcultured on it or on another solution (Table Phal-36). Plantlets should be cultured on yet another substrate (Table Phal-37).

Procedure. After the PLBs have been sectioned, the sections (quarters) should be cultured on the first medium (Table Phal-41). New PLBs should be subcultured on

TABLE PHAL-41. **XE medium for the proliferation of protocorms and protocorm-like bodies of *Phalaenopsis* (Yam et al., 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	500.0	50.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	122.0	12.2 g l^{-1}	10	
4	Dibasic potassium phosphate, K_2HPO_4[c]	30.0	3.0 g l^{-1}	10	
5	Monobasic potassium phosphate, KH_2PO_4[c]	250.0	25.0 g l^{-1}	10	
6	Potassium nitrate, KNO_3	500.0	50.0 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}		
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 2H_2O$	3.0	300.0 mg l^{-1}	10	One solution
(e)	Sodium molybdate, $NaMoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50.0 mg l^{-1}		
	Sugars				
9	Fructose	15.0 g	No stock	No stock	Weigh
10	Glucose	15.0 g	No stock	No stock	Weigh
	Complex additives				
11	Coconut water (CW)[f]	100.0 ml	No stock	No stock	Measure
	Solvent				
12	Water, distilled[g]	To 1000 ml			
	Solidifier				
13	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This combination of K_2HPO_4/KH_2PO_4 buffers the pH of the medium. The use of K_2HPO_4/KH_2PO_4 buffer was first suggested by Professor Hans Burgeff in Germany (Burgeff, 1936 and citations therein). He was one of the earliest students of orchid mycorrhiza and orchid seed germination.

[d]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved.

[e]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[g]Add items 1–8 and 11 to 800 ml of distilled water (item 12), adjust pH to 5.5, add the sugars (items 9 and 10), and bring volume to 1000 ml with distilled water (item 12). Bring the solution to a gentle boil and add the agar (item 13) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave.

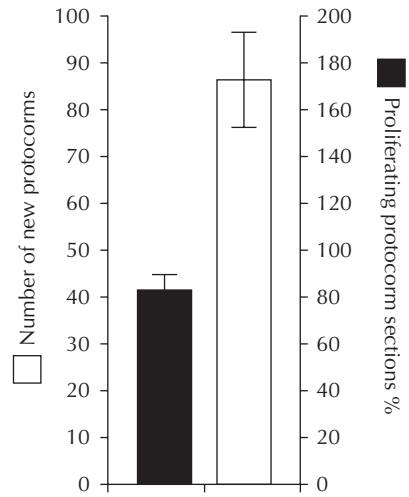


FIG. PHAL-19. **Proliferation of *Phalaenopsis* protocorm sections (modified from Yam et al., 1991).**

XE medium (Table Phal-41) or XER medium (see Table Phal-36). Plantlets should be grown on a third substrate (see Table Phal-37).

Developmental Sequence. Approximately 90% of the sections proliferate on the first medium, with the sections producing about 40 new PLBs each (Fig. Phal-19).

General Comments. The research that led to the formulation of this medium evaluated the effects on proliferation of coconut water (CW), banana homogenate (BH), and 6-(γ-γ-dimethylallylamino)-purine (DMAP) singly and in combinations of two and three of them. DMAP as a single additive completely inhibited proliferation. BH alone and BH plus DMAP reduced the number of protocorms produced by each section relative to controls and the CW-containing medium, but not the percent of sections which proliferated, except when BH was the sole additive. CW did not increase the percent of sections which proliferated except in comparison to a medium that contained only BH. However, it did increase the number of new protocorms per section in comparison to the controls and all media that contained additives. A major advantage of CW is that it brings about considerable proliferation without increasing the number of undesirable mutations.

Proliferation of *Phalaenopsis* Protocorms on a Thidiazuron-containing Medium

PLBs are very similar to protocorms in appearance and development. Therefore a method for protocorm proliferation on a TDZ-containing medium (Ernst, 1994) should also prove to be suitable for PLBs.

Plant Material. Protocorms (those of *Phalaenopsis* Happy Buddah selfed were used in the original research) or PLBs from in vitro cultures can be used.

Surface Sterilization. Protocorms or PLBs from in vitro cultures do not require surface sterilization. However, they should be washed with sterile distilled water.

Culture Vessels. Erlenmeyer flasks, 250-ml capacity containing 50–60 ml medium, or other containers are suitable.

Culture Conditions. Cultures should be maintained at 23–25°C under 12-h photoperiods of about 25 μmol m^{-2} s^{-1} (provided in the original research by F 30TB 30-W Gro-Lux lamps from Sylvania GTE, Versailles, KY, USA) or standard laboratory conditions.

Culture Media. PLBs should be proliferated on XER medium supplemented with 1.25 μmol TDZ l^{-1} (Table Phal-42). To bring about differentiation and growth, new protocorms or PLBs should be cultured on a TDZ-free medium (Table Phal-43). A banana-homogenate-containing medium (see Table Phal-37) should be used for plantlet development.

Procedure. Protocorms or PLBs should be placed on the TDZ-containing medium (Table Phal-42) for approximately 6 months for proliferation. When new protocorms or PLBs form they should be moved to the TDZ-free medium (Table Phal-43). Plantlets are cultured on a banana-homogenate-containing medium (see Table Phal-37).

Developmental Sequence. Proliferation takes place on the first medium (Table Phal-42). Shoots and roots form on the second (Table Phal-43) and plantlets grow on the third (see Table Phal-37).

General Comments. This procedure is simple and efficient. However, use of hormones should be avoided if possible since they can cause undesirable mutations. Therefore the previous procedure (Yam et al., 1991) is preferable.

TABLE PHAL-42. **Thidiazuron-containing XER medium for the proliferation of *Phalaenopsis* protocorms and protocorm-like bodies (Ernst, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$[b]	500.0	50.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	122.0	12.2 g l^{-1}	10	
4	Dibasic potassium phosphate, K_2HPO_4[c]	30.0	3.0 g l^{-1}	10	
5	Monobasic potassium phosphate, KH_2PO_4[c]	250.0	25.0 g l^{-1}	10	
6	Potassium nitrate, KNO_3	500.0	50.0 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}2H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $NaMoO_4{\cdot}2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	0.5	50.0 mg l^{-1}		
	Cytokinin				
9	Thidiazuron (TDZ)	0.275	275 mg 100 ml^{-1} 95% ethanol[f]		
	Sugar				
10	Fructose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g,h]	To 1000 ml			
	Solidifier				
12	Gelrite[g,h]	5.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This combination of K_2HPO_4/KH_2PO_4 buffers the pH of the medium. The use of K_2HPO_4/KH_2PO_4 buffer was first suggested by Profesor hans Burgeff in Germany (Burgeff, 1936 and citations therein). He was one of the earliest students of orchid mycorrhiza and seed germination.

[d]The original recipe does not include EDTA as a chelating agent. It is included here because iron salts tend not to stay in solution. Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. The XER original recipe for this medium calls for 28 mg ferrous sulfate, $FeSO_4{\cdot}7H_2O$ l^{-1}. The difference (0.2 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored. Sticklers for accuracy can add 28 mg l^{-1}.

[e]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The XER medium is described in two papers. One deals with proliferation of *Phalaenopsis* protocorms (Yam et al., 1991). The other reported on the culture of *Phalaenopsis* flower-stalk nodes and protocorm proliferation (Ernst, 1994). Both papers state that the microelements used are those of Nitsch and Nitsch (1956). Only one of the papers (Yam et al., 1991) lists the minerals which were added. The formulation is the same as the one in this table and very different from the combination used by Nitsch and Nitsch (1956). A list of microelements is not given in the paper on the thidiazuron-containing medium. It only states that the XER medium contains "the trace elements of Nitsch & Nitsch, 1956." Therefore it is not clear if the microelements in that version of XER is the same as the one in this table. The microelements in the Nitsch and Nitsch (1956) medium are ferric citrate·$5H_2O$, 10 mg l^{-1}; $MnCl_2{\cdot}4H_2O$, 2.5 mg l^{-1}; H_3BO_3, 2 mg l^{-1}; $ZnSO_4{\cdot}7H_2O$, 0.05 mg l^{-1}; $CoCl_2{\cdot}6H_2O$, 0.03 mg l^{-1}; $CuCl_2{\cdot}2H_2O$, 0.015 mg l^{-1}, and $NaMoO_4{\cdot}2H_2O$, 0.025 mg l^{-1}.

[f]If the cytokinin fails to dissolve add a few drops of 0.1 N HCl. Keep frozen between uses.

[g]Add items 1–9 to 900 ml of distilled water (item 11), adjust pH to 5.5, add sugar (item 10), and bring volume to 1000 ml with distilled water (item 11). If the medium is to be solidified with agar bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. If the medium is to be solidified with Gelrite, gellan gum, or Phytagel (item 12) the solidifier should be added according to the instructions in the following footnote.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite (item 12)] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Phytagel (gellan gum, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into a 2-l Erlenmeyer flask and autoclave. Following the autoclaving use a sterile pipette to add the auxin to the medium while it is still liquid and warm, but no longer hot. Swirl or stir the medium with a sterile stirrer to mix the auxin thoroughly and pour the medium into presterilized culture vessels.

TABLE PHAL-43. **XER medium for the induction of shoots on *Phalaenopsis* protocorms and protocorm-like bodies (Ernst, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$[b]	500.0	50.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	122.0	12.2 g l^{-1}	10	
4	Dibasic potassium phosphate, K_2HPO_4[c]	30.0	3.0 g l^{-1}	10	
5	Monobasic potassium phosphate, KH_2PO_4[c]	250.0	25.0 g l^{-1}	10	
6	Potassium nitrate, KNO_3	500.0	50.0 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}		
(b)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4{\cdot}2H_2O$	3.0	300.0 mg l^{-1}	10	One solution
(e)	Sodium molybdate, $NaMoO_4{\cdot}2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	0.5	50.0 mg l^{-1}		
	Sugar				
9	Fructose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f,g]	To 1000 ml			
	Solidifier				
11	Gelrite[g]	5.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This combination of K_2HPO_4/KH_2PO_4 buffers the pH of the medium. The use of K_2HPO_4/KH_2PO_4 buffer was first suggested by Profesor hans Burgeff in Germany (Burgeff, 1936 and citations therein). He was one of the earliest students of orchid mycorrhiza and seed germination.

[d]The original recipe does not include EDTA as a chelating agent. It is included here because iron salts tend not to stay in solution. Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. The XER original recipe for this medium calls for 28 mg ferrous sulfate, $FeSO_4{\cdot}7H_2O$ l^{-1}. The difference (0.2 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored. Sticklers for accuracy can add 28 mg l^{-1}.

[e]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The XER medium is described in two papers. One deals with proliferation of *Phalaenopsis* protocorms (Yam et al., 1991). The other reported on the culture of *Phalaenopsis* flower-stalk nodes and protocorm proliferation (Ernst, 1994). Both papers state that the microelements used are those of Nitsch and Nitsch (1956). Only one of the papers (Yam et al., 1991) lists the minerals which were added. The formulation is the same as the one in this table and very different from the combination used by Nitsch and Nitsch (1956). A list of microelelemnts is not given in the paper on the thidiazuron-containing medium. It only states that the XER medium contains "the trace elements of Nitsch & Nitsch, 1956." Therefore it is not clear if the microelements in that version of XER is the same as the one in this table. The microelements in the Nitsch and Nitsch (1956) medium are ferric citrate·$5H_2O$, 10 mg l^{-1}; $MnCl_2{\cdot}4H_2O$, 2.5 mg l^{-1}; H_3BO_3, 2 mg l^{-1}; $ZnSO_4{\cdot}7H_2O$, 0.05 mg l^{-1}; $CoCl_2{\cdot}6H_2O$, 0.03 mg l^{-1}; $CuCl_2{\cdot}2H_2O$, 0.015 mg l^{-1}; and $NaMoO_4{\cdot}2H_2O$, 0.025 mg l^{-1}.

[f]Add items 1–8 to 900 ml of distilled water (item 10), adjust pH to 5.5, add sugar (item 9), and bring volume to 1000 ml with distilled water (item 10). If the medium is to be solidified with agar bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. If the medium is to be solidified with Gelrite, gellan gum, or Phytagel (item 11) the solidifier should be added according to the instructions in the following footnote.

[g]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite (item 11)] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Phytagel (gellan gum, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave. After the gellan gum (Phytagel, Gelrite) has dissolved, pour the medium into a 2-l Erlenmeyer flask and autoclave. Following the autoclaving use a sterile pipette to add the auxin to the medium while it is still liquid and warm, but no longer hot. Swirl or stir the medium with a sterile stirrer to mix the auxin thoroughly and pour the medium into presterilized culture vessels. Solidifier is not added to liquid media.

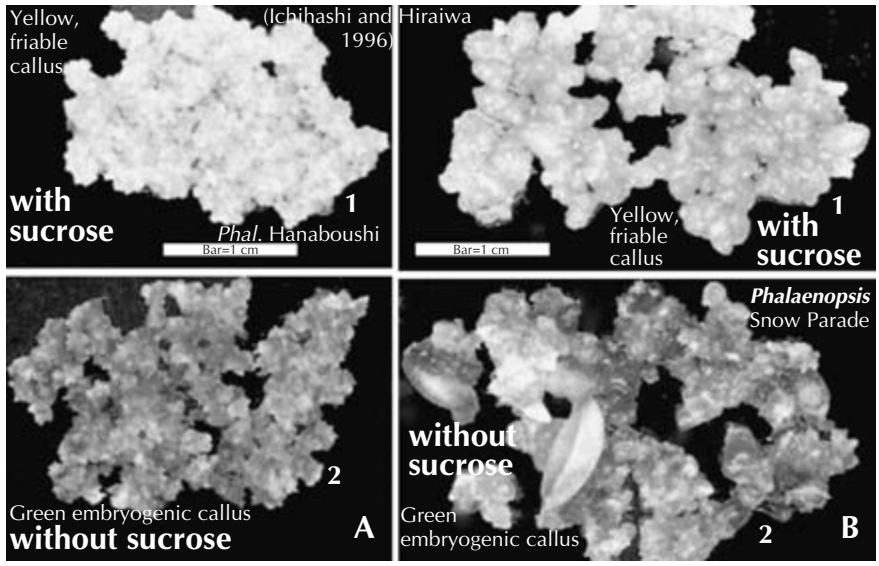


FIG. PHAL-20. ***Phalaenopsis*** **callus. Above. Yellow, friable callus in medium with sucrose (A1 and B1). Below. Green embryogenic callus in sucrose-free medium (A2 and B2). (Ichihashi and Hiraiwa, 1996.)**

Maintenance of Embryogenic Callus of *Phalaenopsis*

Depending on the method being used to culture it, an embryogenic callus can produce protoplasts, embryoids, and/or PLBs, all three of which can be used for genetic engineering or propagation. The effects of solidifiers, sugars, sugar alcohols, and coconuts on an embryogenic callus were studied at the Department of Life Sciences, Aichi University of Education in Japan (Ichihashi and Hiraiwa, 1996). The method used for *Doritaenopsis* is also suitable for *Phalaenopsis* (Fig. Phal-20). This procedure is also suitable for *Darwinara*, *Doritaenopsis*, *Neofinetia falcate*, and *Phalaenopsis* (Ichihashi et al., 1993; Ichihashi and Hiraiwa, 1996).

In Vitro Flowering by *Phalaenopsis* Pink Leopard 'Petra'

Vegetative propagation of *Phalaenopsis* Pink Leopard 'Petra' and the in vitro induction of flowers (Fig. Phal-21) were carried out using the method for *Doriella* Tiny (Duan and Yazawa, 1994*b*, 1994*c*, 1995*a*).

Culture Media. One of the reports states that "explants were transplanted into the Vacin and Went and Hyponex media supplemented with 5 mgl^{-1} [sic] BA and 25 gl^{-1} [sic] sucrose." It is not clear from this statement whether the medium used for *Phalaenopsis* was the same as the one employed for *Doriella* (see Table Drlla-1) or

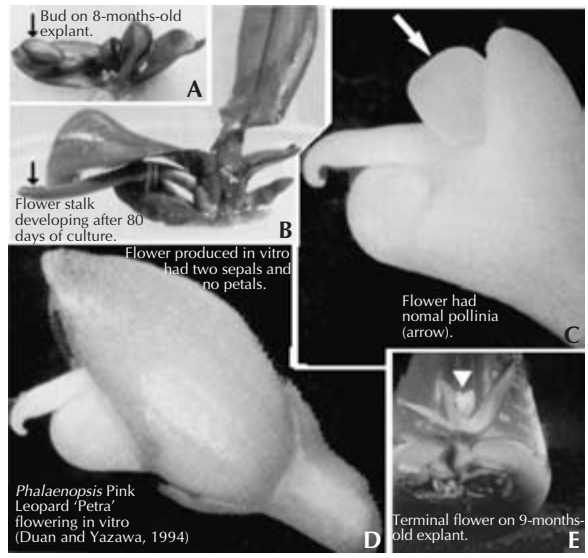


FIG. PHAL-21. **In vitro flowering of *Phalaenopsis* (Duan and Yazawa, 1994*b*).**

TABLE PHAL-44. **Hyponex medium for the culture of *Phalaenopsis* Pink Leopard 'Petra' stem sections (Duan and Yazawa, 1994*b*)**[a]

Component	Amount l^{-1}	Comments
Hyponex[b]	3.5 g	Add all components to 700 ml of water, adjust pH to 5.6, raise volume to 1000 ml, bring to a gentle boil, add agar with stirring and keep stirring until it is dissolved, pour into culture vessels, and autoclave
Peptone	2.0 g	
Coconut water	150.0 ml	
Sucrose	25.0 g	
Agar	10.0	
Water	To 1000 ml	

[a]This is one of two possible media. The other contains peptone (see Table Drlla-1).
[b]One possible source for Hyponex is www.hyponex.co.jp.

a slightly different one which does not contain peptone (Table Phal-44). The Vacin and Went medium (Vacin and Went, 1949) used for *Doriella* contains 10 mg BA l^{-1}. Therefore the solution utilized for *Phalaenopsis* (Table Phal-45) is clearly different.

Procedure. Following sterilization, the explants should be cultured on Hyponex medium (see Tables Drlla-1 or Phal-44) until they develop vegetative buds. These buds should be excised and cultured on the same solution (see Tables Drlla-1 or Phal-44) to increase the number of explants. For flower-bud formation, shoots with 3–4 leaves and 3–4 cm tall are moved to Vacin and Went medium (Table Phal-45). To enhance root formation, after flowering it may be necessary to move the shoots to a root-inducing medium (see Table Drlla-3). Plantlets can be cultured on standard Vacin and Went medium (see Table Drlla-4) for further development.

TABLE PHAL-45. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of bud-bearing *Phalaenopsis* Pink Leopard 'Petra' explants to enhance leaf growth (Duan and Yazawa, 1994*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Cytokinin				
8	Benzyladenine (BA)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e]	1	Or weigh
	Complex additive				
9	Coconut water[f]	150.0 ml	No stock	No stock	Measure
	Sugar				
10	Sucrose	25.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 11) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]If the cytokinin fails to dissolve add a few drops of 0.1 N KOH or NaOH. Keep frozen between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and preservatives (that may have undesirable effects on cultures).

[g]Add items 1, 3–7, and 9 to the 500–700 ml of distilled water (item 11) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 11), adjust pH to 5.6, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the cytokinin to the autoclaved, warm and still liquid medium, swirl or stir with a sterile tool and distribute medium into preautoclaved culture vessels. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

Developmental Sequence. The explants produce vegetative buds on the Hyponex medium (see Tables Drlla-1 or Phal-44). Flower buds form after transfer to Vacin and Went (Table Phal-45). The plants flower on Vacin and Went medium (Table Phal-45).

General Comments. This seems to be a simple yet effective procedure for in vitro flower induction in *Phalaenopsis*. It may also prove to be effective for *Doritis* and *Kingiella* (*Kingidium*). Unlike an unusual and bizarre report about *Dendrobium* (Goh, 1996), the authors provide full details about their procedure. They should be commended for doing it.

Use of Carbon Sources to Affect Growth and Development of Protocorm-like Bodies Derived from Callus

Orchid seeds and seedlings vary in their ability to utilize different carbohydrates and their responses to them (Arditti, 1967, 1979, 1992; Arditti and Ernst, 1984; Ernst and Arditti, 1990). The same is true for embryonic callus of *Phalaenopsis* Wedding Promenade and *Phalaenopsis* Hanaboushi × *Phalaenopsis equestris* Ilocos (Islam, Hiraiwa and Ichihashi, 1997). These differences can be used in micropropagation (Islam et al., 1998). When equimolar levels of carbohydrates were added to new *Phalaenopsis* (NP) medium (Ichihashi, 1992*a*, 1992*b*), the following were observed:

1 Few PLBs generated plantlets (Fig. Phal-22) and most produced yellowish or greenish callus-like bodies (CLBs). Nearly 80% of the unrooted shoots and 50% of the rooted plantlets formed yellowish-greenish CLBs.
2 PLBs multiplied on maltose-containing medium and produced a few plantlets (Fig. Phal-22). Approximately 44% of unrooted shoots and 24% of rooted plantlets produced green PLBs at the bases of plantlets.
3 On sorbitol-containing medium, most PLBs produced plantlets (Fig. Phal-22) and a few new PLBs.

To control growth and development, callus-derived PLBs should be placed on NP medium that contains sucrose, maltose, or sorbitol (Table Phal-46).

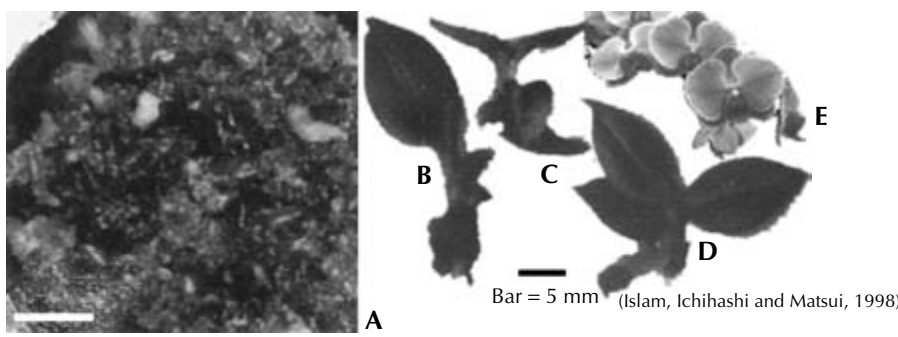


FIG. PHAL-22. **A–D. *Phalaenopsis* callus (A) and plantlets grown on different carbohydrates: sucrose (B), maltose (C), or sorbitol (D). E. Flowers of Phalaenopsis Golden Promenade. (Park et al., 2000.)**

TABLE PHAL-46. **New *Phalaenopsis* (NP) medium (Ichihashi, 1992*a*, 1992*b*) with different carbohydrates for the control of development of *Phalaenopsis* protocorm-like bodies from callus (Islam et al., 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	32.0	3.2 g l^{-1}	10	
2	Ammonium sulfate, $(NH_4)_2SO_4$	303.9	30.39 g l^{-1}	10	
3	Calcium nitrate, $Ca(NO_3)_2$	637.6	63.76 g l^{-1}	10	
4	Magnesium nitrate, $Mg(NO_3)_2 \cdot 6H_2O$	256.4	25.64 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	424.6	42.46 g l^{-1}	10	
6	Monopotassium phosphate, KH_2PO_4	462.7	46.27 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.415	41.5 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar or sugar alcohol				
14	Maltose ***or***	20.0 g	No stock	No stock	Weigh
	Sucrose ***or***	20.0 g	No stock	No stock	Weigh
	Sorbitol	10.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Gelrite[g,h]	3.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Only one of these carbohydrates should be added to the medium depending on the desired results: sucrose, few plants and callus-like bodies (CLBs); maltose, new PLBs and a few plantlets; sorbitol, mostly plantlets, few new PLBs (see text for details).

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.6 ± 0.1, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Add Gelrite as described in the footnote below. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Phytagel (gellan gum, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved, pour the medium into culture vessels and autoclave.

General Comments. It is important to note that equal weights of 6-carbon (sorbitol in this case) or 12-carbon (sucrose and maltose) carbohydrates are not equivalent because they represent different molarities even if they contain the same amount of carbon. For example 20 g of sorbitol is equivalent to 111.01 mmol, whereas 20 g of sucrose or maltose amount to half that at 58.43 mmol. The different molarities affect the osmolarity of media and these differences can have an effect on explants, tissues, PLBs, and plantlets. When larger sugars (tri- and oligosaccharides) are included in media, additional complications may be caused by the ability of the plants to take up, utilize, and/or hydrolyze the different sugars. Therefore Professor Ichihashi and his associates should be commended for using equimolar carbohydrate levels. However, in research with sugars that are readily utilized by orchids (like sucrose, maltose, and sorbitol) comparisons should include both equal carbon and equimolar levels. The hydrolysis of these sugars by orchids should also be studied.

Production of Plants from Callus-derived Protoplasts of *Phalaenopsis*

Callus of *Phalaenopsis* Hanaboushi × *Phalaenopsis equestris* 'Ilocos' was cultured on new *Phalaenopsis* (NP) medium (Ichihashi, 1992*a*, 1992*b*) supplemented with sucrose and coconut water (CW). It was then transferred to NP medium without CW and sucrose for 3 weeks and after that submerged for 90 min in an enzyme solution at 25°C with shaking at 77 strokes min^{-1} "under scatter light." A washing solution was added to the enzyme protoplast preparation and the mixture was filtered through "a stainless sieve of 66 g m [sic] pore size to remove undigested cell clumps and centrifuged at 100× *g* for 5 min."

Fresh washing solution was added to the sediment, mixed with ficoll (www.sigmaaldrich.com) solution, and centrifuged at 230× *g* for 30 min. A layer of protoplasts which formed after the centrifugation was taken up with a pipette, washed with washing solution, suspended in culture medium, and centrifuged at 100× *g* for 5 min.

The protoplast-containing solution was mixed with NP medium supplemented with sorbitol and was cultured at 25°C in the dark. Survival was 89% after 1 week and 60% at the end of 4 weeks. Cell divisions were first observed after 1 week; these increased in number during the fourth week and led to "colony formation and identified plant regeneration." These findings indicate that sorbitol is suitable as an osmoticum for the culture of *Phalaenopsis* protoplasts (Hirose et al., 1998).

Shoot Production from *Phalaenopsis* Flower Stalk Buds on Thidiazuron-containing Medium

TDZ was used primarily as a cotton defoliant until its cytokinin-like properties became known (Mok et al., 1982). Within a relatively short time after that it was shown to induce shoot formation in a number of plants (see Chen and Piluek, 1995 for a short review) and orchids (Ernst, 1994). This led to its use in the micropropagation of *Phalaenopsis* by researchers at Kasetsart University in Thailand and Guizhou Agricultural College in China (Chen and Piluek, 1995).

Plant Material. Nodes, each containing one bud, from flower stalks of *Phalaenopsis* hybrids should be used.

Surface Sterilization. Flower stalks should be washed with household detergent and tap water, rinsed, swabbed with 70% ethanol (73–74 ml 95% ethanol diluted to 100 ml with distilled water), and cut into nodes each with one bud. These nodes should be submerged in 10% Clorox [10 ml Clorox or another household bleach containing sodium hypochlorite diluted to 100 ml with distilled water and containing 2 drops of Tween 20 (sources: www.sigmaaldrich.com, www.fishersci.com, www.vwr.com)] for 25 min. The bracts that cover the buds should be removed after that under sterile conditions with sterilized forceps and the nodes should be immersed in 5% Clorox (5 ml Clorox or another household bleach diluted to 100 ml with distilled water and each 80 ml containing 2 drops of Tween 20) for 15 min. Three rinses with sterile distilled water should follow the immersion. The nodes should be cut to a total length of 1.0–1.5 cm after that (0.5–0.75 cm above and below the bud) and inserted into the medium.

Culture Vessels. Glass bottles, 2.5 cm in diameter and 9.0 cm in height containing 15 ml medium, were used in the original research. Other containers are also suitable.

Culture Conditions. Cultures should be maintained at 23 ± 1°C under 12-h photoperiods of 12 μmol m^{-2} s^{-1} or standard laboratory conditions. Liquid cultures must be placed on a rotary shaker at 120 rpm.

Culture Media. Solid Vacin and Went (VW) medium (Vacin and Went, 1949) containing 15% (v/v) coconut water (CW) supplemented with 5 μmol TDZ l^{-1} (Table Phal-47) is used to induce the formation of adventitious buds on the nodes. The adventitious buds produce shoots on VW containing 15% CW (VWCW; Table Phal-48). These shoots develop roots on VWCW with 10 g sucrose l^{-1} (Table Phal-49). When cultured on VWCW supplemented with 10 μmol TDZ (Table Phal-50), shoots bearing two leaves produce new growths. These newly formed shoots grow roots on VWCW containing 10 g sucrose l^{-1} (Table Phal-49). Shoot tips from shoots with 3–4 leaves, when cultured in liquid VWCW (Table Phal-51) for 4 weeks and then transferred to solid VWCW (Table Phal-48), produce PLBs 1 month after the transfer. These PLBs can be proliferated on CW-containing XER medium (see Table Phal-36) or cultured on new Dogashima (ND) medium (see Table Phal-34) for plantlet production. ND and XER media are similar in some aspects. Plantlets should be cultured on XER medium containing banana pulp homogenate (see Table Phal-37).

Procedure. Nodes are placed on the first medium (Table Phal-47) and maintained on it until they produce adventitious buds. These buds should be moved to VWCW (Table Phal-48) until they produce shoots. When the shoots develop two leaves they can be moved to the third solution for root formation (Table Phal-49) or VWCW with 10 μmol TDZ (Table Phal-50) to bring about the growth of additional growths. Tips of shoots with three leaves cultured in liquid VWCW for 4 weeks and solid medium (Table Phal-48) for a month produce PLBs. PLBs proliferate on XER medium containing CW (see Table Phal-36) or produce plantlets on ND medium

TABLE PHAL-47. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of *Phalaenopsis* flower-stalk nodes (Chen and Piluek, 1995)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Cytokinin				
8	Thidiazuron	1.1	110 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
9	Coconut water (CW)[f]	150.0 ml	No stock	No stock	Measure
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 11) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]If the cytokinin does not dissolve add a few drops of 0.1 N HCl. Keep frozen between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[g]Add items 1 and 3–9 to the 500–700 ml of distilled water (item 11) which contain the calcium phosphate (item 2), raise volume to 900 ml with distilled water (item 11), adjust pH to 5.3, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

TABLE PHAL-48. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for shoot induction from adventitious buds on *Phalaenopsis* flower-stalk nodes (Chen and Piluek, 1995)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water (CW)[e]	150.0 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 10), adjust pH to 5.3, add sugar (item 9), and raise volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

TABLE PHAL-49. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the development of shoots induced from adventitious buds on *Phalaenopsis* flower-stalk nodes (Chen and Piluek, 1995)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water (CW)[e]	150.0 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 3), bring volume to 900 ml with distilled water (item 10), adjust pH to 5.3, add sugar (item 9), and raise volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

TABLE PHAL-50. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for shoot induction on shoots produced by adventitious buds on *Phalaenopsis* flower-stalk nodes (Chen and Piluek, 1995)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Cytokinin				
8	Thidiazuron (TDZ)	2.2	220 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
9	Coconut water (CW)[f]	150.0 ml	No stock	No stock	Measure
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 11) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]If the cytokinin does not dissolve add a few drops of 0.1 N HCl. Keep frozen between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[g]Add items 1 and 3–9 to the 500–700 ml of distilled water (item 11) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 11), adjust pH to 5.3, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

TABLE PHAL-51. **Liquid Vacin and Went (VW) medium (Vacin and Went, 1949) for the induction of protocorm-like bodies from the tips of three leaf-bearing shoots derived from adventitious buds on *Phalaenopsis* flower-stalk nodes (Chen and Piluek, 1995)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water (CW)[e]	150.0 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 10), adjust pH to 5.3, add sugar (item 9), adjust volume to 1000 ml with distilled water (item 10), pour the solution into culture vessels, and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

(see Table Phal-34). Plantlets grow well on XER medium containing banana homogenate (see Table Phal-37).

Developmental Sequence. Nodes produce adventitious buds on the first medium (Table Phal-47). These buds produce shoots on VWCW (Table Phal-48) which develop two leaves. On being moved to the third solution (Table Phal-49) these shoots form roots. They produce additional shoots on VWCW with 10 µmol TDZ (Table Phal-50). PLBs are produced by tips excised from shoots with three leaves and cultured in liquid VWCW for 4 weeks and solid medium for a month (Table Phal-48). When

cultured on CW-containing XER medium (see Table Phal-36) these PLBs proliferate. If cultured on ND medium (see Table Phal-34) they produce plantlets. The plantlets grow well on XER medium containing banana homogenate (see Table Phal-37).

General Comments. This is a multifaceted procedure that makes excellent use of *Phalaenopsis* stalk nodes, utilizes imaginatively a cytokinin which was relatively new at the time, and employs several modifications of one medium to very good advantage. Like Dr. R. Ernst, the authors used information about the culture of *Phalaenopsis* flower-stalk nodes and TDZ cleverly and formulated a novel way of propagating this popular orchid.

Effects of Solidifiers, Coconut Water, and Carbohydrates on Embryogenic Callus of *Phalaenopsis*

Some components are added to media almost due to inertia. They are used simply because: (1) media formulations list them, (2) others have used them, and/or (3) they have been used for a long time. The same is true for their concentrations. Not many investigators devote time and effort to refining media by studying some of their components. Such a study was carried out by Professor Syoichi Ichihashi and his associates at the Department of Life Science, Aichi University of Education. They used the P medium (Table Phal-52) as a basal solution to study the effects of carbohydrates and their concentrations, coconut water, and solidifiers and their levels on embryogenic callus of *Phalaenopsis* (Islam et al., 1997).

Carbohydrates

A study of D-fructose, D-galactose, D-glucose, lactose, maltose, mannitol, L-rhamnose, D-sorbitol, sucrose, and trehalose showed that they had different effects.

- A point sometimes ignored and not always fully appreciated, but properly emphasized by Professor Ichihashi and his associates, is that "osmotic pressure of a medium seems to affect callus growth considerably." This is very important both practically and as a concept because equal weights of monosaccharides and polysaccharides represent different molarities. The fact is that "molar equivalents of monosaccharides supply half the energy of disaccharides." In other words, 20 g of glucose or fructose (or any other 6-carbon monosaccharide or polyol) are not the same as 20 g of sucrose. The "addition of 10 g l^{-1} glucose and/or fructose is equivalent osmotically to 20 g l^{-1} sucrose" or 20 g maltose or 20 g trehalose.

  Another important point is that orchids may differ in their ability to take up or hydrolyze sugars, especially longer-chain oligosaccharides or polysaccharides. This means that seedlings, PLBs, or callus on 20 g each of monosaccharides (glucose or fructose, for example), disaccharides (sucrose or maltose, for instance), trisaccharides (e.g., raffinose, which consists of glucose, fructose, and galactose or maltotriose, made of three glucose molecules) are subject to different osmotic concentrations and carbon availability. They probably take up and/or hydrolyze

TABLE PHAL-52. **P medium (Ichihashi and Hiraiwa, 1996; Islam et al., 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium phosphate, $NH_4H_2PO_4$[b]	922.4	92.24 g l^{-1}	10	
2	Calcium nitrate, $CaNO_3 \cdot 4H_2O$	236.2	23.62 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	246.5	24.65 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	606.6	60.66 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	272.2	27.22 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	310.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.0125	1.25 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.0125	1.25 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.415	41.5 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.125	12.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
13	Coconut water[f]	100.0 ml	No stock	No stock	Measure
	Sugar				
14	Sucrose	10 or 20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Gelrite[g]	4.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The balances of cations and anions in this medium are: $NH_4^+ : K^+ : Ca^{2+} : Mg^{2+} = 40 : 40 : 10 : 10$; $NO_3^- : H_2PO_4^- : SO_4^- = 40 : 50 : 10$. Total concentration of major ions is 20 milliequivalents l^{-1}.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

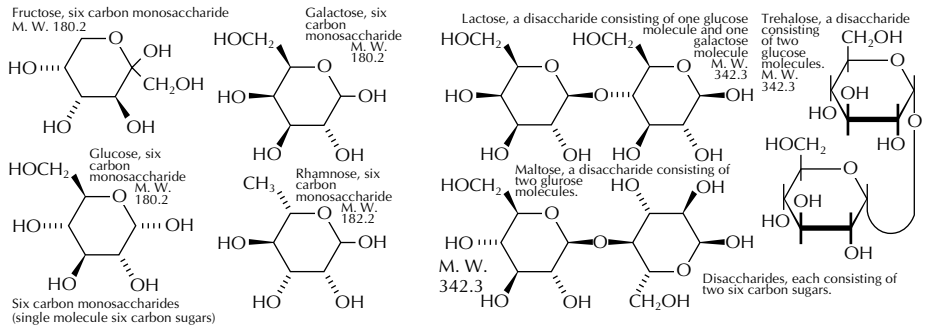
[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Iron and Na_2EDTA concentration is the same as in the Murashige–Skoog medium.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses. The levels of glycine, *myo*-inositol, and vitamins are the same as in the Murashige–Skoog medium.

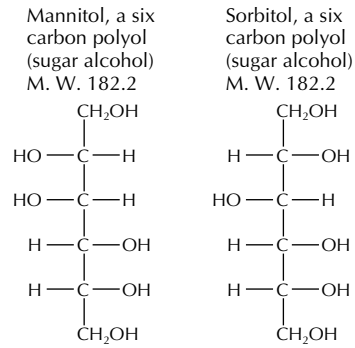
[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures). The most commonly used amount of coconut water is 150 ml l^{-1}.

[g]Add items 1–13 to 750 ml of distilled water (item 15), adjust pH to 5.6 ± 0.1, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Gelrite or gellan gum (available as such from www.caissonlabs.com and as Phytagel (from www.sigmaaldrich.com) is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Phytagel (gellan gum, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gun (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave. Following the autoclaving use a sterile pipette to add the auxin to the medium while it is still liquid and warm, but no longer hot. Swirl or stir the medium with a sterile stirrer to mix the auxin thoroughly and pour the medium into presterilized culture vessels. Solidifier is not added to liquid media. As a rule amino acids (item 8), hormones (none present in this medium), and vitamins (items 10–12) are added to the hot solution under sterile conditions with sterilized pipettes, mixed well, and the medium is then distributed into preautoclaved culture vessels. In this instance all components are added before the medium is autoclaved.



Equal and unequal concentrations

- 20 g disaccharide ≠ 20 g monosaccharide ≠ 20 g trisaccharide ≠ 20 g 6-carbon polyol in terms of molarity and osmolarity, nor physiologically.
- 20 g disaccharide = 10 g monosaccharide = 30 g trisaccharide = 10 g 6-carbon polyol in terms of molarity and osmolarity, and physiologically.



the mononsaccharides and disaccharides with relative ease even if at different rates. But the trisaccharides or longer sugars would be more difficult to break down and/or absorb, which means that the orchids may not have enough sugar.

- "Callus growth on a medium containing glucose and/or fructose was better than sucrose when both glucose and/or fructose were added at 10 and 20 g l^{-1}."
- "Although growth rate among varieties different [differed] to some extent callus of most varieties grew best on media containing mannitol, sorbitol, maltose, and trehalose. . . . Growth was better on media supplemented with rhamnose, fructose, or glucose as energy sources. Lactose and sucrose also favored callus growth in many varieties . . . .Galactose was not a good energy source for callus. . . . Sucrose is the most commonly used energy source for nutrient media . . . for plant tissue culture *in vitro*." But it is not always effective.
- "Callus growth on sucrose supplemented medium remained friable and yellow or pale green . . ."
- ". . . in sugar free medium and/or sorbitol or mannitol containing medium callus changed color into green and developed somatic embryos."
- "The callus can utilize many kinds of sugar and/or sugar alcohols as sources of carbohydrates. Maltose, sorbitol, mannitol, or trehalose supported callus growth better that other energy sources tested. Rhamnose, fructose, or glucose were utilized well by calli." However, there were differences in preference for energy sources by different species.
- Galactose was inhibitory to callus growth.

Solidifiers

Agar is an algal product that has been used for a very long time to solidify culture media. Gelrite (Phytagel, gellan gum) is an extracellular polysaccharide produced by *Pseudomonas elodea*, which came into use as a media solidifier in the 1980s. The effects of solidifier concentrations on callus growth were "insignificant" after 4 weeks of culture. However, after 8 and 12 weeks callus growth was "significantly" better in a medium that was solidified with 4 g gellan gum l^{-1}. Gellan gum was better than agar as a solidifier for the P medium (Table Phal-52). Similar effects were observed with other media and orchids (Huang, 1988; Ichihashi, 1992*a*).

Coconut Water

Several levels of coconut water are used in micropropagation culture media with 150 ml l^{-1} being the most common. However, in this case 100 ml l^{-1} "favored callus growth" (Islam et al., 1997).The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). Water from unripe (green) nuts is preferable. If unripe nuts are not available, water from ripe (brown) ones can be used. It is advisable to filter the water. Canned or commercially frozen coconut water must not be used because it may contain sugars and/or preservatives.

In Vitro Propagation of *Phalaenopsis* via the Culture of Cytokinin-induced Nodes

Every micropropagation method for *Phalaenopsis* has advantages and disadvantages. A method using cytokinin-induced nodes was developed to overcome some shortcomings (Duan et al., 1996).

Plant Material. One-year-old seedlings or plantlets should be treated with BA to bring about stem elongation.

Surface Sterilization. Seedlings growing in vitro do not require surface sterilization. They should be washed with sterile distilled water to remove residual medium.

Culture Vessels. Erlenmeyer flasks, 200- or 250-ml capacity containing 70 or 80 ml medium, respectively, should be used for elongation. Culture tubes, 25×150 mm, containing 20 ml medium are suitable for culturing stem sections. Other containers can also be used.

Culture Conditions. The original cultures were maintained at $25 \pm 2°C$ under 16-h photoperiods of 2500 lx provided by fluorescent lamps (type not described). Standard culture room conditions are also suitable.

Culture Media. The elongation of seedlings or plantlets and the production of adventitious buds occur on Hyponex medium containing 5–10 mg BA l^{-1} (Table Phal-53). For maximal production of shoots, node sections should be cultured on different BA levels (Table Phal-54):

- Basal nodes, no BA in medium.
- Second nodes, 10 mg BA l^{-1}.
- Third nodes, 5 mg BA l^{-1}.
- Top nodes, 0–10 mg BA l^{-1}. Only 10% of these nodes produce shoots on any BA level (0, 5, 10, or 20 mg BA l^{-1}). They may not be worth culturing or should be cultured only if there is a shortage of plant material.

Shoots produce roots on a BA-free Hyponex medium supplemented with 2 g peptone l^{-1} (Table Phal-55). Rooted plants should be potted in sphagnum moss.

TABLE PHAL-53. **Benzyladenine (BA)-containing Hyponex medium for elongation of *Phalaenopsis* seedlings or plantlets (Duan et al., 1996)**

Component	Amount l^{-1}	Remarks
Hyponex[a]	3.5 g	Add all components to 900 ml of water. Adjust pH to 5.6, raise volume to 1000 ml, bring to a gentle boil, add agar with stirring and keep stirring until it is dissolved, pour into culture vessels, and autoclave
Benzyladenine (BA)[b]	5 or 10 mg	
Sucrose	25.0 g	
Water	To 1000 ml	
Agar	10.0	

[a]One possible source for Hyponex is www.hyponex.co.jp.

[b]To prepare a stock solution, dissolve 500 mg benzyladenine (BA) in 100 ml 95% ethanol. If it fails to dissolve add a few drops of 0.1 N HCl or NaOH or KOH. Add 1 ml per liter of medium for 5 mg BA l^{-1} or 2 ml for 10 mg BA l^{-1}. Keep frozen between uses.

TABLE PHAL-54. **Hyponex medium for the culture of stem sections of *Phalaenopsis* seedling or plantlet-stem sections (Duan et al., 1996)**

Component	Amount l^{-1}	Comments
Hyponex[a]	3.5 g	Add all components to 700 ml of water. Adjust pH to 5.6, raise volume to 1000 ml, bring to a gentle boil, add agar with stirring and keep stirring until it is dissolved, pour into culture vessels, and autoclave
Benzyladenine (BA)[b]	5 or 10 mg	
Sucrose	25.0 g	
Water	To 1000 ml	
Agar	10.0	

[a]One possible source for Hyponex is www.hyponex.co.jp.

[b]To prepare a stock solution dissolve 500 mg benzyladenine (BA) in 100 ml 95% ethanol. If it fails to dissolve add a few drops of 0.1 N HCl or NaOH or KOH. Add 1 ml per liter of medium for 5 mg BA l^{-1} or 2 ml for 10 mg BA l^{-1}. Keep frozen between uses. Basal or top sections should be cultured on BA-free medium. For second sections add 5 mg BA l^{-1} (1 ml of stock solution). Third sections should be cultured on medium containing 10 mg BA l^{-1} (2 ml stock solution).

TABLE PHAL-55. **Hyponex medium for root induction on shoots derived from stem sections of *Phalaenopsis* seedlings or plantlets (Duan et al., 1996)**

Component	Amount l^{-1}	Comments
Hyponex[a]	3.5 g	Add all components to 900 ml of water. Adjust pH to 5.6, raise volume to 1000 ml, bring to a gentle boil, add agar with stirring and keep stirring until it is dissolved, pour into culture vessels, and autoclave
Peptone	2.0 mg	
Sucrose	25.0 g	
Water	To 1000 ml	
Agar	10.0	

[a]One possible source for Hyponex is www.hyponex.co.jp.

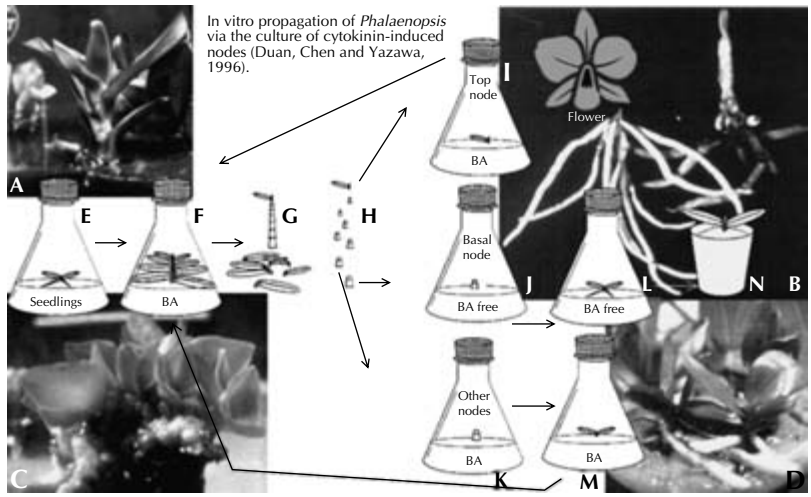


FIG. PHAL-23. **In vitro propagation of *Phalaenopsis*. A. Seedling with an elongated stem on Hyponex medium containing 5 mg BA l^{-1} after 90 days of culture. B. Defoliated seedling on BA-free medium after 90 days of culture. C. Formation of shoots and multiple adventitious buds on a second node-stem section on Hyponex medium with 5 mg BA l^{-1} after 70 days of culture. D. Rooted plantlets on BA-free Hyponex medium. E–N. Micropropagation procedure. (Duan et al., 1996.)**

Procedure. Seedlings (Fig. Phal-23A, E) or plantlets are grown on a medium which contains 5–10 mg BA l^{-1} for 90–135 days until they elongate (Fig. Phal-19F) and are defoliated (Fig. Phal-19B, G). The elongated stems (about 1 cm in length) are cut into four sections: top (including the apex), second, third, and basal sections (Fig. Phal-19H–K). When plantlets are produced they should be moved to the peptone-containing, BA-free medium for rooting (Fig. Phal-19C, L, M). Rooted plants are potted.

Developmental Sequence. Plantlets and/or seedlings elongate on the first medium (Table Phal-53). Sections produce shoots on the second medium (Table Phal-54). The shoots develop roots on the third medium (Table Phal-55).

General Comments. This is a clever and efficient procedure whose major drawback could be the high levels of BA in the first medium. Such high levels of any hormone may induce undesirable mutations. Therefore this method should be approached with caution until the effects of the high BA levels become known.

Propagation of *Phalaenopsis* Protocorm-like Bodies in Liquid Medium

The growth and multiplication of PLBs have been described as two rate-limiting steps in the mass, rapid, clonal propagation of *Phalaenopsis*. A liquid culture method that increases PLB multiplication and development was formulated at Shizuoka University in Japan (Park et al., 1996).

Plant Material. The paper which describes the procedure is entitled "Efficient propagation of protocorm-like bodies of *Phalaenopsis* in liquid medium" (Park et al., 1996). Despite this "*Doritaenopsis* (*Doritaenopsis* × *Phalaenopsis* cultivar) was used to obtain PLB's." One-node flower-stalk sections were cultured in MS agar medium until the shoots which developed from the nodes formed 1–3 leaves. Segments taken from the apical, median, and basal areas of the fully expanded uppermost leaves were also cultured on MS agar medium at 25°C under 16-h photoperiods of 6.25–7.5 μmol m^{-2} s^{-1} (sources not described). Presumably PLBs obtained from these sections were used to develop the procedure.

Surface Sterilization. PLBs from cultures in vitro do not require surface sterilization.

Culture Vessels. Erlenmeyer flasks, 200-ml capacity containing 100 ml medium, were used "to investigate the effects of the liquid media on the multiplication ratio of PLB." Also:

> . . . to investigate the propagation of PLB in bioreactors, several reactors, a turbine blade reactor (TBR, Sakura Co. Ltd., Tokyo), bubble column of 45 mm in diameter and 500 mm in length, and cotton plate design were used. The TBR had a working volume of 1 liter . . . rates of agitation and aeration were 200 rpm and 0.5 vvm (volume of gas per volume of liquid per minute) as a low oxygen supply and the other 300 rpm and and 1.5 vvm as a high oxygen supply . . . the minumum agitation . . . was kept at 200 rpm . . . bubble column with a working volume was operated at two kinds of cultural conditions: the aeration rate was 0.17 vvm as a low oxygen supply, and 1.67 vvm as a high oxygen supply. (Park et al., 1996)

A cotton plate bioreactor (Fig. Phal-24) consisted of a:

> . . . small Petri dish . . . 85 mm in diameter . . . placed upside down on a plate [actually in the drawing this looks like a larger Petri dish] 120 mm in diameter. Two kinds of cotton were used: One was non-adsorbent cotton which had uneven surface; the other, adsorbent (500G, Nishio Eizai Co Ltd., Nagoya) with an even surface. The cotton pad . . . 140 mm in diameter was laid on the small plate as a support for PLBs. These cotton plates were soaked or submerged [in] liquid medium during the culture period. Fifteen excised PLBs were placed on each cotton plate. . . . The

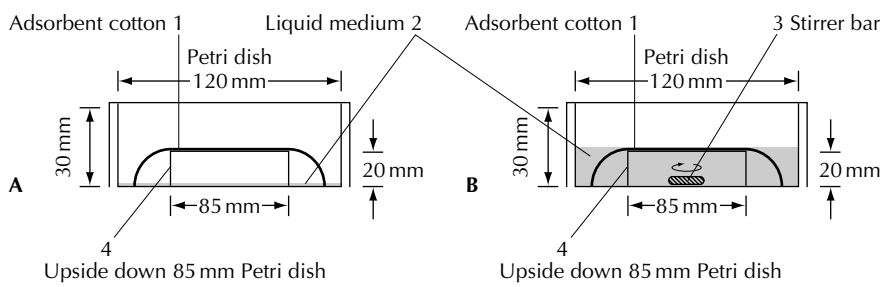


FIG. PHAL-24. **Cotton plate reactors. A. Reactor with cotton plate only. The cotton plate was soaked with medium. B. Reactor with cotton plate and stirrer bar. The cotton plate was submerged in the medium. (Park et al., 1996.)**

> thickness of the adsorbent cotton was varied from 1 to 5 mm, as was the culture [medium] volume increasing [it] to 150 ml under which conditions the PLBs were submerged. (Park et al., 1996)

Culture Conditions. Cultures should be maintained at 25°C under 16-h photoperiods of 6.25–7.50 μmol m^{-2} s^{-1}. Standard laboratory conditions are also suitable. Liquid cultures should be placed on a rotary shaker at 6 rpm.

Culture Medium. Liquid MS medium (Murashige and Skoog, 1962) containing 20% coconut water (Table Phal-56) should be used to wet the cotton layers.

Procedure. PLBs of 5–6 mm are sectioned to obtain sections 1–2 mm in size. These are placed on the 6-mm-thick cotton layers wetted with 7.2 ml g^{-1} cotton. The medium should not be stirred.

Developmental Sequence. The PLBs multiply on the cotton.

General Comments. PLBs multiply when this procedure is used, but they also increase in number when simpler methods are employed. This rather complicated and probably expensive (due to the equipment it requires) protocol does not seem to offer enough or even any advantages to warrant its use. It is presented here only because it was published.

***Phalaenopsis amabilis* from Illustrations of Orchidaceous Plants by Thomas Moore (London, 1857)**

TABLE PHAL-56. **Vacin and Went (VW) medium (Vacin and Went, 1949) for proliferation of protocorm-like bodies in a reactor (Park et al., 1996)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additive				
8	Coconut water (CW)[e]	200.0 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 2), adjust pH to 5.2–5.4, add sugar (item 9), and raise volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

Propagation of *Phalaenopsis* through the Culture of Node Sections from Inflorescences following Senescence of the Flowers

If inflorescences are taken for micropropagation before the flower buds open, or while flowers are at their prime, the decorative and horticultural value of the donor plant will be lost. This can be avoided if flower stalks are taken after the flowers senesce and abscise (Jiménez and Guevara, 1996).

Plant Material. Inflorescence axes should be taken after senescence of the flowers. Apical buds, as well as median nodes below the flowers and basal ones, can all be used.

Surface Sterilization. The stems should first be washed with tap water and a mild detergent (this step is not listed in the original paper, but is advisable), wiped with cheese cloth (or gauze) wetted with 95% ethanol, and then cut into sections which have 2 cm of stem above and below the node. These sections should be soaked in a 1 : 10 dilution (or 10%) of a household bleach (9 or 10 ml, respectively, of bleach such as Clorox which contains 6 or 5.25% sodium hypochlorite and diluted to 100 ml with distilled water with a drop of Tween 80 or mild household detergent; this is 0.53% sodium hypochlorite) for 15 min and then rinsed with sterile distilled water (the rinse is not included in the original paper, but is advisable nevertheless). All steps after this should be carried out under sterile conditions. The bracts which cover the buds should be removed after that; the sections should be immersed with agitation in 5% (0.263 sodium hypochlorite) household bleach (5 ml diluted to 100 ml with distilled water with one drop of Tween 80 or a mild household detergent) for 10 min and washed three times with sterile distilled water. The stem on each side of the bud should be trimmed after the last wash so that only 1.0–1.5 cm remain above and below the bud. The cut on the basal end should be diagonal to facilitate insertion into the medium.

Culture Vessels. Culture tubes, 18 × 150 mm containing 15 ml of medium, covered with aluminum foil (cotton buns or plastic caps can also be used), were used in the original research for initial cultures. Other containers are also suitable. Baby food jars containing 25 ml medium were used to culture plantlets or shoots that form in the test tubes. Erlenmeyer flasks or other containers filled to 20–30% of their capacity with medium can be used instead of the jars.

Culture Conditions. The research cultures were maintained at 26–30°C under 24-h photoperiods of 2600 lx (source not described). Standard culture room conditions are also suitable.

Culture Media. A substantially modified MS medium (Murashige and Skoog, 1962) should be used initially (Table Phal-57) to establish the cultures. Shoots which fail to form roots and/or do not develop well, must be moved to another modification of MS (Table Phal-58). Plantlets can be potted in vermiculite in covered plastic containers for a week. The covers should be removed for part of the time in the second week.

TABLE PHAL-57. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of node sections from flower stalks after the flowers have senesced (Jiménez and Guevara, 1996)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	2.9	0.29 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinins				
11	Benzylaminopurine (BAP)	3.0	300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
12	Kinetin	0.86	86 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Purine				
13	Adenine	5.0	500 mg 100 ml^{-1} 95% ethanol[e]	1	
	Vitamins				
14	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
16	Thiamine (vitamin B_1)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar alcohol				
17	Manitol	10.0 g	No stock	No stock	Weigh
	Complex additive				
18	Coconut water (CW)	200.0 ml	No stock	No stock	Measure
	Sugars				
19	Fructose	10.0 g	No stock	No stock	Weigh
20	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
21	Water, distilled[g]	To 1000 ml			
	Solidifier				
22	Agar[g]	8.0 g	No stock	No stock	Weigh
	Darkening agent				
23	Activated charcoal[h]	0.5 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin, or cytokinins or purine do not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–18 to 700 ml of distilled water (item 21), adjust pH to 5.2, add the sugars (items 19 and 20), and raise volume to 1000 ml with distilled water (item 21). Bring the solution to a gentle boil and add the agar (item 22) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved add the charcoal (item 23) with constant stirring to disperse it completely and dispense the solution into culture vessels. As a rule auxins, cytokinins, purines, and vitamins should be added after autoclaving, but in this case the original report indicates that they were added before the medium was autoclaved. If baby food jars are used as culture vessels the caps should be put on loosely to prevent possible explosions in the autoclave. Or, a hole should be drilled in each cap and stuffed with cotton. Agar is not added to liquid media.

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

TABLE PHAL-58. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the rooting of shoots derived from sections from flower stalks after the flowers have senesced (Jiménez and Guevara, 1996)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol[f]	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[g]	To 1000 ml			
	Solidifier				
15	Agar[g]	8.0 g	No stock	No stock	Weigh
	Darkening agent				
16	Activated charcoal	500.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]Inositol and *meso*-inositol are other names for this substance.

[g]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH to 5.5, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, add the activated charcoal (item 16) with vigorous stirring of the solution to mix it wel, distribute the medium to culture vessels, and autoclave.

[h]Only activated pure vegetable charcoal should be used. Two possible sources are www.sigmaaldrich.com and http://search.wako-chem.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances.

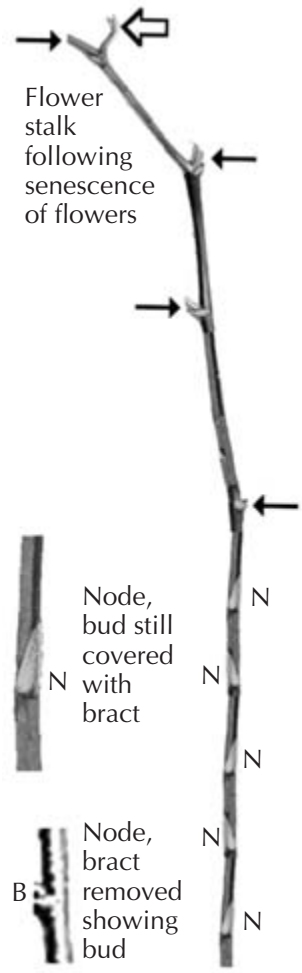


FIG. PHAL-25. **Flower stalk of *Phalaenopsis* following senescence of flowers and excised nodes before (middle left) and after (bottom left) the removal of the bract which covers the bud. Solid arrow, remnant of flower; open arrow, shoot tip; B, bud; N, node. (Jiménez and Guevara, 1996.)**

Procedure. Inflorescence axes taken after senescence of the flowers should be wiped with gauze (or cotton) dipped quickly in 95% ethanol, and then cut into sections with 2.0 cm of stalk above and below the node, surface-sterilized, and trimmed leaving 1.0–1.5 cm of stem on each side of the bud (Fig. Phal-25). The cuts below the buds should be slanted. Apical buds, as well as median nodes below the flowers and basal ones, can be used. The sections are inserted into the first medium with the bud touching the agar (Table Phal-57) following surface sterilization. Those that become contaminated can be removed from the culture vessels, resterilized, and inserted in fresh medium. Sections which form shoots or plantlets are moved to the same medium in baby food jars or other containers. Shoots which fail to develop roots or do not grow well are moved to the second medium (Table Phal-58).

Developmental Sequence. Buds on sections from the basal or apical portions of the stalk may fail to grow. Sections from the middle part of the stalk will mostly produce vegetative shoots. A few will produce floral axes. When excised and cultured, these floral axes produce vegetative shoots. Plantlets form following the transfer of shoots to fresh first medium (Table Phal-57) or the second medium (Table Phal-58). They can be transferred to a greenhouse after 4–6 months of culture.

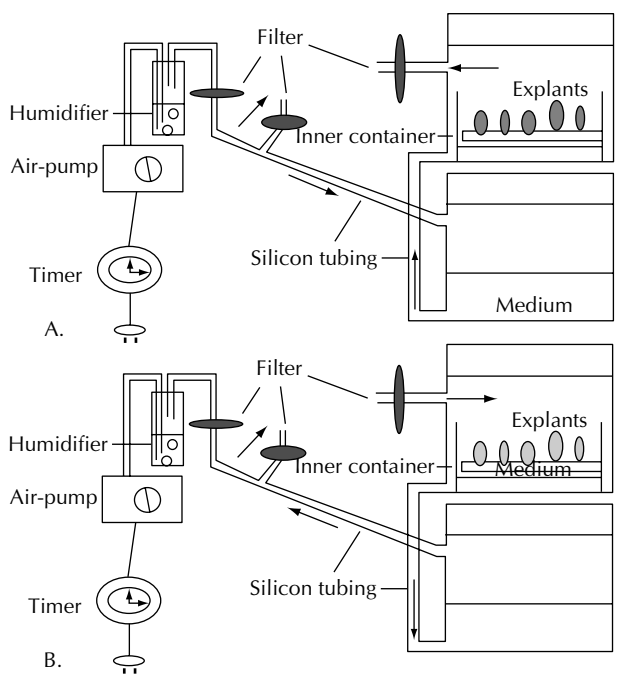
General Comments. The use of inflorescences after flowers have senesced as the explant source is ingenious. Shoots produced can be excised and cultured. Roots usually appear after leaf formation. If roots do not form the shoots should be cultured on medium devoid of hormones to induce rooting.

Differences in the Formation of Shoots and Protocorm-like Bodies from Axillary Buds and Shoot Tips of *Phalaenopsis*

See the procedure for *Doritaenopsis* (see p. 686, Vol. I; Yamazaki et al., 1997).

Abnormal Protocorm-like Bodies and Plantlets of *Doritaenopsis* and *Phalaenopsis*

Abnormal PLBs and plantlets are produced by cultures of *Doritaenopsis* and *Phalaenopsis*. The culture period and types of clones used are among the factors associated with this phenomenon (Park and Paek, 1999).



Operation mode of API bioreactor. Arrows represent the airflow or culture medium movement. A. Transfer of medium from the reservoir to the culture container. B. Transport of medium from the culture container to the reservoir.

Mass Propagation and Plantlet Production of *Phalaenopsis* Protocorm-like Bodies in a Bioreactor

Standard micropropagation protocols produce a relatively small number of plants, which means that multiplication of these plants is somewhat slower than may be required by marketing needs. To overcome this problem, a bioreactor-based, mass, rapid propagation system was developed at Chungbuk National University in Korea (Park et al., 2000). Since this method is different from standard micropropagation procedures, presentation of the procedure here will not be in the format used for other protocols.

Plant Material. Flower-stalk sections, 2 cm long, sterilized for 10 min in 0.2% mercuric chloride, and washed with sterile distilled water were cultured in solid MS medium (Murashige and Skoog, 1962) containing 45 g sucrose l^{-1} and 3 mg BA l^{-1}. Leaves produced by these explants were cut into sections, 5×10 mm, which were cultured on MS with 45 g sucrose l^{-1}, 1 mg NAA l^{-1}, and 15 mg BA l^{-1} (this is a high concentration which can cause undesirable mutations). PLBs produced by these explants after 8 weeks were cut transversely, and sections, ca. 2 mm in size, bearing apical meristems were used as explants for proliferation. There is no information in the original paper about the culture vessels and conditions that were used in these steps of the procedure.

PLB Proliferation in Flasks. Approximately 25 sections of PLBs (500 mg), cultured in 250-ml Erlenmeyer flasks containing 100 ml Hyponex medium (Kano, 1965) supplemented with 1% (probably weight/volume) potato homogenate (Table Phal-59), produced 10 PLBs per section after 8 weeks of culture. The Hyponex medium used here is described as "6.5N – 4.5P – 19K 1 g l^{-1} + 20N – 20P – 20K 1 g l^{-1}." This suggests that there are at least two different formulations of Hyponex and that 1 g of each should be used per liter. This water-soluble fertilizer is hard to find in the USA. It is available from www.hyponex.co.jp. Those who intend to use this procedure should purchase two formulations: 6.5N–4.5P–19K and 20N–20P–20K. No information is given on how to prepare the potato homogenate. One way would be to homogenize the required amount by putting cubed potatoes in a liquid medium during initial preparation. The culture conditions are 25 ± 2°C under white fluorescent lights of 60 μmol m^{-2} s^{-1} intensity. Photoperiods were probably 24 h long. Flasks must be placed on a horizontal gyrotory shaker at 100 rpm.

TABLE PHAL-59. **Hyponex medium for the proliferation of protocorm-like bodies (Park et al., 2000)**

Component	Amount l^{-1}	Comments
Hyponex[a]	3.5 g	Add all components to 900 ml of water. Adjust pH to 5.6, raise volume to 1000 ml, bring to a gentle boil, add agar with stirring and keep stirring until it is dissolved, pour into culture vessels, and autoclave
Potato homogenate	10.0 g	
Sucrose	25.0 g	
Water	To 1000 ml	
Agar	10.0 g	

[a]One possible source for Hyponex is www.hyponex.co.jp.

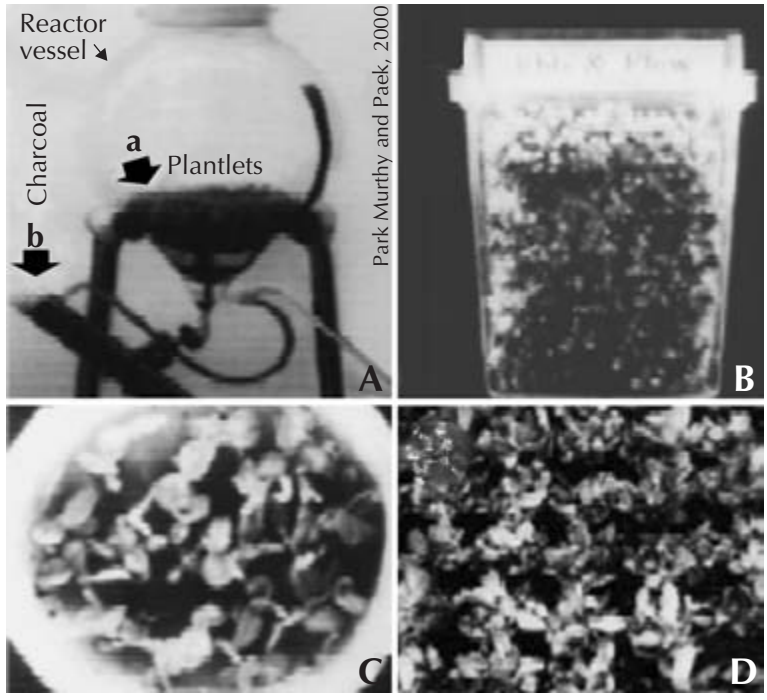


FIG. PHAL-26. **A. PLBs and plantlets proliferating in a temporary immersion bioreactor. B. PLBs harvested from the bioreactor. C. Plantlet production from PLBs on Hyponex medium. D. Acclimatized plantlets. (Park et al., 2000.)**

PLB Proliferation in a Bioreactor. Approximately 500 sections of PLBs (10 g) cultured in temporary immersion cultures, produced 13 PLBs per section after 8 weeks of culture in the same medium (Table Phal-59) and conditions as above. The sections should be placed on a plastic net inside the reactor vessel (Fig. Phal-26A). The system should be designed to immerse the sections in medium for 5 min, followed by 2 h without solution. PLBs harvested after 8 weeks (Fig. Phal-26B) should be cultured (Fig. Phal-26C) on Hyponex medium with 45 g sucrose l^{-1} and 0.5% activated charcoal (www.sigmaaldrich.com) l^{-1} (Table Phal-60) in polystyrene vessels, 115 × 50 mm (Tong Yang Moolsan C., Seoul, Korea; other containers are also suitable) at 25 ± 2°C under 16-h photoperiods of 60 μmol m^{-2} s^{-1} (light sources not described).

TABLE PHAL-60. **Hyponex medium for the proliferation of protocorm-like bodies (Park et al., 2000)**

Component	Amount l^{-1}	Comments
Hyponex[a]	3.5 g	Add all components to 900 ml of water. Adjust pH to 5.6, raise volume to 1000 ml, bring to a gentle boil, add agar with stirring and keep stirring until it is dissolved, pour into culture vessels and autoclave.
Potato homogenate	10.0 g	
Sucrose	45.0 g	
Water	To 1000 ml	
Agar	10.0	
Activated charcoal	5.0	

[a]One possible source for Hyponex is www.hyponex.co.jp.

Standard laboratory conditions are also suitable. The plantlets should be subcultured every 4 weeks. When they reach a height of 3–4 cm, the plantlets should be potted (Fig. Phal-26D) in a mix consisting of peat moss and perlite (in a ratio of 1 : 1) and grown in a greenhouse under 60–70% relative humidity, at 25°C during 16-h days of 500 μmol m^{-2} s^{-1} and at 15°C at night.

General Comments. This bioreactor-based protocol makes possible large-scale rapid micropropagation. If a bioreactor is not available, a relatively large number of PLBs can be proliferated in flasks under agitation. An unanswered question is whether this procedure might induce undesirable mutations.

Plant Production from Callus Culture of *Phalaenopsis*

This procedure is not micropropagation in the strictest sense because seedlings are induced to form callus, which serves as a source for PLBs that produce plantlets (Y.-C. Chen et al., 2000). The protocol is presented here because it can also be used to propagate plantlets which are produced clonally.

Plant Material. Seeds of self-pollinated *Phalaenopsis* Nebula, harvested 120 days after pollination, were germinated on quarter-strength MS salts (Murashige and Skoog, 1962) supplemented with 100 mg *myo*-inositol l^{-1}, 50 g banana pulp l^{-1}, 1 mg peptone l^{-1}, and 2 g charcoal l^{-1}, and solidified with 3 g Gelrite l^{-1} at 25°C under 16-h photoperiods of 10 μmol m^{-2} s^{-1} provided by daylight fluorescent tubes (FL-30D/29, 40W, China Electric Co., Taipei). Protocorms, 1–2 months old, produced by these seeds were used for callus induction. Other protocorms or PLBs obtained from culture of explants can also be used.

Surface Sterilization. Protocorms or PLBs from cultures in vitro do not require surface sterilization.

Culture Vessels. Culture tubes, Erlenmeyer flasks, and other containers can be used.

Culture Conditions. During the callus induction phase, cultures should be maintained at 26 ± 2°C in the dark. The culture of PLBs, as well as plantlet induction and maintenance, should be under 16-h photoperiods of 28–36 μmol m^{-2} s^{-1}, presumably provided by the same light source as above.

Culture Media. To induce callus, 2-month-old PLBs or seed-derived protocorms should be cultured on half-strength MS medium (Murashige and Skoog, 1962) supplemented with TDZ and peptone (Table Phal-61). The callus can be maintained on half-strength MS with peptone, 2,4-D, and TDZ (Table Phal-62). For PLB and plantlet production, callus should be cultured on hormone-free half-strength MS (Table Phal-63).

Procedure. Seeds are germinated, and when protocorms are formed they are moved to the first medium (Table Phal-61) for callus initiation. Callus is maintained on the second medium (Table Phal-62). Sections of callus are moved to the third

TABLE PHAL-61. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for callus induction from protocorms or protocorm-like bodies of *Phalaenopsis* (Y.-C. Chen et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Complex additive				
11	Peptone	1.0 g	No stock	No stock	Weigh
	Cytokinin				
12	Thidiazuron (TDZ)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N KOH.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.2, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Add the Gelrite (item 18) according to the instructions in footnote *h*. When the Gelrite (item 18) is completely dissolved, pour the solution into culture vessels and autoclave. Gelrite is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved, pour the medium into culture vessels and autoclave.

TABLE PHAL-62. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the maintenance of callus produced by protocorms of *Phalaenopsis* (Y.-C. Chen et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Complex additive				
11	Peptone	1.0 g	No stock	No stock	Weigh
	Auxin				
12	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
13	Thidiazuron (TDZ)	0.5	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
14	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
16	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Gelrite[g,h]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the cytokinin or auxin does not dissolve, add a few drops of 0.1 N HCl or KOH, respectively.

[g]Add items 1–16 to 900 ml of distilled water (item 18), adjust pH to 5.2, add sugar (item 17), and raise volume to 1000 ml with distilled water (item 18). Add the Gelrite (item 19) according to the instructions in footnote *h*. When the Gelrite (item 19) is completely dissolved, pour the solution into culture vessels and autoclave. Gelrite is not added to liquid media.

[h]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved, pour the medium into culture vessels and autoclave.

TABLE PHAL-63. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the induction and culture of protocorm-like bodies and plantlets from callus produced by protocorms or PLBs of *Phalaenopsis* (Y.-C. Chen et al., 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	Monosodium phosphate, NaH_2PO_4	170.0	17.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
9	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Complex additive				
11	Peptone	1.0 g	No stock	No stock	Weigh
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[e]	To 1000 ml			
	Solidifier				
17	Gelrite[e,f]	2.2 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Add items 1–16 to 900 ml of distilled water (item 16), adjust pH to 5.2, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Add the Gelrite (item 17) according to the instructions in footnote *f*. When the Gelrite (item 17) is completely dissolved, pour the solution into culture vessels and autoclave. Gelrite is not added to liquid media.

[f]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved, pour the medium into culture vessels and autoclave.

solution (Table Phal-63) for PLB and plantlet production. Plantlets are potted in sphagnum.

Developmental Sequence. Seedling protocorms produce callus on the TDZ-containing solution (Table Phal-61). The callus does not differentiate on the substrate that contains TDZ and 2,4-D (Table Phal-62). PLBs and plantlets form on the hormone-free medium (Table Phal-63).

General Comments. Since the quality of seedlings is not known, there is no point or need to propagate them clonally. However, this method can probably be used to propagate PLBs obtained through the culture of explants from mature plants of known quality.

Micropropagation of *Phalaenopsis* via the Culture of Leaf Tissue

Some red *Phalaenopsis* hybrids are reported to be difficult to propagate. A method using leaf tissues was developed to overcome these difficulties (Myint et al., 2001).

Plant Material. Fully expanded leaves, 5–8 × 2.5–3.5 cm in size, of red *Phalaenopsis* seedlings growing in pots were used in the original research. The leaves must be surface-sterilized before sections 0.5 m^2 in size can be cultured.

Surface Sterilization. The leaves should be washed with running water and a mild detergent, rinsed with distilled water, wiped with gauze or cotton wetted with 70% ethanol (73–74 ml 95% ethanol diluted to 100 ml with distilled water), immersed in 1% sodium hypochlorite (17 or 19 ml of a household detergent which contains 5.25 or 6% sodium hypochlorite, respectively, diluted to 100 ml with distilled water) for 15 min, and rinsed with sterile distilled water 3–4 times.

Culture Vessels. To induce PLBs, leaf sections should be cultured on a cotton platform, with 5 cm^2 surface and 1 cm thick, placed in a 100-ml Erlenmeyer flask and wetted with 30 ml liquid medium. For propagation, PLBs produced by the leaf sections should be cultured on 6 ml of solid medium in 15 × 180 cm culture tubes. Erlenmeyer flasks, 100-ml capacity containing 20 ml medium, can also be used.

Culture Conditions. Leaf sections should be maintained at 25 ± 1°C, in the dark for the first 2 weeks, and after that under 16-h photoperiods of 27 μmol m^{-2} s^{-1} provided by fluorescent lamps. The original paper does not describe the culture conditions for the propagation of PLBs. However, it is reasonable to assume that the conditions used under illumination for leaf sections and production of plantlets will be suitable. For plantlet regeneration, PLB sections should be maintained at 25 ± 1°C under 16-h photoperiods of 27 μmol m^{-2} s^{-1}. Standard culture room conditions will probably be suitable for cultures under illumination.

Culture Media. For PLB formation, leaf sections should be cultured in liquid modified Vacin and Went medium (Vacin and Went, 1949) on a cotton support

TABLE PHAL-64. **Modified Vacin and Went (VW) medium (Vacin and Went, 1949) for the induction of protocorm-like bodies on *Phalaenopsis* seedling leaf explants (Myint et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	10.0	1 g 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	Benzyladenine (BA)	10.0	1 g 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 11) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[f]Keep frozen between uses.

[g]Add items 1 and 3–9 to the 500–700 ml of distilled water (item 11) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 11), adjust pH to 5.2–5.4, add sugar (item 10), raise volume to 1000 ml with distilled water (item 11), pour the solution into culture vessels which have been lined with cotton on the inside, and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

containing relatively high levels of NAA and BA (Table Phal-64). PLBs proliferate on agar-solidified Vacin and Went medium containing lower levels of the same hormones (Table Phal-65). To bring about shoot formation, PLB sections should be cultured on cotton in liquid new Dogashima (ND) medium (Tokuhara and Mii, 1993) also supplemented with BA and NAA (Table Phal-66). Shoots and plantlets should be cultured on cotton platforms in liquid Hyponex medium containing the same additives as the ND medium (Table Phal-67).

Procedure. The leaves are sectioned under sterile conditions after surface sterilization. Sections of 0.5 cm^2 are cultured on the first medium (Table Phal-64), abaxial surface in contact with the medium, first in the dark for 2 weeks and after that under illumination (16-h photoperiods of 27 $\mu mol\ m^{-2}\ s^{-1}$). PLBs produced on this medium

TABLE PHAL-65. **Modified Vacin and Went (VW) medium (Vacin and Went, 1949) for the proliferation of protocorm-like bodies produced by *Phalaenopsis* seedling leaf explants (Myint et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	Benzyladenine (BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 11) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[f]Keep frozen between uses.

[g]Add items 1 and 3–9 to the 500–700 ml of distilled water (item 11) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 11), adjust pH to 5.2–5.4, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

are proliferated on the second medium (Table Phal-65). When PLBs reach a diameter of 5–6 mm they are sectioned "into 3–4 pieces [3–4 mm in size] either transversely or dorsally." The sections are cultured on the third medium (Table Phal-66). Shoots which form on this medium are grown on the fourth solution (Table Phal-67).

Developmental Sequence. The sections produce PLBs on the first medium (Table Phal-64). These PLBs proliferate on the second solution (Table Phal-65). PLB sections produce shoots and plantlets on ND medium (Table Phal-66). Plantlets and shoots grow further on Hyponex medium (Table Phal-67).

TABLE PHAL-66. **Modified new Dogashima (ND) medium (Tokuhara and Mii, 1993) for shoot formation by *Phalaenopsis* protocorm-like bodies produced by seedling leaves (Myint et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	480.0	48.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	470.0	47.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	550.0	55.0 g l^{-1}	10	
5	Potassium chloride, KCl	150.0	15.9 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	200.0	20.0 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.5	50.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	3.0	300.0 mg l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}		
(f)	Sulfuric acid, concentrated	0.5 µl	0.5 ml		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	50.0 mg l^{-1}		
	Amino acid				
9	*l*-cysteine	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
10	*myo*-inositol	100.0	No stock	No stock	Weigh
	Purine				
11	Adenine	1.0	100 mg in 100 ml acidified 70% ethanol[e,f]	1	
	Auxin				
12	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,g]	1	
	Cytokinin				
13	6-Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,g]	1	
	Vitamins				
14	Biotin	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Calcium pantothenate	1.0	100 mg 100 ml^{-1} 70% ethanol[e,h]	1	
16	Niacin (nicotinic acid)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
17	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
18	Thiamine (vitamin B_1)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
19	Potato homogenate[i]	30.0 g	No stock	No stock	Weigh
	Sugar				
20	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
21	Water, distilled[j]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 7a) and the iron salt (item 7b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Dissolve the adenine in up to 30 ml 0.1 N hydrochloric acid and make up the solution to 100 ml with 95% ethanol.

[g]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[h]Dissolve the calcium pantothenate in 30 ml distilled water and make up the solution to 100 ml with 95% ethanol.

[i]The original paper does not contain instructions regarding the preparation of potato homogenate. One possibility is to peel a potato, cube it and place 30 of the cubes in a homogenizer (like a domestic blender), add 100 ml of distilled water and homogenize the mixture.

[j]Add items 1–19 (see footnote *i* for preparation of the potato homogenate) to 700 ml of distilled water (item 21), adjust pH to 5.2–5.4, add sugar (item 20), adjust volume to 1000 ml with distilled water (item 21), pour the solution into culture vessels which have been lined with cotton on the inside, and autoclave. The amino acid (item 9), hormones (items 12 and 13), and vitamins (items 14–18) are not heat-stable and are often added to autoclaved and still warm and liquid media with sterilized pipettes and mixed well before distribution to preautoclaved culture vessels. In this case all components of the medium are autoclaved.

TABLE PHAL-67. **Modified Hyponex/Kyoto medium (Tsukamoto et al., 1963) for the culture of *Phalaenopsis* plantlets (Myint et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Hyponex[b]	3.5 g	No stock	No stock	Weigh
	Auxin				
2	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[c,d]	1	
	Cytokinin				
3	Benzyladenine (BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[c,d]	1	
	Complex additive				
4	Potato homogenate[e]	30.0 g	No stock	No stock	
	Sugar				
5	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
6	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.
[b]Available from www.hyponex.co.jp.
[c]Keep frozen between uses.
[d]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively. This stock solution may be at the limit of solubility of BA. If a precipitate forms shake well before dispensing.
[e]The original paper does not contain instructions regarding the preparation of potato homogenate. One possibility is to peel a potato, cube it and place 30 of the cubes in a homogenizer (like a domestic blender), add 100 ml of distilled water and homogenize the mixture.
[f]Add items 1–4 to 700 ml of distilled water (item 6), adjust pH to 5.0 (Tsukamoto et al., 1963) to 5.4, add sugar (item 5), adjust volume to 1000 ml with distilled water (item 6), pour into culture vessels which have been lined with cotton, and autoclave.

General Comments. The original experiments were carried out with leaves taken from seedlings. Since the quality of seedlings is not known there is usually no reason to propagate them. Therefore, it seems reasonable to assume that the seedlings were used only as a model system. This method should also prove suitable for the culture of segments taken from leaves of plantlets produced from explants such as flower-stalk nodes, for example. The concentrations of NAA and BA (10 mg l^{-1} of each) in the first medium (Table Phal-64) are relatively high. At such levels these hormones may induce undesirable mutations.

Rapid Propagation of *Phalaenopsis* through the Culture of Leaves Produced by Flower-stalk Explants in Vitro

There is an ever-increasing demand for clonally propagated *Phalaenopsis* to meet market needs for both cut flowers and pot plants of this orchid. A method using young leaves produced by flower-stalk explants was developed in the laboratory of Professor Kee-Yoeup Paek at Chungbuk University, Cheongiu, Korea (Park et al., 2002).

Plant Material. Flower-stalk nodes, second to the fourth nodes, each with a lateral bud should be used as explants. Sections, 2 cm long, must be surface-sterilized by

immersing them for 20 min in 3% sodium hypochlorite (50 or 57 ml of household bleach containing 6.0 or 5.25% sodium hypochlorite, respectively, diluted to 100 ml with distilled water). They should be washed three times with sterile distilled water after that and placed in culture. The medium, 20 ml in 25 × 250-mm culture tubes, should be MS solution (Murashige and Skoog, 1962) containing 45 g sucrose l^{-1}, supplemented with 20.2 μmol (4.55 mt) BA l^{-1} and solidified with 7 g agar l^{-1}. Leaves which develop following 4 weeks in culture are used as explants. After removal of the first leaf, the nodes are moved to fresh medium where they develop additional leaves which are also harvested and cultured.

Surface Sterilization. Leaves taken from in vitro cultures do not require surface sterilization.

Culture Vessels. Plastic Petri dishes, 90 mm diameter and 15 mm high containing 30 ml of medium, should be used for the induction of PLBs. Erlenmeyer flasks, 100-ml capacity containing 30 ml medium, should be used for PLB proliferation. Larger flasks, 250-ml or even 500-ml capacity, containing medium equal to 25–30% of their capacity, are more suitable for plantlet production. Plants are potted in 11-cm pots.

Culture Conditions. Cultures should be maintained at 25 ± 1°C under 16-h photoperiods of 30 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes. Potted plants should be grown in a greenhouse at 30/25°C (day/night) under 16-h photoperiods of 500 μmol m^{-2} s^{-1}.

Culture Media. For PLB production, leaves should be cultured on half-strength MS medium (Table Phal-68) containing BA and NAA. PLBs proliferate and plantlets grow on Hyponex medium supplemented with potato homogenate, peptone, and charcoal (Table Phal-69). Plantlets should be potted in a mixture of peat moss and perlite (in a ratio of 1 : 1). They should be fertilized with Hyponex (6.5N : 4.5P : 19K) fertilizer (www.hyponex.co.jp).

Procedure. The young leaves are cut into 5 × 10 mm sections and cultured, 25 explants per Petri dish, with the abaxial surface in contact with the first medium (Table Phal-68). For proliferation, PLBs produced in the Petri dishes are cut in half and basal sections are cultured with the cut surface in contact with the second medium (Table Phal-69). When plantlets reach a height of 3–4 cm they are potted in peat moss in 11-cm pots, grown in a greenhouse, and fertilized with Hyponex.

Developmental Sequence. Leaf explants produce PLBs when cultured on the first medium (Table Phal-68) in Petri dishes. The PLBs proliferate and produce plantlets on the second medium (Table Phal-69).

General Comments. This procedure can accelerate micropropagation of outstanding clones without endangering the original plant. However, the high concentration of BA (88.8 μmol or 20 mg l^{-1}) may be problematic because such cytokinin levels may cause undesirable mutations.

TABLE PHAL-68. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for induction of protocorm-like bodies on explants of leaves produced by flower-stalk nodes in vitro (Park et al., 2002)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	20.0	No stock	No stock	Weigh
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.2, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. As a rule the amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) are added after a medium has been autoclaved. However, all components of this medium are added before sterilization. Agar is not added to liquid media.

TABLE PHAL-69. **Modified Hyponex medium (Tsukamoto et al., 1963) for proliferation of protocorm-like bodies and plantlet production (Park et al., 2002)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Hyponex[b]	1.0 g	No stock	No stock	Weigh
	Complex additives				
2	Potato homogenate[c]	30.0 g	No stock	No stock	
3	Peptone	2.0 g	No stock	No stock	
	Sugar				
4	Sucrose	20.0 g	No stock	No stock	Weigh
	Darkening agent				
5	Charcoal[d]				
	Solvent				
6	Water, distilled[e]	To 1000 ml			
	Solidifier				
7	Agar	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Available from www.hyponex.co.jp.

[c]The original paper does not contain instructions regarding the preparation of potato homogenate. One possibility is to peel a potato, cube it and place 30 of cubes in a homogenizer (like a domestic blender), add 100 ml of distilled water, and homogenize the mixture.

[d]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances.

[e]Add items 1–3 to 700 ml of distilled water (item 6), adjust pH to 5.2, add sugar (item 4) and adjust volume to 1000 ml with distilled water (item 6). Bring the solution to a gentle boil and add the agar (item 7) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour it in the charcoal slowly with constant stirring. When the charcoal has been mixed in completely pour the medium into culture vessels and autoclave.

Isolation of *Phalaenopsis* Protoplasts

The method used to isolate protoplasts of *Cattleya skinneri*, *Cymbidium* Kenny 'Wine Color,' *Dendrobium* Malones 'Hope,' *Epidendrum radicans*, and *Paphiopedilum insigne* are also suitable for *Phalaenopsis*. The species used in this research was *Phalaenopsis amabilis* (Yasugi, 1990).

Micropropagation of *Phalaenopsis* through the Culture of Shoot Tips of Flower Stalk Buds

A procedure developed for *Doritaenopsis* is also suitable for *Phalaenopsis* (Tokuhara and Mii, 1993).

Effects of Complex Organic Additives on the Growth of *Phalaenopsis* Callus

Findings on the effects of complex organic additives on the growth of *Doritaenopsis* and *Neofineia* callus (Ichihashi and Islam, 1999) are also relevant for *Phalaenopsis*.

Biotechnology and Bioengineering of *Phalaenopsis*

Biotechnology and bioengineering as they relate to *Phalaenopsis* (Chen et al., 1990*a*, 1990*b*, 1990*c*, 1991, 1995, 1998; Hsie et al., 1992; Belarmino and Mii, 2000) are beyond the scope of this book, but relevant papers are listed here for those who are interested in the subject.

Encapsulation of *Phalaenopsis* Shoot Tips

Shoot tips were excised under filter-sterilized antioxidant, and then dried, placed in an alginate solution, and dispensed into calcium chloride (Senarath et al., 2003).

In Vitro Morphogenesis of *Phalaenopsis*

Protocorms were induced to form callus, which produced embryo-like structures (Chang et al., 2001).

Micropropagation of *Phalaenopsis* Under Light-emitting Diode (LED) Illumination

"Highest shoot and root fresh weight were observed in plantlets grown under 80% red + 20% blue LED" (Tanaka et al., 2001).

Plant Regeneration from Protocorm-like Bodies Produced by Etiolated Leaves of *Phalaenopsis aphrodite*

Etiolated leaves of *Phalaenopsis aphrodite* were induced to produce PLBs and subsequently plantlets on a medium that contained 0.3% sucrose, 0.5 mg BA l^{-1} and 0.5 mg TDZ l^{-1} (Hsu and Chen, 2003).

Effect of Ultrasonicated Water Treated with Quartz Porphyry "Bakuhan-seki" (USW-B) on Organogenesis in Protocorm-like Bodies

The best induction of shoots with roots occurred on a medium containing 3% USW-B (Shimasaki et al., 2003*a*).

Effects of Environmental Factors on the Growth of *Phalaenopsis* Plantlets

Plantlet growth was best under high intensity illumination, blue and red light-emitting diodes, and increased carbon dioxide levels with or without sucrose (Shin et al., 2001).

Reduction of Medium and Protocorm Browning during Culture of *Phalaenopsis* in Vitro

Calcium chloride, malic acid, and α-tocopherol reduced medium browning and total phenolics. Treatment with calcium chloride, citric acid, and malic acid reduced phenylalanine ammonia-lyase activity and total phenolics in protocorms. It is reasonable to assume that these substances will have the same effects on PLBs (Shin et al., 1999).

Carbohydrate Sources for Somatic Embryogenesis and the Effects of Yeast Extract and Benzyladenine

Most culture media contain 10–30 g sucrose l^{-1} as a carbon (carbohydrate or energy) source. Even when other sugars are used, the amounts added to media are often the same. This is a mistake because equal weights of different sugars may represent different molarities. Molarity is a manner of expressing concentration. It is the number of moles of solute (in this case sugar) dissolved in 1 l of solution. The unit in this case is moles of sugar per liter of medium.

The expression *molarity* = moles of sugar/liter of medium is very different from *concentration* = weight of sugar/liter of medium.

The reason is Avogadro's number, which is the number of atoms or molecules in a mole of a chemical substance, that is 6.022×10^{23}. For example, 20 g of sucrose (MW 342.30) is 20/342.30 = 0.05843 mol, which is $0.0584 \times 6.022 \times 10^{23}$ molecules. An equal weight (20 g) of glucose (MW 180.16) is 20/180.16 = 0.111.01 mol, or twice the molarity of sucrose, which means $111.01 \times 6.022 \times 10^{23}$ molecules. And, the number of molecules determines the osmolarity of a solution.

Osmolarity is the osmotic pressure which is exerted by a solution (in this case sucrose or glucose) that is in contact with pure water across a semipermeable membrane [i.e., a membrane that prevents movement of the solute (in this case the sugar), but allows free passage of the water] compared to pure water. It is dependent on the number of particles in a solution but independent of their nature. For example, a solution of 1 mol of sucrose or glucose dissolved in 1 l of water has an osmolarity of 1 osmol (osm) l^{-1}. This means that in the example above, the osmolarity of a solution of 20 g glucose l^{-1} is twice that of 20 g sucrose l^{-1}. And, since osmolarity has an effect on cells in addition to or other than that of carbon levels, the effects of 20 g sucrose l^{-1} and 20 g glucose l^{-1} will be different.

Very few investigators, even those studying the effects and utilizations of different sugars on orchid seedlings or plantlets, have paid attention to the influence of osmolarity (for one example see Ernst and Arditti, 1990, even if the intent in this case was to carry out follow-up experiments with equimolar levels of the same sugars). Therefore, a comparative study of the effects of equimolar levels (58.4 mmol, which is 20 g of sucrose, maltose, and lactose or 10 g of glucose, fructose, and sorbitol) of carbon sources on PLBs of *Phalaenopsis* and *Doritaenopsis* (Tokuhra and Mii, 2003) is both interesting and welcome. The results show that:

1 The highest number of PLBs was produced on glucose.
2 PLB formation without callus proliferation takes place on maltose and sorbitol.

3 Callus proliferation on glucose and sucrose is the same.
4 Proliferation of callus on sucrose occurs without PLB formation.
5 Only half as much callus proliferation occurs on fructose as on glucose.
6 Lactose is an inadequate carbon source.
7 There is no PLB formation and callus proliferation on lactose.

Other findings are that:

1 Yeast extract (YE) at 0.1–1 g l^{-1} enhances cell proliferation. It inhibits cell proliferation and PLB formation at 10 g l^{-1}.
2 BA at a level of 0.4 μmol (0.1 mg l^{-1}) increases callus proliferation slightly, but inhibits PLB formation.
3 Plantlets are produced on a medium containing 58.4 mmol (20 g) sucrose l^{-1} and 1 g YE l^{-1}.
4 The most efficient PLB formation by cell suspension cultures of *Phalaenopsis* Snow Parade occurs on a medium containing 29.2 mmol (10 g) sucrose l^{-1}.
5 PLB formation by cell suspension cultures of *Phalaenopsis* Wedding Promenade takes place on 14.6 mmol (5 g) sucrose l^{-1}.

PHALAENOPSIS AT HOME (Williams and Williams, 1894)
(From the *Gardens' Chronicle*.)

Pholidota

Pholidota chinensis can be propagated from leaf tips by the method used for *Acampe rigida* (Yam, 1989). Root tips of *Pholidota cantonensis* and *P. chinesis* produce callus when cultured by the method used for *Bletilla striata* (Yam, 1989).

Phragmipedium

Although not as popular as *Paphiopedilum* hybrids, species and crosses of *Phragmipedium* are of some interest to orchid growers.

Aseptic Propagation of *Phragmipedium*

According to one report "axillary bud explants . . . obtained from *Phragmipedium* Sedenii, *Phragmipedium* Sedenii 'Candidulum' and *Phragmipedium caricinum* [were] sterilized by routine techniques [and] . . . cultured on Reinert & Mohrs' [sic] medium supplemented with varying levels of NAA and zeatin . . ." (Stokes et al., 1975). Plants produced by this method reportedly flowered 18 months after the start of culture.

This paper provides very few details and leaves the reader with many questions.

Clonal Propagation of *Phragmipedium* Sedenii through the Culture of Flower Buds

A procedure for the clonal propagation of *Phragmipedium* Sedenii was developed at the Institute for Soil Science and Plant Nutrition (F. Penningfeld, an expert in orchid mineral nutrition, was its director at the time) at Weihenstephan, Germany (Fast, 1979). The procedure is similar to the method for clonal propagation of *Oncidium* from flower-stalk tips (Fast, 1973*b*).

Plant Material. The uppermost bud and inflorescence tip above it are taken from the inflorescence.

Surface Sterilization. The sheath is removed and the explant is surface-sterilized by immersing it in 1.5% household bleach (approximately 30 ml Clorox diluted to 100 ml with distilled water) for 15 min. This concentration seems high, and it is possible that the household bleach used in the original research is different from Clorox or Purex. Therefore it may be necessary to experiment with several concentrations before sterilizing a desirable clone. After being removed from the sterilizing solution the explant should be rinsed several times with sterile distilled water.

Culture Vessels. Photographs in the original paper show that the cultures were maintained in small (25–50 ml) Erlenmeyer flasks containing 10–25 ml medium.

Culture Conditions. The cultures should be maintained under 16-h photoperiods of 3000 lx (light sources not listed) and temperatures 23–25°C.

Culture Medium. The explants are cultured on medium containing half the concentration of the MS macroelements, the microelements of Nitsch, and hormones (Table Phrag-1).

Procedure. The explants are inserted in the agar medium and allowed to form plantlets. These can be moved to Knudson C or Vacin and Went medium for further development (see Tables Aranda-7 and Cym-5).

Developmental Sequence. The explants show growth after 1 month, and several plantlets form within 3–4 months. PLBs can also develop at that time. Undisturbed plantlets form roots in 4 months.

General Comments. This is a useful and safe procedure for the propagation of *Phragmipedium* that could perhaps serve as a starting point for research that may lead to a similar method for *Paphiopedilum*.

TABLE PHRAG-1. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for the culture of *Phragmipedium* flower buds (Fast, 1979)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220	22 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950	95 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3O_3	0.5	500 mg l^{-1}	1	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	25 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	3	300.0 mg l^{-1}		
(d)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	25 mg l^{-1}		
(e)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5	500 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-Inositol	50	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.1	40 mg 100 ml^{-1} 95% ethanol[f,g]	0.25	
	Cytokinin and related substance				
11	Benzylaminopurine (BAP)	10	1000 mg 100 ml^{-1} 95% ethanol[f,g]	1	
12	Adenine sulfate[h]	40	No stock	No stock	Weigh
	Vitamins				
13	Niacin (nicotinic acid)	0.5	100 mg 100 ml^{-1} 95% ethanol[g]	0.5	
14	Pyridoxine (vitamin B_6)	0.5	100 mg 100 ml^{-1} 95% ethanol[g]	0.5	
15	Thiamine (vitamin B_1)	0.1	40 mg 100 ml^{-1} 95% ethanol[g]	0.25	
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[i]	To 1000 ml			
	Solidifier				
18	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved. The original paper used a commercial preparation (Iron Chelate Fetrilon from BASF) that may not be available outside Germany.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin do not dissolve, add a few drops of KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Combine hormone and vitamin alcohol solutions (items 10, 11, and 13–15) in a small autoclaved vial and add the adenine to this solution at least 20 min before mixture is to be added to medium. Shake well several times. Let stand and shake again. Pour mixture into hot, still liquid medium. If powder remains on the vial, wash it into culture medium with sterile distilled water.

[i]Add items 1–7 and 9 to 900 ml distilled water (item 17), adjust pH as required, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil and add agar (item 18) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid, hormones, and vitamins (items 8, and 10–15) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

Pleione

> PLEIONE—The Indian Crocus—is a genus of terrestrial orchids originating from Taiwan, India and mainland China. Large, handsome flowers are produced before the leaves and both arise from basal pseudobulbs. As a result of collecting difficulties . . . species most in demand are represented by single clones which are functionally self-incompatible and must therefore be propagated vegetatively. Some . . . species and hybrids are . . . in demand and . . . must be vegetatively propagated as clones. The slow rate of vegetative reproduction pointed to the need for a rapid micropropagation system for this genus, which was said by Morel (1971*a*, 1971*b*) to be amenable to the same techniques as for *Cymbidium*. . . . However, . . . the *Cymbidium* techniques were not successful when applied to *Pleione*. . . . (Weatherhead and Harberd, 1980)

Therefore, a more suitable procedure was developed at the Department of Plant Science, University of Leeds, England (Weatherhead and Harberd, 1980). This research was a harbinger of things to come since Weatherhead, now deceased, at the Botany Department, University of Hong Kong, developed an impressive research program dealing with orchid seed germination and tissue culture.

Plant Material. Shoot tips 2–3 mm long are taken from lateral shoots 1.0–1.5 cm long excised from the bases of pseudobulbs.

Surface Sterilization. In the original research the shoots were "surface-sterilized in 5% (v/v) sodium hypochlorite (British Drug Houses) for 15 min and then rinsed three times in sterile distilled water." This concentration is probably similar to the one that could be obtained by diluting 5 ml Clorox to 100 ml with distilled water.

Culture Vessels. Test tubes, Petri dishes, Erlenmeyer flasks, or other containers can be used.

Culture Conditions. Cultures should be maintained under 16-h photoperiods of 2000 lx at 27 ± 2°C.

Culture Medium. Knudson C medium with charcoal (Table Pln-1) is used for all stages.

Procedure. Place the explants on the culture medium and allow them to develop PLBs. Section these into quarters and move to fresh medium in Petri dishes, where proliferation takes place and clumps of PLBs form. Individual PLBs can be separated from the clumps and either sectioned again or cultured separately and allowed to form shoots. Move each shoot with a basal piece of PLB attached to a test tube where roots can be expected to form within 4 weeks. The resulting plantlets can be treated as seedlings and potted in community pots.

Developmental Sequence. The PLBs originate from divisions of the epidermal cells (Fig. Pln-1A) of the shoot-tip explant. They also develop basal rhizoids. Following the sectioning of the initial PLBs, new ones arise also from divisions of the epidermal

TABLE PLN-1. **Modified Knudson C medium (Knudson, 1946) for the micropropagation of *Pleione* (Weatherhead and Harberd, 1980)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
	Iron[c]				
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Sugar				
7	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
8	Water, distilled[d]	To 1000 ml			
	Solidifier				
9	Agar[e]	12–15 g	No stock	No stock	Weigh
	Darkening agent				
10	Activated charcoal[f]	3 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[d]Add items 1–6 to 900 ml distilled water (item 8), adjust pH to 5.0, add sugar (item 7), and adjust volume to 1000 ml with distilled water (item 8). Bring solution to a gentle boil and add agar (item 9) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, add charcoal, stir to mix it well, and dispense solution into culture vessels and autoclave. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower pH if necessary.

[e]Add agar to solid medium only.

[f]The original paper reports that the medium was "supplemented with activated charcoal 0.3% w/v (Fisons Scientific Apparatus) . . ." but does not indicate the type of the charcoal. Our experience is that vegetable charcoal is preferable.

cells (Fig. Pln-1B). They have a basal ring of rhizoids. The development of plantlets from PLBs is similar to that of seedlings (Fig. Pln-1C–F).

General Comments. This procedure is simple and effective. It can produce approximately 100 plants from one shoot-tip explant in about 20 weeks. Its major advantage is that it uses the same simple medium for all steps. A less obvious but nevertheless very important advantage is the extremely well written paper that reports it.

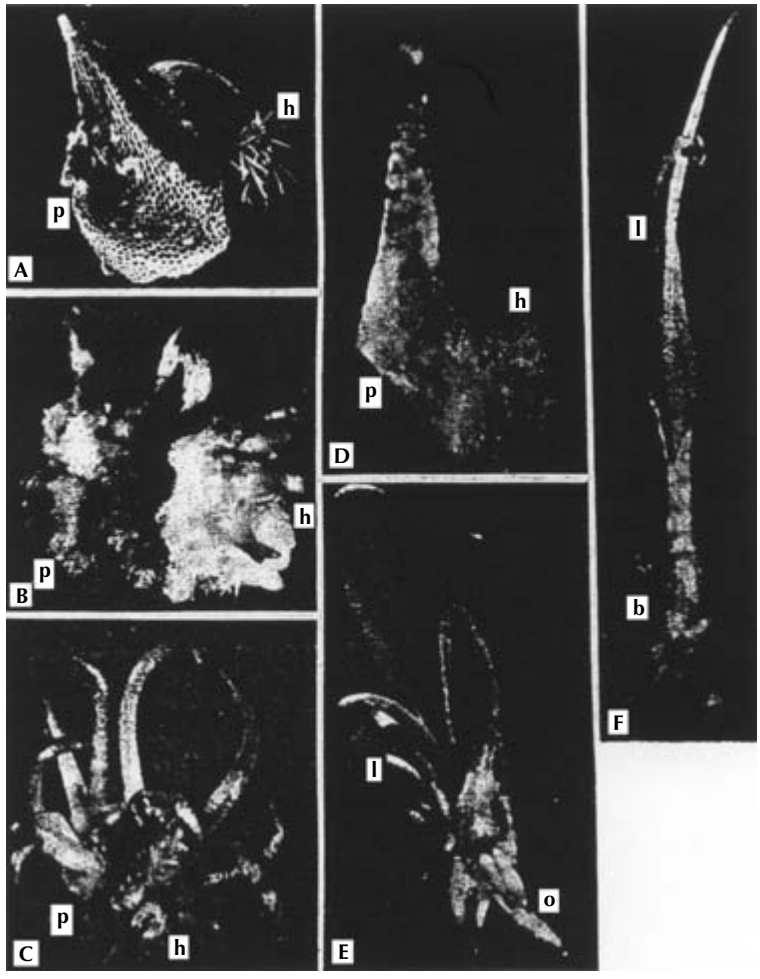


FIG. PLN-1. **PLBs and plantlet development in *Pleione*. A. A PLB (p) formed by division of the epidermal cells of the meristem; basal rhizoids (h) are clearly evident. B. A clump of PLBs (p) formed from the epidermal cells of a PLB section; each PLB has a ring of rhizoids (h). C. Several shoots arising from an individual PLB (p); rhizoids (h) are evident at the base. D. A shoot with a basal section of a PLB (p) and rhizoids (h). E. A plantlet (l) with a root (o). F. A plantlet (l) with a basal pseudobulb (b). (Courtesy Dr. M. A. Weatherhead, Botany Department, University of Hong Kong.)**

Pogonia

Species which at one time were part of *Pogonia* are currently included in *Cleistes*, *Triphora*, and *Isotria*. Because of this, *Pogonia* is a small genus now. The name is derived from the Greek πωγων (beard) or πωνωνας (*pogonias*) which means "bearded" and refers to the bearded crest on the labellum.

Micropropagation of *Pogonia japonica* through the Culture of Rhizome Segments

Pogonia japonica is a terrestrial species found in China, Japan, and Korea. It grows in lowland, barren, sunny bogs. Disruption of natural habitats due to land reclamation threatens this species with extinction. A micropropagation method (Fig. Pog-1) was developed at the Mukaishima Orchid Center and Hiroshima University to allow for the reintroduction of the species for conservation purposes (Takahashi and Kondo, 1998).

Plant Material. Seeds of *P. japonica* collected from a wild population in Higashi-Hiroshima City, Japan were surface-sterilized with 1.2% (v/v) sodium hypochlorite [presumably domestic bleach but it is not clear if the percentage refers to a bleach solution (as, for example, 1.2 ml bleach solution diluted to 100 ml) or to its active component (as, for example, 20 ml bleach diluted to 100 ml), but it is probably the latter] containing a few drops of Tween 20 (from www.sigmaaldrich.com, www.vwr.com, www.fishersci.com, www.thomassci.com, and other sources) or mild household detergent. The seeds were then rinsed three times with sterile distilled water and germinated on Hyponex medium (Kano, 1965) consisiting of 3 g Hyponex l^{-1} (www.hyponex.co.jp), 2 g peptone l^{-1} (www.sigma-aldrich.com, www.caissonlabs.com, www.duchefa.com, www.phytotechlab.com), 3% sucrose (w/v), and 0.3% Gelrite (w/v; www.sigmaaldrich.com, www.caissonlabs.com, www.duchefa.com, www.phytotechlab.com) at a pH of 5.8. The seeds germinated

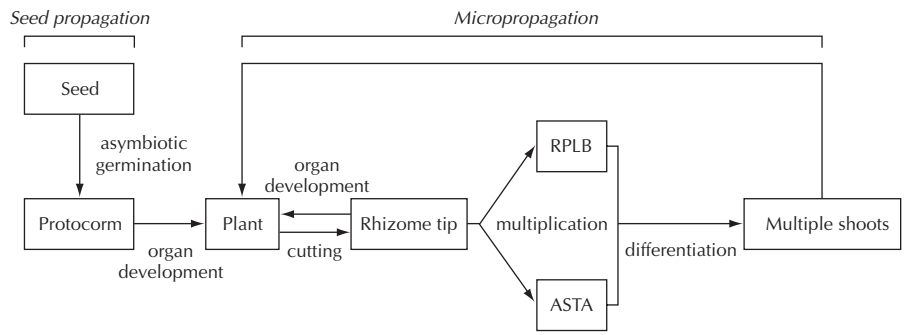


FIG. POG-1. **Propagation of *Pogonia japonica* through seed germination and tissue culture. ASTA, abnormal shoot-tip aggregation; RPLB, rhizome-derived protocorm-like bodies. (Takahashi and Kondo, 1998.)**

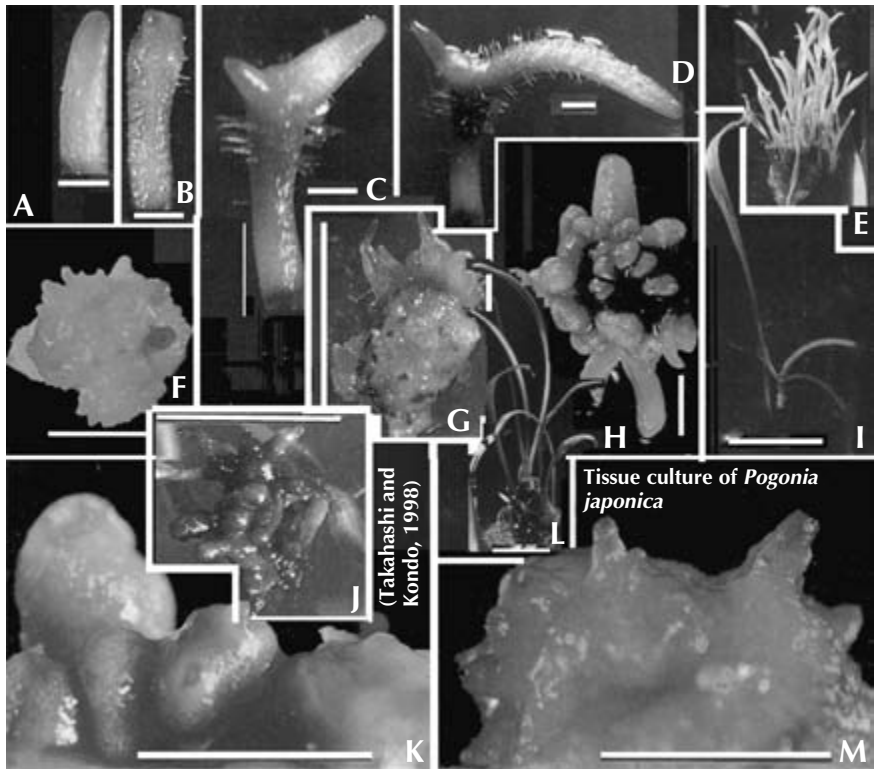


FIG. POG-2. **Micropropagation of *Pogonia japonica*. A. Rhizome-tip explant at the start of culture. B. Explant after 7 days of culture showing the start of adventitious bud formation. C. Newly formed rhizomes on a rhizome-tip explant after 14 days of culture. D. New rhizomes developing after 21 days. E. Plantlets produced from abnormal shoot-tip aggregation. F. Abnormal shoot-tip aggregation. G. Plantlets starting to develop from an abnormal shoot-tip aggregation. H. PLBs. I. A 60-day-old culture showing a well-developed juvenile plant. J. Plantlets starting to develop from a PLB. K. PLBs. L. Plantlets that formed from a PLB. M. Abnormal shoot-tip aggregation. Bars: 1 mm for A–D; 2 mm for F, H, K, M; 10 mm for E, G, I, J, L. (Takahashi and Kondo, 1998.)**

in 90–200 days and the seedlings were allowed to grow for 100 days. Rhizome tips, 2–3 mm long, were taken from these seedlings and cultured (Fig. Pog-2A).

Surface Sterilization. Explants taken from seedlings growing in vitro do not require surface sterilization.

Culture Vessels. Culture tubes, 30 × 200 mm containing 20 ml medium, were used in the original research. Other containers filled to 25–30% of their volume with medium are also suitable.

Culture Conditions. Seedling cultures were maintained at 24 ± 1°C under continuous illumination of 1000 lx (source not indicated). Standard laboratory conditions will

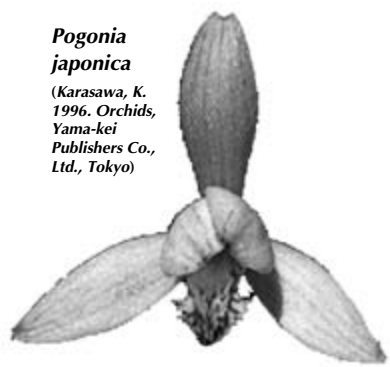
probably also be suitable. Explant cultures in liquid medium were placed on a rotary shaker at 2 rpm at 24 ± 1°C under 2000–10,000 lx of continuous illumination (source not given).

Culture Media. Explants should be cultured on liquid MS medium (Murashige and Skoog, 1962) containing 0.02 mg l^{-1} each of BA and NAA (Table Pog-1) for about 2 months until clusters of PLBs are formed (Fig. Pog-2H, K). The PLBs should be moved to solid hormone-free MS (Table Pog-2) for plantlet formation (Fig. Pog-2J, L).

Procedure. Rhizome-tip explants are cultured in liquid medium (Table Pog-1) under gentle agitation for 2 months or until PLBs are formed. These PLBs are moved to the solid medium (Table Pog-2) for plantlet formation.

Developmental Sequence. When the proper media and sequence are used (Table Pog-1 followed by Table Pog-2), normal PLBs (Fig. Pog-2H, K) and then plantlets (Fig. Pog-2J, L) will form. If the rhizome explants (Fig. Pog-2A) are placed on solid medium they form adventitious buds (Fig. Pog-2B), then new rhizomes (Fig.2-1C, D), and eventually plantlets (Fig. Pog-2I). Abnormal aggregations (Fig. Pog-2F, M), bodies (Fig. Pog-2G), and plantlets (Fig. Pog-2E) can form if the medium and/or conditions are not correct.

General Comments. As published, this procedure is for the micropropagation of seedlings and not of selected clones (Fig. Pog-1). However, since its purpose is to propagate a species for conservation and reintroduction this is not a major problem or limitation. And since orchids are generally very heterogenous, genetic pauperization of a population reintroduced in this manner is not likely.



Pogonia japonica
(*Karasawa, K. 1996. Orchids, Yama-kei Publishers Co., Ltd., Tokyo*)

TABLE POG-1. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Pogonia japonica* rhizome explants (Takahashi and Kondo, 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.02	2 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	0.02	2 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.8, add sugar (item 15), bring volume to 1000 ml with distilled water (item 16), pour the solution into culture vessels, and autoclave. Agar is not added to liquid media. Amino acids, hormones, and vitamins are generally not heat-stable. Therefore it is preferable to sterilize them by filtration or make the stock solutions in alcohol and add them after autoclaving. In this case they are autoclaved.

TABLE POG-2. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for production of *Pogonia japonica* plantlets (Takahashi and Kondo, 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[f]	To 1000 ml			
	Solidifier				
15	Gelrite[f,g]	3.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH to 5.8, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Add Gelrite as indicated in footnote *g*. When the Gelrite is completely dissolved pour the solution into culture vessels and autoclave. The amino acid (item 8) and vitamins (items 10–12) are not heat-stable. Therefore they are often sterilized through autoclaving or their stock solutions are made in alcohol and added after autoclaving. In this case the amino acid and vitamins are autoclaved.

[g]Gellan gum [available as such from www.caissonlabs.com and as Phytagel (www.sigmaaldrich.com) or Gelrite] is an agar substitute which produces a firm, clear, and colorless solid substrate at concentrations lower than those of agar. It requires divalent cations to form a gel. Most plant tissue culture media contain enough Ca^{2+} and Mg^{2+} for gelation. Dilute media may require additional calcium or magnesium salts or higher concentrations of gellan gum. Gellan gum (Phytagel, Gelrite) must be added slowly with vigorous stirring to liquid medium at room temperature to eliminate the formation of lumps. If gellan gum (Phytagel, Gelrite) is added to warm or hot water or medium it will form clumps and will not gel properly after autoclaving. The gelling temperature of gellan gum (Phytagel, Gelrite) is 27–31°C. Typical concentrations for standard media are 1.5–2.5 g gellan gum (Phytagel, Gelrite) l^{-1}, but up to 10 g l^{-1} can or may have to be used for media which must be very hard or contain low cation levels. After the gellan gum (Phytagel, Gelrite) has dissolved pour the medium into culture vessels and autoclave.

Potinara

The procedures used for *Cattleya* and *Laeliocattleya* (Huang, 1984) can also be employed for *Potinara*.

Renades

The first *Renades* hybrid was produced in Hawaii half a century ago. It was *Renades* Kaulani (*Renanthera monachica* × *Aerides fieldingii*) registered in 1955 by Kirsch. According to the *Sander's One-Table List of Orchid Hybrids (1946–1960)* (Sander and Wreford, 1961) this could be either Oscar M. Kirsch or Rose B. Kirsch, both of the same address in Honolulu. Despite its vintage, *Renades* is not a very popular line of hybrids.

Clonal Propagation of Renades through the Culture of Inflorescences

Renades Arunoday [the hybrid name is spelled "Arunodoya," "Arunodya," "Arunodaya," and "Aronoday" in Ahmed et al. (1998), whereas the spelling in Sander's list is "Arunoday"] was produced by the Orchid Research Centre (ORC), State Forest Research Institute, Forest Department, Tipi Bhalukpong, Arunachal Pradesh, India and was registered in 1990 by S. N. Hegde of the same institution. The hybrid was described as having "commercial value for its long inflorescences with 18–20 . . . pale pink flowers with deep rose spots on petals and red blotches on the lobes of [the] lip" (Ahmed et al., 1998). Despite this description *Renades* is not grown as a cut-flower orchid in South East Asia, perhaps because the available hybrids do not seem to be as long lasting and as free flowering as the ones which are already in cultivation. "In order to multiply this ornamental hybrid on a mass scale" a micropropagation method using young inflorescences was developed at the ORC (Ahmed et al., 1998).

Plant Material. Young inflorescences, 8–10 mm long, were harvested, surface-sterilized and, after the final washing, "bracts were removed very carefully and inoculated . . ." (Ahmed et al., 1998). The statement in quotation marks here suggests that the bracts were placed in culture. However, the context of the report and a photograph (Fig. Rnds-1A) indicate that the young inflorescence was cultured. Therefore it may be advisable to place both the bracts and the young inflorescences in culture.

Surface Sterilization. The inflorescences should be cleansed with tap water and a soft brush (a used toothbrush is suitable) and Teepol (www.sigmaaldrich.com) or a mild household detergent and then rinsed with distilled water 3–5 times. They should then be dipped in 70% ethanol (73–74 ml 95% ethanol diluted to 100 ml with distilled water) for 3 min, washed with sterile distilled water three times, immersed in saturated calcium hypochlorite solution (7 g powdered calcium hypochlorite suspended in 100 ml distilled water, stirred vigorously, allowed to stand for 3–5 min, agitated

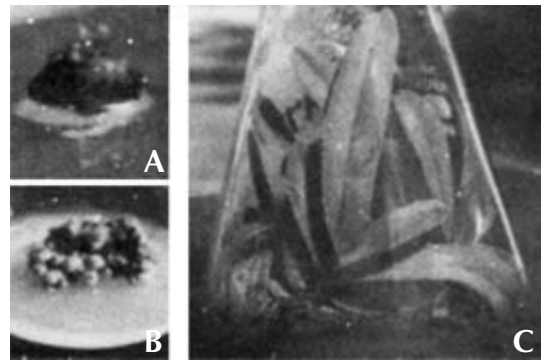


FIG. RNDS-1. **Culture of a *Renades* Arunoday inflorescence. A. Callus formation on the node area. B. Callus. C. Plantlets. (Ahmed et al., 1998.)**

again, left undisturbed for another 3–5 min, and then decanted or filtered; the liquid must be used within 12 h) for 5min and rinsed three times with sterile distilled water.

Culture Vessels. Erlenmeyer flasks were used in the original research. Other containers are also suitable. Smaller containers (culture tubes or 50- or 125-ml Erlenmeyer flasks containing medium equal to 25–35% of their volume) should be used for the initial explants and to subculture callus. Larger flasks (250- or 500-ml capacity) can be used for plantlet production.

Culture Conditions. Cultures should be maintained at 25 ± 2°C and 16-h photoperiods of 2500 lx provided by fluorescent tubes or under standard laboratory conditions.

Culture Media. For callus induction, explants should be cultured on Vacin and Went medium (Vacin and Went, 1949) supplemented with coconut water, calcium pantothenate, pyridoxine hydrochloric acid (HCl), thiamine HCl, and NAA (Table Rnds-1). The callus produces plantlets on Vacin and Went medium with coconut water, banana homogenate, and an additive described as "starch potato" (Table Rnds-2). It is not clear if this is a particular type of potato or potato starch. Therefore, those who use this method are advised to try one version of the medium with potato homogenate and another with potato starch.

Procedure. Explants are taken from plants, surface-sterilized, and cultured on the first medium (Table Rnds-1). Callus which forms on these explants develops plantlets on the second medium (Table Rnds-2).

Developmental Sequence. Callus (Fig. Rnds-1A, B) is formed on the first medium and plantlets (Fig. Rnds-1C) develop on the second.

General Comments. This seems to be a productive micropropagation method which could perhaps be used for this hybrid genus as well as its parent genera,

TABLE RNDS-1. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of young inflorescences of *Renades* Arunoday (Ahmed et al., 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Vitamins				
8	Calcium pantothenate	0.25	25 mg in 100 ml 95% ethanol[e]	1	
9	Niacin	1.25	125 mg in 100 ml 95% ethanol[e]	1	
10	Pyridoxine HCl	0.25	25 mg in 100 ml 95% ethanol[e]	1	
11	Thiamine HCl	0.25	25 mg in 100 ml 95% ethanol[e]	1	
	Auxin				
12	Naphthaleneacetic acid (NAA)	0.1	10 mg in 100 ml 95% ethanol[e,f]	1	
	Complex additive				
13	Coconut water[g]	150.0 ml	No stock	No stock	Measure
	Solvent				
14	Water, distilled[h]	To 1000 ml			
	Solidifier				
15	Agar[h]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 14) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Keep frozen between uses.

[f]If the auxin fails to dissolve add a few drops of 0.1 N KOH.

[g]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[h]Add items 1 and 3–13 to the 500–700 ml of distilled water (item 14) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 14), adjust pH to 5–5.4, raise the volume to 1000 ml with distilled water (item 14), bring the solution to a gentle boil, and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable. Hormone (item 12) and vitamins (items 8–11) are not heat-stable and are usually dissolved in alcohol or filter-sterilized and added after autoclaving. However in this case they are added before the medium is autoclaved.

TABLE RNDS-2. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for plantlet production from callus produced by young inflorescences of *Renades* Arunoday (Ahmed et al., 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Complex additives				
8	Coconut water[e]	150.0 ml	No stock	No stock	Measure
9	Ripe banana[f]	100.0 g	No stock	No stock	Weigh
10	Potato starch[g]	2.0	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[h]	To 1000 ml			
	Solidifier				
12	Agar[h]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 11) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[f]Opinions differ regarding the use of banana in culture media. The predominant opinion is that only homogenized pulp of ripe banana should be used. Other views are that: (1) unripe (green) bananas are preferable, (2) the peel rather than the pulp should be used, (3) unpeeled banana homogenate should be incorporated in media, and (4) slices, ripe or unripe, peeled or unpeeled, rather than homogenate are most effective. Opinions also differ regarding which is the most effective banana variety (Gros Michel, Raja, Pisang Susu, and others). Since there is no experimental evidence to support any view the best approach is to follow the recommendations in the original reports and/or use what is suggested there.

[g]Potato starch is available from www.spicyglobe.com or www.sigmaaldrich.com.

[h]Pour the 500–700 ml of distilled water (item 11) which contain the calcium phosphate (item 2) into a homogenizer (a domestic type is appropriate), add items 1 and 3–10 and homogenize thoroughly. Bring volume to 900 ml with distilled water (item 11), adjust pH to 5–5.4, raise the volume to 1000 ml with distilled water, bring the solution to a gentle boil, and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

Renanthera and *Aerides*. It is unfortunate that the paper which describes it is not more detailed, written with more attention to detail, and edited with greater care.

Micropropagation of *Renades* through the Culture of Leaf Explants

Renades Arunoday was produced by the Division of Orchidology, State Forest Research Institute in Arunachal Pradesh, India. A micropropagation method using leaf explants was developed at the same institute (Sinha and Hegde, 1999).

Plant Material. Leaves, 0.8–2.0 cm in length, were taken from seedlings of *Renades* Arunoday growing in vitro. Whole leaves (Fig. Rnds-2Aa) and leaf-base segments (Fig. Rnds-2Ac, Ad) produce the best results. However, callus is also produced along the entire leaf base (Fig. Rnds-2Aa, Ac).

Surface Sterilization. Explants taken from seedlings growing in vitro do not require surface sterilization. However, they should be washed with sterile distilled water to remove medium residues.

Culture Vessels. Erlenmeyer flasks were used in the original research. Smaller flasks (125-ml flask containing 30 ml of medium) are suitable for initial cultures. Larger flasks (250- or 500-ml capacity containing medium equal to 25–30% of their volume) should be used for larger callus masses or plantlet production.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 14-h photoperiods of 2000 lx (the illumination sources used in the original research were not described). Standard laboratory conditions will probably also prove to be suitable.

Culture Media. Leaf explants should be cultured on half-strength MS medium (Murashige and Skoog, 1962) containing 1 mg BA l^{-1} and 0.5 mg NAA l^{-1} (Table Rnds-3). Callus and PLBs produced on this medium should be cultured on half-strength MS medium (Murashige and Skoog, 1962) containing 1 mg BA l^{-1} and 1 mg NAA l^{-1} (Table Rnds-4).

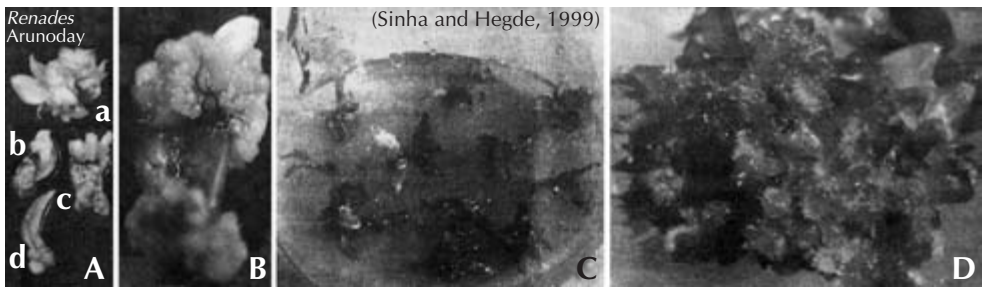


FIG. RNDS-2. **Culture of *Renades* leaves. A. Callus and plantlet formation on the entire leaf surface (a), blade and base (b), tip and base (c), and base (d). B. Formation of callus on the leaf tip and base. C. Well-developed plantlets. D. Platelets formed from PLBs. (Sinha and Hegde, 1999.)**

TABLE RNDS-3. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Renades* Arunoday seedling leaves (Sinha and Hegde, 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, pour the solution culture vessels and autoclave. Amino acid (item 8), hormones (items 10 and 11) and vitamins (items 12–14) are not heat-stable and are usually added to the hot solution under sterile conditions with sterilized pipettes. However in this case all components are mixed before autoclaving.

TABLE RNDS-4. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of seedling-leaf-derived *Renades* Arunoday callus and protocorm-like bodies for plantlet production (Sinha and Hegde, 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Amino acid (item 8), hormones (items 10 and 11) and vitamins (items 12–14) are not heat-stable and are usually added to the hot solution under sterile conditions with sterilized pipettes. However in this case all components are mixed before autoclaving.

Procedure. Leaves and leaf explants are cultured on the first medium (Table Rnds-3) until callus masses and PLBs are formed. PLBs should be moved to the second medium (Table Rnds-4) for plantlet production.

Developmental Sequence. Leaves and leaf explants start to swell within 4 weeks of the start of culture. PLBs, callus, and leaf primordia are produced within 28 weeks.

General Comments. This seems to be an effective and productive protocol with only one drawback: The explants are taken from seedlings, which means that the procedure cannot be used to select desirable clones. However, if explants are taken from plantlets produced through the previous procedure, selection of clones is possible.

A question unrelated to the procedure is the reason why *Renades* was chosen as the name of this hybrid rather than the better sounding *Aeridanthera*, which is another possibility as the name of a bigeneric hybrid is derived by combining the names of the parent genera.

Names of Media

Media names have interesting origins and histories. Some were named by those who formulated them. Examples are two different and unrelated P media (Hoppe and Hoppe, 1987*a*, 1987*b*, 1988; Ichihashi and Hiraiwa, 1996), B5 medium (Gamborg et al., 1968), new *Phalaenopsis* (NP) medium, new Dogashima medium (Tokuhara and Mii, 1993), ND medium (Tsukazaki et al., 2000), media B (Knudson B, KB; Knudson, 1922) and C (Knudson C, KC; Knudson, 1946; Knudson also used the Pfeffer solution and may have designated it as A) and GD medium (Thomale, 1954, 1957).

Subsequent users named several media for those who published or formulated them. This is the case with formulations by Nitsch and Nitsch (for obtaining haploid *Nicotiana*, simplified Knop, M_2, N_1) which are usually referred to as Nitsch or Nitsch and Nitsch followed by a date; Vacin and Went or VW for Emil Vacin and Frits W. Went's solution 5 (Vacin and Went, 1949); Murashige–Skoog medium or MS for Toshio Murashige and Folke Skoog's revised medium (Murashige and Skoog, 1962); Linsmaier and Skoog medium or LS for Elfrede Linsmaier and a Folke Skoog's revised medium or RM 1964 (Linsmaier and Skoog, 1964); Mitra, Prasad, and Roychowdhury or MPR medium for an orchid substrate (Mitra et al., 1976); and Norstog medium for Knut Norstog's barley medium II (Norstog, 1973).

Some researchers named their media after themselves. This is the case with media XE (experimental Ernst; Yam et al., 1991), XER (experimental Ernst, Robert; Ernst, 1994), RE (Robert Ernst; Ernst, 1982), REM (Robert Ernst medium; Ernst, 1986), and WS (Wannakrairoj, Surawit; Wannakrairoj and Tanyasonti, 1996).

When a medium is modified it is customary to indicate this (i.e., "modified X") rather than assign a new name.

Renanetia

Micropropagation of *Renanetia* through the Culture of Flower Bud Explants

The methods used for *Vandofinetia* (Kishi et al., 1997*a*, 1997*b*) can also be used for *Renanetia*.

Renantanda

The first cross in this hybrid genus was *Renantanda* Sanderi (*Renanthera imshootiana* × *Vanda suavis*) in 1935. Since then a large number of additional hybrids have been produced involving as parents *Renanthera coccinea*, *Renanthera monachica*, *Renanthera storiei*, *Vanda insignis*, *Vanda spathulata*, and *Vanda limbata* among species, as well as a number of hybrids.

Clonal Propagation of *Renantanda* through the Culture of Leaf Explants

Leaves are preferable as sources of explants for clonal propagation since their removal does not seriously damage or endanger the plant. A method for the clonal propagation of a *Renantanda* hybrid was developed at the National University of Singapore (Goh and Tan, 1982).

Plant Material. Young leaves of mature plants measuring 1–3 mm (generally the topmost three leaves) are removed and cultured.

Surface Sterilization. The leaves are washed with sterile distilled water, wiped thoroughly with 95% ethanol, immersed in 10% Clorox (10 ml Clorox diluted to 100 ml with distilled water) for 10 min, and rinsed with sterile distilled water.

Culture Vessels. Erlenmeyer flasks should be used.

Culture Conditions. In the original research the cultures were maintained under natural daylight (filtered through a window) with 12-h photoperiods of approximately 2500 lx at 28 ± 2°C. Liquid cultures are placed on a shaker at 80 lateral oscillations per minute for 15 h daily.

Culture Media. Initial culture is in a modification of the Vacin and Went medium (Table Rntda-1). Callus and PLBs produced in this medium are transferred to an unmodified Vacin and Went solution (see Table Aranda-7).

Procedure. Place the explants on the first medium (Table Rntda-1), and allow them to develop callus and PLBs. Move these to the second medium (see Table Cym-5) for leaf, root, and plantlet formation. These plantlets can be cultured to a larger

TABLE RNTDA-1. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of *Renantanda* leaf explants (Goh and Tan, 1982)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric citrate, $FeC_6H_5O_7 \cdot 52H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water[e]	200 ml	No stock	No stock	Weigh
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Coconut water can be replaced with 1×10^{-6} mol benzyladenine and 1×10^{-6} mol 2,4-dichlorophenoxyacetic acid.

[f]Add items 1 and 3–8 to the 500 ml distilled water (item 10) that contains item 2, adjust pH to 5.2–5.5, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense the medium into culture vessels, and autoclave. Omit agar for liquid medium.

size on the Knudson C (see Table Aranda-7) or Vacin and Went (see Table Cym-5) media.

Developmental Sequence. Papillae develop on the leaves after 2 weeks in culture. After 8 weeks the leaves show a water-soaked appearance near the area of the papillae, and callus forms at the base. PLBs form after 10 weeks and also following subculture. Two forms of PLBs can be seen. Larger PLBs are part of a loosely arranged tissue, while smaller ones form a more compact callus. The first leaves appear 2–3 weeks following subculture, and the roots are seen 2–3 weeks after that.

General Comments. Contamination was high during the initial experiments (54% in one case). However, success (i.e., uncontaminated cultures) was also high (more than 70%). Since leaves are easy to obtain, this procedure should prove of great value to those who wish to propagate *Renantanda* clones.

Clonal Propagation of *Renantanda* through the Culture of Shoot Tips

A method for the culture of *Renantanda* shoot tips was developed at the Botany Department, University Kebangsaan Malaysia, Bangi, Selangor, Malaysia (Abdul Ghani pers. comm.*; Abdul Ghani et al., 1992).

Plant Material. Shoot tips 2 mm long are excised from mature plants.

Surface Sterilization. Shoots should be washed with water and a mild detergent. (Teepol from the Shell Chemical Co., Kuala Lumpur, Malaysia, was used in the original research.) The washed shoots are rinsed with running tap water for 2 h, the upper young leaves are removed, and shoot tips with several leaf primordia are cut. These shoots should be submerged in 10% household bleach (e.g., 10 ml Clorox diluted to 100 ml with distilled water) for 15 min. After that they are moved for another 15 min to 5% bleach (5 ml Clorox diluted to 100 ml with distilled water). The sterilized shoot tips are then placed in a sterile Petri dish and all remaining leaves and leaf primordia are removed to expose the shoot apex. These shoot tips should be placed in 1% bleach (1 ml Clorox diluted to 100 ml with distilled water) for 10 min and rinsed with sterile distilled water for 5 min before the tips are excised.

Culture Vessels. Erlenmeyer flasks, of 100-ml capacity and containing 50 ml medium, were used in the original research. Flat-bottom culture vials, 2.5×10 cm and containing 20 ml medium, were used for plantlet induction. Other containers can also be used.

Culture Conditions. Liquid cultures should be placed on a gyrorotatory shaker at 100 rpm. In the original research all cultures were maintained under 8-h photoperiods of 2000 lx (provided by NEC daylight fluorescent tubes FL 20SSDD/18) at $25 \pm 2°C$.

Culture Media. Liquid Yamada II (YII) medium containing coconut water (Table Rntda-2) is suitable for the induction of PLBs. The PLBs proliferate on modified sugar-free liquid Vacin and Went (VW – s) medium (Table Rntda-3). Plantlets form on solid sugar-containing VW medium (VW + s; Table Rntda-3).

Procedure. Culture the excised shoot tips in the YII medium until PLBs form. Move the PLB to the VW – s medium for proliferation. When enough PLBs become available, move them to the VW + s medium for plantlet induction.

Developmental Sequence. PLBs form in the first medium (YII), proliferate on the second (VW – s), and produce plantlets on the third (VW + s).

* I thank Dr. Abdul Karim Adbul Ghani for allowing me to use in the first edition what was unpublished material at the time.

TABLE RNTDA-2. **Modified Yamada II medium as used for the culture of *Renantanda* explants (Abdul Ghani, pers. comm.)**

Item number	Component	Amount per liter
1	Gaviota fertilizer no. 67[a]	2.5 g
2	Peptone[b]	1.75 g
3	Coconut water[c]	200 ml
4	Tomato juice[d]	100 ml
5	Sucrose	15 g
6	Distilled water[e]	To 1000 ml
7	Agar[e]	14 g

[a]Blue Sky Agricultural Supply Co., (PTE), Ltd., Kuala Lumpur, Malaysia.

[b]Sigma Chemical Co., St. Louis, MO, USA.

[c]From green nuts; water from ripe nuts should also prove suitable.

[d]Prepared by removing seeds from 100–150 g fresh ripe tomatoes, homogenizing pulp, and filtering homogenate. The seeds must be removed since they may contain growth inhibitors. Do not use tomato juice from commercial sources since it may contain preservatives that could inhibit growth.

[e]Add items 1–4 to 600 ml distilled water and adjust pH to 5.2. Dissolve sugar (item 5), and bring total volume to 1000 ml with distilled water (item 6). Bring solution to a gentle boil, and add agar (item 7) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels, and autoclave. Omit agar if preparing liquid medium.

TABLE RNTDA-3. **Vacin and Went medium for the germination of orchid seeds (Vacin and Went, 1949) as modified for the proliferation of *Renantanda* protocorm-like bodies and for plantlet induction (Abdul Ghani, pers. comm.)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric citrate, $FeC_6H_5O_7 \cdot 5H_2O$	28	No stock		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water from young nuts	200 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose[e]	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Omit sugar from the PLB proliferation medium (referred to in text as VW – s). Add it only to the medium for plantlet induction (VW + s).

[f]Add items 1 and 3–8 to the 500 ml distilled water (item 10) that contains item 2; adjust pH to 5.2, add sugar (item 9), but only to medium used for plantlet induction; adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels, and autoclave. Omit agar for liquid medium.

General Comments. This is an efficient medium for mass rapid clonal propagation of *Renantanda* that does not contain hormones (except for those present in the coconut water). Such media are less apt to induce mutations.

Isolation and Culture of *Renantanda* Protoplasts

The development of methods for the isolation, culture, and fusion of protoplasts of a number of plants led to the development of similar procedures for *Renantanda* at the School of Biological Sciences, University Sains Malaysia, Penang, Malaysia, and the Institute for Plant Nutrition, Justus Liebig University, Giessen, West Germany (Teo and Neumann, 1978*b*, 1978*c*; Teo, 1980).

Plant Material. Protocorms of *Renantanda* Rosalind Cheok (*Vanda* Gilbert Triboulet × *Renanthera storiei*) were used as protoplast sources in the original research.

Surface Sterilization. Surface sterilization is not necessary for protocorms raised under aseptic conditions.

Culture Vessels. Information on culture vessels is not provided, but it is reasonable to assume that the containers used for *Angraecum eburneum* (Price and Earle, 1984) would be suitable.

Culture Conditions. Incubate protocorms for 18–20 h (temperature and illumination not described). Culture protoplasts under diffuse light (intensity and photoperiods not given) at 25°C.

Culture Media. The enzyme solution consists of 2% cellulase, 1% Macerozyme, 0.5% pectinase, 0.5% potassium dextransulphate (all w/v), and 12.75 g sorbitol per 100 ml distilled water. For sources of enzymes and equipment see Appendix 2 and procedures for *Angraecum giryamae*, *Barlia robertiana*, and *Ophrys lutea*. Protoplasts are cultured in a modified Knudson C medium (Table Rntda-4). It is not clear from the original paper whether the medium is liquid or solid. However, work with other plants and orchids indicates that it must be liquid. Viability is determined by treating the protoplasts with 0.5% fluorescein acetate and observing them after 5 min under a microscope with light of 435-nm wavelength. Viable protoplasts fluoresce bright yellow against a red background.

Procedure. Place protocorms in the enzyme solution for 18–20 h and filter the resulting cell suspension through a 90-μm sieve. Remove the cells from the sieve with sterile medium (Table Rntda-4), and culture.

Developmental Sequence. In freshly isolated protoplasts the chloroplasts are distributed throughout the cytoplasm, but can also be seen in the periphery. Raphide-containing idioblasts also occur in the suspension. Approximately 40% of the protoplasts are viable and fluoresce. "After about a week nearly 10% of the protoplasts [divide and form] clusters of 4–6 cells. Clusters of 80–100 cells can remain alive for up to 10 weeks" (Teo and Neumann, 1978*b*).

TABLE RNTDA-4. **Knudson C medium for the germination of orchid seeds (Knudson, 1946) modified for the culture of *Renantanda* protoplasts (Teo and Neumann, 1978*b*, 1978*c*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
	Iron[c]				
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxin				
7	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.442	176.8 mg 100 ml^{-1} 95% ethanol[d]	0.25	
	Cytokinin				
8	Kinetin	0.215	86 mg 100 ml^{-1} 95% ethanol[d]	0.25	
	Sugar alcohol				
9	Sorbitol	12.75 g	No stock	No stock	Weigh
	Sugar				
10	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[e]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[d]Keep refrigerated or frozen between uses.

[e]Add items 1–6 and 9 to 850 ml distilled water (item 11), adjust pH to 5.0–5.5, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11), pour solution into a 2-l flask, and autoclave. Add hormones (items 7 and 8) to the autoclaved medium under sterile conditions and dispense into preautoclaved culture vessels. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. A dilute solution of hydrochloric acid (HCl) should be used to lower the pH if necessary.

General Comments. The authors (Teo and Neumann, 1978*b*, 1978*c*; Teo, 1980) have fused protoplasts of *Dendrobium* with *Dendrobium*, *Phalaenopsis* with *Phalaenopsis*, *Renantanda* with *Renantanda*, and *Dendrobium* and *Phalaenopsis* with *Renantanda* by treating them with polyethylene glycol. However, despite optimistic predictions there are no reports of plants being produced from fused or unfused protoplasts.

Renanthera

The genus *Renanthera* consists of approximately 15 species which are found in South East Asia. Plants are large monopodials with long stems. The gynostemium and pollinaria resemble those of *Vanda* and *Aerides*. That is why some *Renanthera* species were included in these genera at one time or another.

Micropropagation of *Renanthera imschootiana* through the Culture of Shoot Tips and Leaf Explants

Renanthera imschootiana (Fig. Ren-1A), also known as the Red *Vanda*, is of major horticultural importance both as an orchid which is grown for its own flowers and as a parent of many popular hybrids. At present it is an "extremely rare and endangered epiphytic orchid [in] north-eastern India . . . [and] Burma [now Myanmar] . . . due to indiscriminate collection and severe habitat loss . . . [therefore] large scale multiplication in botanic gardens to meet trade demands has been recommended . . ." (Seeni and Latha, 1992). A method designed to accomplish this aim was developed at the Plant Biotechnology Unit, Tropical Botanic Garden and Research Institute, Palode, Trivandrum, India (Seeni, 1990; Seeni and Latha, 1992).

Plant Material. Stem cuttings, each with six leaves, should be taken from mature plants. Following surface sterilization, shoot tips of 0.5 cm, and whole leaves,

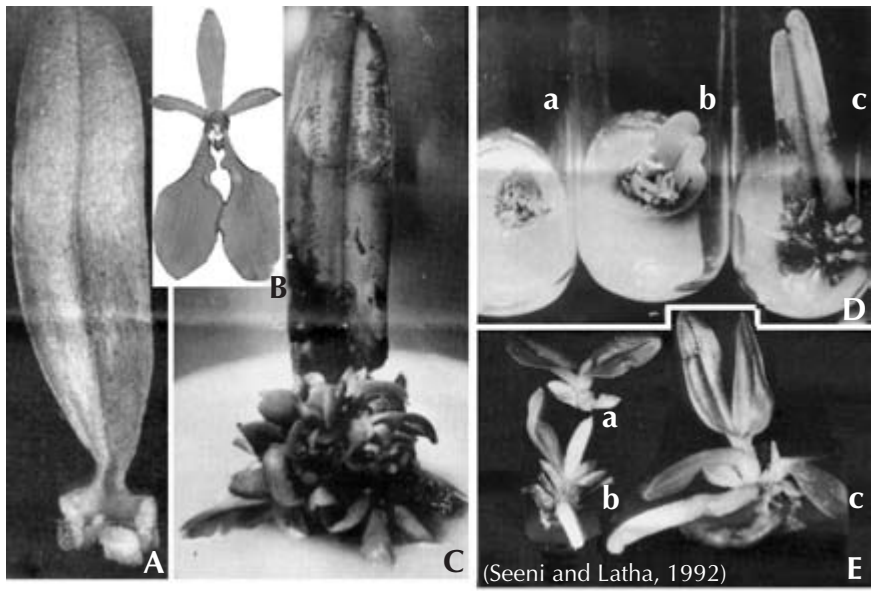


FIG. REN-1. **Micropropagation of *Renanthera imschootiana*. A. Swelling at the leaf base and early stages of vegetative bud formation after 4 weeks of culture. B. *Renanthera imschootiana* flower. C. Vegetative buds at the base of a leaf. D. Formation of vegetative buds on the first (a), second (b) and third (c) leaf of a stem. E. Plantlet development showing leaf and root formation (a–c).**

1.8–6.7 cm in length, including their sheathing bases measuring 3–8 cm should be cultured (Fig. Ren-1A, C, D).

Surface Sterilization. The stem sections should be washed with running tap water and soaked "for more than 15 min in 1% (v/v) commercial detergent (Teepol, Glaxo India, Pvt. Ltd., Bombay)" (Seeni and Latha, 1992). Teepol is also available from www.sigmaaldrich.com. A mild household detergent is also suitable. "Following the removal of the lowermost leaf" the sections should be dipped in 70% ethanol (73–74 ml 95% ethanol diluted to 100 ml with distilled water) for 30 s, immersed in 0.1% mercuric chloride ($HgCl_2$; available from most chemical supply houses, this reagent should be treated with caution since it is dangerous to humans and animals), and rinsed three times with sterile distilled water. All remaining leaves should be removed one by one following the rinse under sterile conditions, until the shoot tip with 1–2 leaf primordia is exposed. The shoot tip, ca. 5 mm in length, should be excised after that. Leaves and shoot tips should be immersed separately in 0.05% $HgCl_2$ for 2 min and washed three times with sterile distilled water.

Culture Vessels. Culture tubes, 25×150 mm, should be used for initial cultures, but larger vessels are better suited for plantlets.

Culture Conditions. Cultures should be maintained at $25 \pm 2°C$ under 15-h photoperiods of 25–50 $\mu mol\ m^{-2}\ s^{-1}$ provided by fluorescent tubes (type not described in the original paper) or standard culture room conditions.

Culture Media. Leaves should be placed first on Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) supplemented with BA, NAA, and peptone (Table Ren-1). Vegetative buds produced on this solution should be subcultured onto a substrate containing coconut water and pulp from ripe bananas (Table Ren-2). Shoots which develop on this medium form roots on a third version of MPR medium which contains BA, NA, and activated charcoal (Table Ren-3). Shoot tips should be cultured initially on a different modification of MPR (Table Ren-4).

Procedure. Leaf explants are planted vertically (Fig. Ren-1A, C, D) so that the leaf bases, which measure 3–8 mm, are buried 3–5 mm deep in the first version of MPR (Table Ren-1). Vegetative buds (Fig. Ren-1A, C) that form on this medium are moved to the second substrate (Table Ren-2) for shoot development. Shoots from the second medium are moved to the third solution (Table Ren-3) for rooting and plantlet formation (Fig. Ren-1E). When plantlets are large enough they are potted in community pots. Shoot tips are placed on the fourth medium (Table Ren-4). If any survive, the resulting tissues should be treated like those produced by leaf explants (Tables Ren-2 and Ren-4).

Developmental Sequence. Both leaves and shoot tips exude phenolics that cause browning of the medium. These compounds do not seem to have an ill effect on leaves, which produce 3–5 vegetative buds at their bases within 5 weeks. Up to 10 buds may be produced within 10–12 weeks on the first medium (Table Ren-1). As many as 40 shoots may develop after 12 weeks of subculture on the second medium

TABLE REN-1. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Renanthera imschootiana* leaves (Seeni, 1990; Seeni and Latha, 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
15	Benzyladenine (BA)	10.0	500 mg 100 ml^{-1} 95% ethanol[e,f]	2	
	Complex additive				
16	Peptone	2.0 g	No stock	No stock	Weigh
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH (or NaOH) or HCl, respectively.

[g]Add items 1–16 to 900 ml of distilled water (item 18), adjust pH to 5.6, add sugar (item 17), and raise volume to 1000 ml with distilled water (item 18). Bring the solution to a gentle boil and add the agar (item 19) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

TABLE REN-2. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for shoot production from *Renanthera imschootiana* leaf- and shoot-tip-derived vegetative buds (Seeni, 1990; Seeni and Latha, 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4{\cdot}H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2{\cdot}6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4{\cdot}5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2{\cdot}4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4{\cdot}7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Complex additives				
14	Coconut water (CW)[f]	100.0 ml	No stock	No stock	Measure
15	Ripe banana homogenate (BH)[g]	35.0 g	No stock	No stock	Weigh
16	Peptone	2.0 g	No stock	No stock	Weigh
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[h]	To 1000 ml			
	Solidifier				
19	Agar[h]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[g]Opinions differ regarding the use of banana in culture media. The predominant opinion is that only homogenized pulp of ripe banana should be used. Other views are that: (1) unripe (green) bananas are preferable, (2) the peel rather than the pulp should be used, (3) unpeeled banana homogenate should be incorporated in media, and (4) slices, ripe or unripe, peeled or unpeeled, rather than homogenate are most effective. Opinions also differ regarding which is the most effective banana variety (Gros Michel, Raja, Pisang Susu, and others). Since there is no experimental evidence to support any view the best approach is to follow the recommendations in the original reports and/or use what is suggested there.

[h]Add items 1–16 to 800 ml of distilled water (item 18) in a homogenizer, homogenize thoroughly, adjust pH to 5.6, add sugar (item 17), and bring volume to 1000 ml with distilled water (item 18). Bring the solution to a gentle boil and add the agar (item 19) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

TABLE REN-3. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the rooting of *Renanthera imschootiana* shoots produced by leaf- and shoot-tip-derived vegetative buds (Seeni, 1990; Seeni and Latha, 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
15	Benzyladenine (BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
16	Peptone	2.0 g	No stock	No stock	Weigh
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Agar[g]	7.0 g	No stock	No stock	Weigh
	Darkening agent				
20	Activated charcoal[g]	1.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH (or NaOH) or HCl, respectively.

[g]Add items 1–16 to 900 ml of distilled water (item 18), adjust pH to 5.6, add sugar (item 17), and bring volume to 1000 ml with distilled water (item 18). Bring the solution to a gentle boil and add the agar (item 19) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, add the darkening agent (item 20) slowly with vigorous stirring. After the darkening agent has been dispersed completely, pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

TABLE REN-4. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Renanthera imschootiana* shoot tips (Seeni, 1990; Seeni and Latha, 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
15	Benzyladenine (BA)	5.0	500 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
16	Peptone	2.0 g	No stock	No stock	Weigh
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH (or NaOH) or HCl, respectively.

[g]Add items 1–16 to 900 ml of distilled water (item 18), adjust pH to 5.6, add sugar (item 17), and bring volume to 1000 ml with distilled water (item 18). Bring the solution to a gentle boil and add the agar (item 19) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

(Table Ren-2), but 24 is more usual. Root initiation occurs within a week of moving the shoots to the third medium (Table Ren-3). Every shoot may have two to four well-developed roots at the end of 4 weeks. Shoot tips did not grow in culture.

General Comments. This protocol seems to be effective and efficient, but the high level of BA (10 mg l^{-1}) requires caution because it may cause undesirable mutations.

Renanthera imschootiana is mentioned in Rex Stout's Nero Wolfe mystery *Fourth of July Picnic* when the oversized Wolfe, upset by waiting for a witness to talk to the police, says: "I've had all the humiliation I can stand. Jumping out of my skin every time the phone rang. Did you notice how quickly I answered your ring upstairs? Afraid, by heaven, afraid to go into the tropical room to look over the *Renanthera imschootiana*!"

Micropropagation of *Renanthera imschootiana* through Axillary-Bud, Leaf-Segment, and Shoot-Tip Culture

As long as shoots are taken from a plant for micropropagation it is a good idea to attempt to culture as many parts of it as possible. A method which uses several explants from one shoot was developed at the Biotechnology Laboratory, Department of Plant Breeding and Genetics, College of Agriculture, Central Agricultural University, Imphal, Manipur, India (Laishram and Devi, 1999).

Plant Material. Shoots, 3–4 cm long, should be removed from mature plants (those used in the original research were grown in a shade house). Older leaves should be removed to expose axillary buds and the shoot tip, all of which should be excised and cultured. Young leaves should be sectioned into apical, median, and basal explants.

Surface Sterilization. Explants should be immersed with gentle agitation in 0.1% mercuric chloride ($HgCl_2$, available from most chemical supply houses, is toxic and should be handled with care) for 20 min and washed five times with sterile distilled water before being placed in the culture.

Culture Vessels. Culture tubes, Erlenmeyer flasks, and other containers can be used.

Culture Conditions. A temperature of 25 ± 2°C and 12-h photoperiods of 2500 lx are suitable, as are standard culture room conditions.

Culture Media. Shoot-tip explant should be cultured in Vacin and Went (VW) medium (Vacin and Went, 1949) supplemented with 20% coconut water (CW), 1 mg NAA l^{-1}, and 2 mg BA l^{-1} (Table Ren-5). VW medium with 20% CW and 1 mg each of NAA and BA per liter (Table Ren-6) should be used to culture axillary buds. Leaf segments should be cultured on VW with 20% CW, 0.1 mg NAA l^{-1} and 1.0 mg BA l^{-1} (Table Ren-7). No medium is suggested for the culture of PLBs to produce plantlets. The initial medium used for each explant may be suitable and should be tested. Other media that can be used are one or more of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables

TABLE REN-5. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of *Renanthera imschootiana* shoot tips (Laishram and Devi, 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	6-Benzylaminopurine (BAP)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
10	Coconut water[g]	200.0 ml	No stock	No stock	Measure
	Sugar				
11	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[h]	To 1000 ml			
	Solidifier				
13	Agar[h]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 12) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[g]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[h]Add items 1 and 3–10 to the 500–700 ml of distilled water (item 12) which contain the calcium phosphate (item 2), raise volume to 900 ml with distilled water (item 12), adjust pH to 5.6, add sugar (item 11), and adjust volume to 1000 ml with distilled water (item 12). Bring the solution to a gentle boil and add the agar (item 13) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of other nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable. Hormones are heat-labile and are usually added after autoclaving, but in this case they are incorporated into the medium before sterilization.

TABLE REN-6. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of *Renanthera imschootiana* axillary buds (Laishram and Devi, 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	6-Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
10	Coconut water[g]	200.0 ml	No stock	No stock	Measure
	Sugar				
11	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[h]	To 1000 ml			
	Solidifier				
13	Agar[h]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 12) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[g]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[h]Add items 1 and 3–10 to the 500–700 ml of distilled water (item 12) which contain the calcium phosphate (item 2), raise volume to 900 ml with distilled water (item 12), adjust pH to 5.6, add sugar (item 11), and adjust volume to 1000 ml with distilled water (item 12). Bring the solution to a gentle boil and add the agar (item 13) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable. Hormones are heat-labile and are usually added after autoclaving, but in this case they are incorporated into the medium before sterilization.

TABLE REN-7. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of *Renanthera imschootiana* leaf sections (Laishram and Devi, 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
8	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	6-Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
10	Coconut water[g]	200.0 ml	No stock	No stock	Measure
	Sugar				
11	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[h]	To 1000 ml			
	Solidifier				
13	Agar[h]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium, 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 12) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[g]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[h]Add items 1 and 3–10 to the 500–700 ml of distilled water (item 12) which contain the calcium phosphate (item 2), raise volume to 900 ml with distilled water (item 12), adjust pH to 5.6, add sugar (item 11), and adjust volume to 1000 ml with distilled water (item 12). Bring the solution to a gentle boil and add the agar (item 13) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable. Hormones are heat-labile and are usually added after autoclaving, but in this case they are incorporated into the medium before sterilization.

Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (see Table Pln-1), or VW (Vacin and Went, 1949) solution (see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C medium that contains this darkening agent (see Table Pln-1, footnote *f*). Media developed for *Renanthera* shoot formation from vegetative buds (Table Ren-2) and the rooting of these shoots (Table Ren-3) may also prove to be suitable.

Procedure. Shoot tips, axillary buds, and leaf sections are cultured on the appropriate media (Tables Ren-5, Ren-6, and Ren-7, respectively). PLBs produced on these media are moved onto one of the other solutions. When plantlets form they should be allowed to grow on the second medium until they become large enough for community pots or subcultured to fresh solution to accelerate the process.

Developmental Sequence. Seventy percent of the shoot tips start to produce PLBs 60 days after the start of culture. Only 10% of the axillary buds produce PLBs, starting 50 days after the cultures were initiated. The median and apical leaf segments do not produce PLBs. Of the basal explants, 50% start to produce PLBs 65 days after being paced on the appropriate medium. Plantlets develop from the PLBs.

General Comments. The use of a single shoot to obtain a shoot tip, several leaf explants, and a number of axillary buds is clever and economical. This procedure seems simple, effective, and productive. The hormone concentrations are relatively low and will probably not induce undesirable mutations.

Renanthera imschootiana was first imported by the firm of Messrs. Sander & Co of St. Albans in Britain. It was named in 1891 by Robert Allen Rolfe after Mr. M. A. van Imschoot of Ghent (Gand), Belgium (Bechtel et al., 1992).

Renanthera comes from the Latin *renes* for kidney and the Greek *anthera* for anther, in allusion to the kidney-shaped pollinia of the first species described by Loureiro in 1790.

Restrepia

When leaves of *Restrepia* that have already flowered are cut to include approximately 5 cm of stem and are planted in a mixture of tree fern and peat or peat only to the level of the leaf axil, they produce plantlets. "The function of the stem is merely to anchor the leaf to ensure that it will remain planted at the proper level" (Webb, 1981). Five different species of *Restrepia* have been propagated in this manner. This suggests that leaves or leaf explants of *Restrepia* could perhaps produce callus, PLBs, and/or plantlets in vitro on an appropriate culture medium. Despite not being an in vitro method for the micropropagation of *Restrepia*, this procedure is included here because it provides a means for relatively fast multiplication of this orchid.

Rhynchostele

A method for the proliferation of *Rhynchostele bictoniense* through the use of seeds and seedling explants was developed at the National University in Mexico (González, no date).

Rhynchostylis

A small genus of approximately four species, *Rhynchostylis* was first described by C. L. Blume in 1825 with the type species being *Rhynchostylis retusa*. The genus is distributed in India, Malaysia, Indonesia, and the Philippines. Some botanists have included all species in the genus *Saccolabium*. This suggests a close relationship, and it is possible that tissue culture procedures developed for one of these genera will also be suitable for the other.

Rhynchostylis gigantea

Monopodial orchids have been more difficult to propagate through tissue culture, especially of their shoot tips, than sympodial ones. The reason for this, at least in part, is probably that they have different or more complex requirements. Thus,

> after reviewing the previous . . . work on tissue and organ culture, . . . an . . . attempt was made to formulate a new medium for culturing tissues of monopodial-type orchids. . . . [The attempt was successful to a limited extent and] even though it cannot be applied easily in the commercial multiplication of this type of orchid at the present time, it may provide a clue for further developments in culturing orchid tissues. (Vajrabhaya and Vajrabhaya, 1970)

Plant Material. In the original experiment, seedlings of *Rhynchostylis gigantea* collected from the wild were utilized. Buds from leaf-covered stems and shoot tips were used as the source of explants.

Surface Sterilization. The procedure employed for *Cymbidium* (Sagawa et al., 1966) is suitable for this species.

Culture Vessels. Employ 21 × 173-mm test tubes, containing medium to a height of 35 mm, placed in an upright position.

Culture Conditions. Maintain cultures under diffuse light of an intensity ranging between 2000 and 3000 lx for 14-h photoperiods. In the original experiments, Philips TL 40/33 and TL 46/54 fluorescent tubes were used in a 1 : 1 ratio. It would seem reasonable to assume that Sylvania Gro Lux (or the equivalent) or a combination of cool white fluorescent tubes and incandescent bulbs would be equally suitable. The temperature should be 24–27°C.

Culture Medium. A semisolid medium formulated especially for this species is used (Table Rhy-1). This medium is also suitable for *Dendrobium* and *Cattleya*.

Procedure. The apical meristem is excised like that of *Cymbidium* (Sagawa et al., 1966). Only five to six explants can be secured from any one seedling of the size used in the original experiments. Place explants on the culture medium.

TABLE RHY-1. **Medium for the culture of shoot-tip and bud explants of *Rhynchostylis gigantea* (Vajrabhaya and Vajrabhaya, 1970)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	500	50 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Potassium chloride, KCl	250	25 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	1.0	1 g l^{-1}	10	One solution
(b)	Cobaltous chloride, $CoCl_2 \cdot 6H_2O$	0.02	2 mg l^{-1}		
(c)	Cupric chloride, $CuCl_2 2 \cdot 2H_2O$	0.01	1 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	2.0	2 g l^{-1}		
(e)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.02	2 mg l^{-1}		
(f)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.1	10 mg l^{-1}		
	Vitamins				
8	Calcium pantothenate	0.25	100 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Pyridoxine (vitamin B_6)	0.25	100 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Thiamine (vitamin B_1)	0.25	100 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
12	Naphthaleneacetic acid (NAA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1.0	
	Amino acid				
13	Glycine	7.5	750 mg 100 ml^{-1} 95% ethanol[e]	1.0	
	Complex additives				
14	Tryptone	500	No stock	No stock	Weigh
15	Coconut water	100 ml	No stock	No stock	Measure
	Sugar				
16	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[f]	To 1000 ml			
	Solidifier				
18	Agar, Difco Bacto[f]	6 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original recipe suggests 50 mg of (Fe-EDTA), a preparation which may not be readily available. The substitute given in this table is widely used in culture media for orchids and other plants. Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved. This can also be described as Fe_2EDTA.

[d]Add the salts to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium.

[e]Keep refrigerated or frozen between uses.

[f]Add items 1–7, 14, and 15 to 800 ml distilled water (item 17); adjust pH to 5.2–5.5, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18). Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour the solution into a 2-l flask and autoclave. Add vitamins (items 8–11), hormone (item 12), and glycine (item 13) to the hot autoclaved solution with sterilized pipettes, mix well by swirling, and distribute medium to preautoclaved culture vessels. Omit agar for liquid media.

Development Sequence. During the first week of culture, most explants enlarge and some portions turn green. From weeks 2 through 4 a few produce new tissues, but some of these die. At the end of 2 months, only one explant in 30 survived in the original experiment. The survivors formed calli of up to 10 mm in diameter. Growth was slow, but no abnormalities were detected after five subcultures. If not subcultured, the calli will produce plantlets.

General Comments. Disadvantages of this method are the low rate of success and the need to destroy an entire plant or growth. However, considering the difficulties encountered with the culture of monopodial orchids, the procedure represents a significant advance in the clonal propagation of desirable clones.

Propagation of *Rhynchostylis retusa* through the Culture of Leaf Segments

"The potential of *Cymbidium* leaf tissues to produce protocorm-like bodies in culture, first demonstrated by Wimber (1965), opened up new possibilities . . . and the list of taxa propagated, using leaf tissues as explants, is . . . growing" (Vij and Pathak, 1990). One species added to this list through research at the Department of Botany, Panjab University, Chandigarh, India, is *Rhynchostylis retusa* (Vij et al., 1984).

Plant Material. Segments 0.5–1.0 cm long from young leaves of 6-month-old seedlings grown in vitro are the only ones that can be cultured successfully. Explants from mature leaves died within 12 weeks of culture.

Surface Sterilization. It is not necessary to sterilize leaves of seedlings that are growing under aseptic conditions in vitro.

Culture Vessels. Culture tubes and/or Erlenmeyer flasks are suitable as culture vessels.

Culture Conditions. Cultures should be maintained under 12-h photoperiods of 3500 lx at 25 ± 2°C.

Culture Media. A modified Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) is used for the initial culture (Table Rhy-2). An unmodified MPR medium (Table Rhy-3) is used for organogenesis.

Procedure. Place the explants on the initial medium (Table Rhy-2), and allow them to remain on it for at least 4 weeks or for as long as is necessary for the development of PLBs. Excise these and place them on the unmodified medium (Table Rhy-3) for leaf and root formation.

Developmental Sequence. Sporadic divisions in some of the epidermal cells are the first signs of growth. Groups of cells with dense cytoplasm also form. These are followed by meristematic activity and bulges that eventually form PLBs. Formation of

TABLE RHY-2. **Modified Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) for the culture of *Rhynchostylis retusa* leaf segments (Vij et al., 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100	10 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	200	20 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180	18 g l^{-1}	10	
5	Monosodium phosphate, NaH_2PO_4	150	15 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	1.0	200 mg 100 ml^{-1} 95% ethanol[e]	0.5	
	Cytokinin				
15	Kinetin (6-furfurylaminopurine)	1.0	200 mg 100 ml^{-1} 95% ethanol[e]	0.5	
	Complex additive				
16	Peptone	300[f]	No stock	No stock	Weigh
	Sugar				
17	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Agar, Difco Bacto[g]	9 g	No stock	No stock	Weigh
	Darkening agent				
20	Activated charcoal[h]	2	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original recipe used 3 ml $Na_2FeEDTA$, a preparation that may not be readily available. The substitute listed here is used very commonly in orchid tissue culture media. Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium.

[e]Keep refrigerated or frozen between uses.

[f]The amount used in the original study is not listed. This is an estimate based on work with *Rhynchostylis* root tips in the same laboratory (Sood and Vij, 1986; Vij et al., 1987). One can experiment by varying the amount from 250 mg to 2 g.

[g]Add items 1–16 to 900 ml distilled water (item 18), adjust pH to 5.6, add sugar (item 17), and adjust volume to 1000 ml with distilled water (item 18). Bring solution to a gentle boil, and add agar (item 19) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, add charcoal (item 20) and stir well to insure complete, uniform mixing. Dispense solution into culture vessels, and autoclave. Omit agar for liquid medium. Media that contain hormones and vitamins should not be autoclaved unless prior experimentation has determined that this has no deleterious effects. This is such a case (Mitra, 1971; Mitra et al., 1976), so the medium may be autoclaved.

[h]The original paper does not describe the charcoal. In our experience, activated vegetable charcoal is preferable to that from animal sources.

TABLE RHY-3. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) for the organogenesis of *Rhynchostylis retusa* leaf explants (Vij et al., 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100	10 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	200	20 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180	18 g l^{-1}	10	
5	Monosodium phosphate, NaH_2PO_4	150	15 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Sugar				
14	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[f]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[f]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original recipe used 3 ml $Na_2FeEDTA$, a preparation that may not be readily available. The substitute listed here is used very commonly in orchid tissue culture media. Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium.

[e]Keep refrigerated or frozen between uses.

[f]Add items 1–13 to 900 ml distilled water (item 15), adjust pH to 5.6, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense solution into culture vessels and autoclave. Omit agar for liquid medium. Media that contain vitamins should not be autoclaved unless prior experimentation has determined that this will have no deleterious effects. This is such a case (Mitra, 1971; Mitra et al., 1976), so the medium may be autoclaved.

PLBs on the initial medium (Table Rhy-2) occurs within 4 weeks. When these PLBs are excised and cultured on the second medium (Table Rhy-3), they differentiate small leaves within 5 weeks and roots following an additional 21 days. Plantlets ready for subculture into community pots develop within a total of 10 weeks.

General Comments. Methods that utilize explants that do not endanger the plant to be propagated are very desirable and necessary. The only problem with this procedure is its inapplicability to mature plants. However, Vij's scientific reputation suggests that he and his associates will overcome this hurdle.

Plantlet Regeneration in Vitro from Root Segments of *Rhynchostylis retusa*

A number of workers (Vij et al., 1987) have shifted their emphasis "toward exploring alternative plant organs for rapid propagation . . . whereby the very existence of the mother plant is not threatened." This has led to a ". . . study [which] deals with the regeneration potential of *Rhynchostylis retusa* roots *in vitro*" (Sood and Vij, 1986).

Plant Material. Root segments 0.5–1.0 cm long without tips, taken from 38-week-old seedlings, are cultured.

Surface Sterilization. It is not necessary to surface-sterilize explants taken from seedlings growing in vitro.

Culture Vessels. Culture tubes and/or Erlenmeyer flasks are suitable as culture vessels.

Culture Conditions. Cultures should be maintained under 12-h photoperiods of 3500 lx at 25 ± 2°C.

Culture Media. Explants can be cultured on several modifications of the Mitra medium, but a modified MS solution (Table Rhy-4) is more suitable for initial culture. Use the same medium or a different modification (Table Rhy-5) for leaf formation and root induction.

Procedure. Place explants with the tips removed on the initial medium (Table Rhy-4), and allow to form PLBs. Move these to the same (Table Rhy-4) or the second medium (Table Rhy-5) for plantlet formation. Transfer well-developed plantlets to community pots.

Developmental Sequence. Explants with intact tips elongate and develop root hairs. Those without tips (root-tip explants whose tips have been removed) produce PLBs within 5 weeks. (Those who use this method for the first time should try both kinds of explants.) Shoots and buds form after 12 weeks. Leaves can be seen on the second medium after approximately 2 months. Complete plants develop after 12 weeks.

General Comments. This procedure is valuable, especially as a starting point for future research. When adapted for use with roots from adult plants, this will be an extremely valuable method. Those who wish to culture roots from mature plants should select plants that have not come into contact with a source of fungi that could penetrate them.

TABLE RHY-4. **Modified MS medium (Murashige and Skoog, 1962) for the initial culture of *Rhynchostylis retusa* root segments (Vij et al., 1987)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1	200 mg 100 ml^{-1} 95% ethanol[f,g]	0.5	
	Cytokinin				
11	Kinetin	1	200 mg 100 ml^{-1} 95% ethanol[f,g]	0.5	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin do not dissolve, add a few drops of dilute KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour the solution into a 2-l flask and autoclave; 2 mg l^{-1} activated vegetable charcoal can be added to hot agar solution and mixed well before pouring into the 2-l flask. Add amino acid, hormones, and vitamins (items 8 and 10–14) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

TABLE RHY-5. **Modified MS medium (Murashige and Skoog, 1962) for leaf and root initiation and plantlet formation from protocorm-like bodies derived from *Rhynchostylis retusa* root segments (Vij et al., 1987)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1	200 mg 100 ml^{-1} 95% ethanol[f,g]	0.5	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
14	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[h]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[h]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]If auxin does not dissolve, add a few drops of dilute KOH.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 15), adjust pH to 5.6, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour the solution into a 2-l flask and autoclave; 2 mg l^{-1} activated vegetable charcoal can be added to hot agar solution and mixed well before pouring into 2-l flask. Add amino acid, hormone, and vitamins (items 8 and 10–13) to hot autoclaved solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

Isolation of *Rhynchostylis* Protoplasts

The method developed for *Acampe praemorsa* was used to isolate 0.7×10^4 and 0.1×10^4 protoplasts from leaves and roots, respectively, of *Rhynchostylis retusa* (Seeni and Abraham, 1986).

Clonal Multiplication of *Rhynchostylis retusa* through Root and Leaf-tip Culture in Vitro

Root and leaf tips taken from "aseptically-grown plantlets" (probably seedlings) of *Rhynchostylis retusa* formed PLBs on a modified Vacin and Went medium supplemented with 1 ppm BA, 1 ppm IAA, and 200 ppm casein hydrolysate (CH). PLBs develop without callus formation. The plantlets proliferate with periodic subculture (every 2 months) on a medium containing 0.5 mg BA, 1 mg IAA, and 0.1 mg 2,4-D per liter. Plantlets develop well on a medium containing 0.1 ppm IAA and 200 ppm CH (Sharma and Chaturvedi, 1988).

Plantlet Production from *Rhynchostylis retusa* Root Explants

Rhyncostylis retusa is found in India, Malaysia, Myanmar, Philippines, Sri Lanka, and Vietnam. It is a popular orchid among growers and is getting rarer in nature. A method using root explants to propagate it was developed by Professor Suraj P. Vij at Panjab University (Vij et al., 1987).

Plant Material. Root-tip segments, 5–10 mm long (Fig. Rhy-1A), should be taken from 38-week-old seedlings growing in vitro.

Surface Sterilization. Explants taken from seedlings growing in vitro do not require surface sterilization.

Culture Vessels. Culture tubes, Erlenmeyer flasks, and other containers are suitable.

Culture Conditions. Cultures should be maintained at $25 \pm 2°C$ under 12-h photoperiods of 3500 lx (source was not listed) or standard laboratory conditions.

Culture Media. MS medium (Murashige and Skoog, 1962) containing 1 mg NAA l^{-1} and 2 g activated charcoal l^{-1} (Table Rhy-6) should be used.

Procedure. Explants are placed on the medium and allowed to form plantlets. If/when the cultures become crowded, plantlets should be separated and subcultured onto the same medium and allowed to reach community pot size.

Developmental Sequence. Root-tip explants (Fig. Rhy-1A) form PLBs within weeks (Fig. Rhy-1B). Vegetative buds (Fig. Rhy-1C) are formed if IAA is used instead of NAA. Leaves (Fig. Rhy-1G) develop after 6 weeks on the medium (Table Rhy-6).

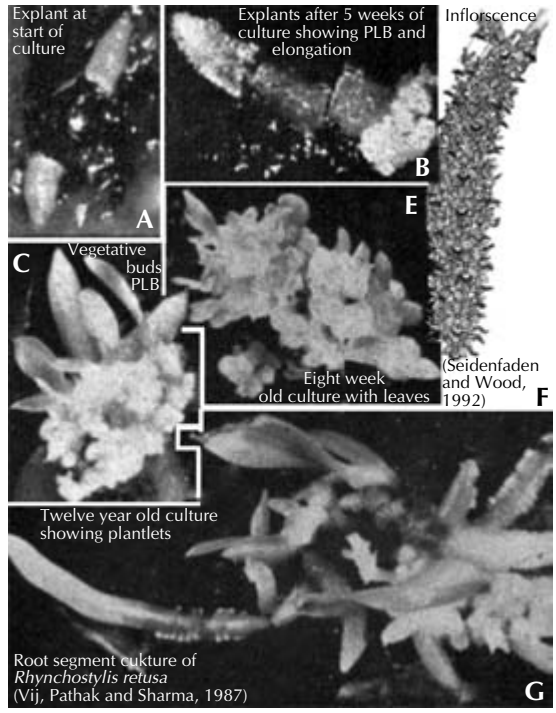


FIG. RHY-1. **Culture of *Rhynchostylis retusa* root explants (Vij et al., 1987).**

Roots appear after 8 weeks. Plantlets with 2–3 leaves and 1–2 roots (Fig. Rhy-1E) can be seen in 12 weeks.

General Comments. Root tips are useful as explants because plants have many of them and their removal does not cause roots to die. If taken from mature plants in pots or in nature, they will harbor mycorrhiza which will contaminate and overrun cultures.

Rhynchostylis from the Greek *rhynchos* (beak) and *stylis* (column) alluding to the beak-like gynostemium (Schultes and Pease, 1963).

TABLE RHY-6. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Rhynchostylis retusa* root explants (Vij et al., 1987)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh
	Darkening agent				
17	Activated charcoal[h]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.6–5.8, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved add the charcoal while stirring continuously. Once the charcoal is completely dispersed, pour the solution into culture vessels and autoclave. Amino acid (item 8), hormone (item 10), and vitamins (items 11–13) are usually added after autoclaving under sterile conditions with sterilized pipettes, but in this case they are added before sterilization. Agar is not added to liquid media.

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances.

Production of *Rhynchostylis retusa* Plantlets from Foliar Peels

Foliar peels offer three advantages as explants. One is their abundance. Another is that their removal does not endanger or even damage the donor plant. And, third, they "are low in endogenous growth hormones . . . and are thus expected to be more sensitive to even slightest changes [in] the quantity and quality of . . . growth regulators" (Vij and Kaur, 1992; Vij, 1994).

Plant Material. In the original research "peels, 1–4 layers in thickness and 0.5 mm in length were . . ." taken from "juvenile leaves [of] *in vitro* raised cultures." It is not clear from this statement whether the "cultures" were of seedlings or plantlets produced through tissue culture. A photograph (Fig. Rhy-2A) suggests that the length should be 0.5 cm.

Surface Sterilization. Explants from in vitro cultures do not require sterilization.

Culture Vessels. Culture tubes, Erlenmeyer flasks, or other containers can be used.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx or standard culture room conditions.

Culture Medium. Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) with 2 mg kinetin l^{-1} (Table Rhy-7) should be used.

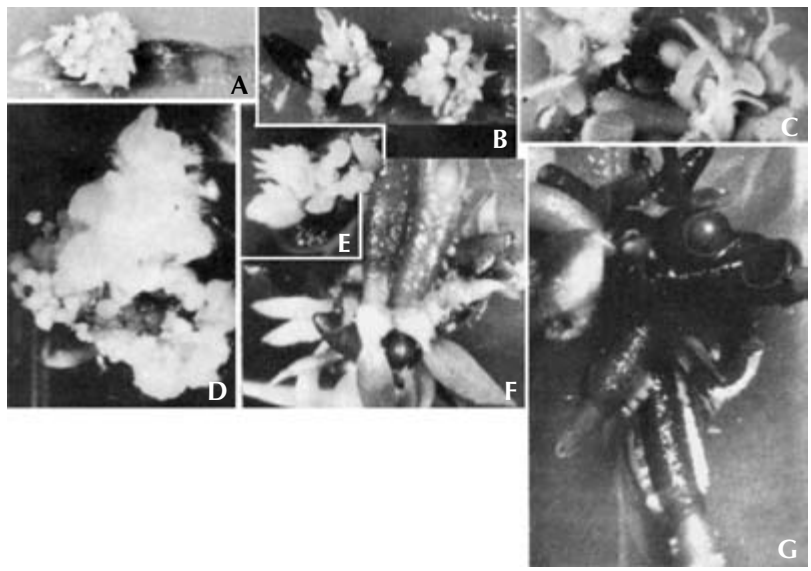


FIG. RHY-2. **Plantlet production from *Rhynchostylis retusa* from foliar peels. A. PLBs. B. Multiplication of PLBs. C. Plantlet formation. D. Organogenetic callus. E. Differentiating PLBs. F. Plantlet formation. G. Root formation and suppressed growth of leaves following prolonged culture on kinetin-containing medium. (Vij and Kaur, 1992.)**

TABLE RHY-7. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Rhynchostylis retusa* leaf peels (Vij and Kaur, 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate $Ca(NO_3)_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Cytokinin				
14	Kinetin	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1 ml	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve add a few drops of 0.1 N HCl.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

Procedure. Peels are placed on the medium (Table Rhy-7) where they form PLBs (Fig. Rhy-2A, B) and eventually plantlets (Fig. Rhy-2C, E–G). An organogenetic callus (Fig. Rhy-2D) forms in the presence of 1 mg 2,4-D l^{-1}. If cultures become crowded, PLBs or plantlets should be subcultured on the same medium.

Developmental Sequence. PLBs are produced by 75% of explants after 4 weeks of culture. Leaves appear in 6 weeks. Roots develop 2 weeks later. Plantlets with two leaves and 2–3 roots are formed after 11 weeks.

General Comments. Explants from seedlings cannot be used for cultivar selection, but this is less important for a species than for hybrids.

Micropropagation of *Rhynchostylis retusa* through the Culture of Leaf Segments

Leaf explants offer two advantages. One is abundant material because plants have many leaves. The second is that the removal of a few leaves does not endanger the donor plant. Explants from leaves of mature plants "copiously released brownish exudates (phenolics?) into the medium (irrespective of the use of A[ctive] C[harcoal]), lost their chlorophyll, and perished within 10–20 w[eeks] without showing any meristematic activity. However, those from seedlings leaves in axenic cultures proliferated along their cut ends . . ." (Vij and Pathak, 1990).

Plant Material. "Young leaves, up to 2 cm in length from . . . 16–40 w[eek] old axenic seedlings . . . were segmented into 0.5–1.0 cm long basal (with leaf base) and apical (with leaf tip) parts and used as explants" (Vij and Pathak, 1990).

Surface Sterilization. Explants from leaves growing in vitro do not require surface sterilization.

Culture Vessels. Culture tubes, Erlenmeyer flasks, and other containers are suitable.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx or standard culture room conditions.

Culture Media. When cultured on hormone-free MS medium (Murashige and Skoog, 1962) darkened with active charcoal (Table Rhy-8), 50% of both apical and basal explants produced an average of ten PLBs (Fig. Rhy-3A–C) per explant (i.e., theoretically 100 leaves can produce 500 PLBs from apical explants and an equal number from basal ones). If IAA, NAA, kinetin, and/or BA are added to the medium alone or in several combinations, the total number of PLBs produced is smaller. PLBs should be moved to MS supplemented with NAA and kinetin (Table Rhy-9) for plantlet (Fig. Rhy-3D) production.

Procedure. The leaves are cut in half, placed on the medium, and cultured on the first medium (Table Rhy-8) until the PLBs produced by the explants are sufficient

TABLE RHY-8. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Rhynchostylis retusa* leaf explants (Vij and Pathak, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[f]	To 1000 ml			
	Solidifier				
15	Agar[f]	9.0 g	No stock	No stock	Weigh
	Darkening agent				
16	Activated charcoal (AC)[g]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH to 5.5, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, add the charcoal (item 16) with constant stirring to insure complete and even distribution, pour the solution into culture vessels, and autoclave. Amino acid (item 8) and vitamins (items 10–12) are heat-labile and are usually added after autoclaving to the hot and still liquid medium under sterile conditions with sterilized pipettes. In this case they are added before sterilization. Agar is not added to liquid media.

[g]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no real difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. Two possible sources are www.fishersci.com and www.vwr.com, but there are many others.

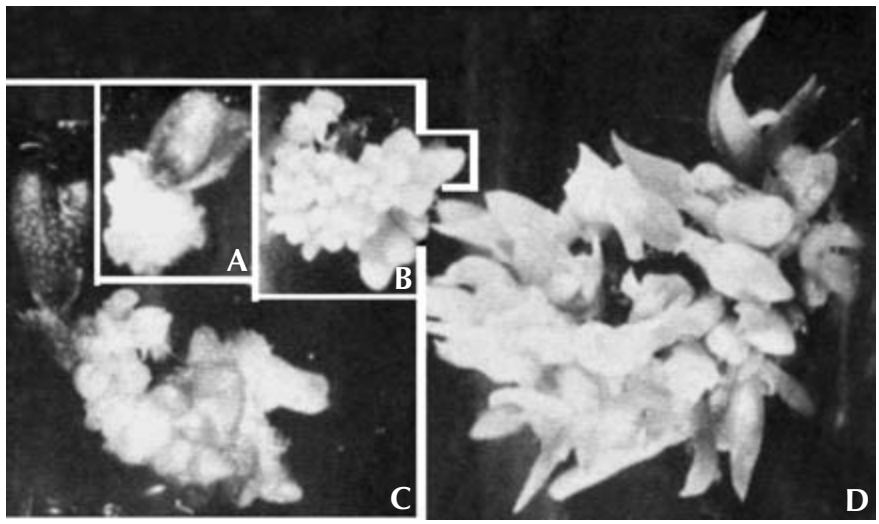


FIG. RHY-3. **Culture of *Rynchostylis retusa* leaf explants. A–C. Multiplication of PLBs. D. Plantlets. (Vij and Pathak, 1990.)**

in number and large enough to be transferred to the second solution (Table Rhy-9). Once the plantlets produced on the second solution are large enough they should be moved to community pots.

Developmental Sequence. Explants produce PLBs (Fig. Rhy-3A–C) on the first medium (Table Rhy-8) 5 weeks after being placed in culture. The first leaf appears after 8 weeks. Roots form 16 weeks after cultures are initiated. Plantlets (Fig. Rhy-3D) develop well on the second substrate (Table Rhy-9).

General Comments. This seems to be a productive method, but like other procedures that utilize explants from seedlings, it cannot be used to propagate desirable clones because the quality of seedlings is not known. However this protocol can be used to propagate *Rhynchostylis retusa* plantlets that have been produced by explants taken from mature plants. The use of a hormone-free initial medium (Table Rhy-8) is an advantage because hormones can induce undesirable mutations, sometimes even when used at low concentrations.

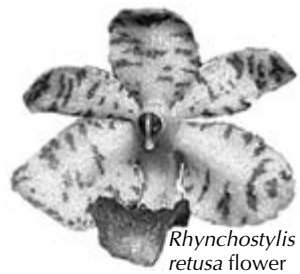

Rhynchostylis retusa flower

TABLE RHY-9. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for plantlet production from leaf-explant-derived protocorm-like bodies of *Rhynchostylis retusa* (Vij and Pathak, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Kinetin	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh
	Darkening agent				
18	Activated charcoal (AC)[h]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–12 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved add the charcoal (item 18) with constant stirring to insure complete and even distribution, pour the solution into culture vessels, and autoclave. Amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) are heat-labile and are usually added after autoclaving to the hot and still liquid medium under sterile conditions with sterilized pipettes. In this case they are added before sterilization. Agar is not added to liquid media.

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

Micropropagation of *Rhynchostylis retusa* through the Culture of Root Explants

Professor Suraj P. Vij and his associates Sanjeev Arora, Parminder Kaur, Kusant Mahant, Promila Pathak, Vishal Sharma, and Anil Sood have been imaginative and effective in identifying explants for orchid micropropagation. Use of root explants for 12 orchid taxa including *Rhynchostylis retusa* is one of their innovations (Vij, 1993).

Plant Material. Initial explants, "young and actively growing roots, with and without well developed caps were . . ." taken from both 16–30-week-old seedlings and mature plants growing under horticultural conditions. They were cut into sections, 5–10 mm long. Explants from mature plants "released brownish exudates (phenolics!) into the medium and perished within 8–10 w[eeks] of inoculation." Several root explants from seedlings proliferated and produced plantlets.

Surface Sterilization. Explants taken from axenically grown seedlings do not require surface sterilization.

Culture Vessels. Culture tubes, Erlenmeyer flasks, and other containers can be used.

Culture Conditions. Cultures should be maintained under 25 ± 2°C under 12-h photoperiods of 3500 lx or standard culture room conditions.

Culture Media. Explants produced PLBs and plantlets on several media including hormone-free MS medium (Murashige and Skoog, 1962) darkened with activated charcoal (Table Rhy-10). This medium is recommended here because the use of hormones should be avoided whenever possible since they can cause (even if not always) undesirable mutations.

Procedure. Explants are placed on MS medium with activated charcoal and allowed to produce PLBs. The PLBs should be subcultured on the same medium for leaf, root, and plantlet production.

Developmental Sequence. Only 25% of the root explants produce PLBs. Explants on MS with NAA, or kinetin and NAA and/or IAA, respond similarly. PLBs, an average of ten per explant, are produced 6 weeks after the start of culture. Leaves (Fig. Rhy-4) appear 1 week later. Roots appear 3 weeks after that (i.e., 10 weeks after cultures were initiated).

General Comments. Despite the relatively low yields, the use of root explants is a good idea because plants have many roots and the removal of a few does not endanger the donor plant. Therefore a micropropagation method using root explants from mature plants would be very useful.

TABLE RHY-10. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Rhynchostylis retusa* leaf explants (Vij, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[f]	To 1000 ml			
	Solidifier				
15	Agar[f]	9.0 g	No stock	No stock	Weigh
	Darkening agent				
16	Activated charcoal (AC)[g]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH to 5.5, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved add the charcoal (item 16) with constant stirring to insure complete and even distribution, pour the solution into culture vessels and autoclave. Amino acid (item 8) and vitamins (items 10–12) are heat-labile and are usually added after autoclaving to the hot and still liquid medium under sterile conditions with sterilized pipettes. In this case they are added before sterilization. Agar is not added to liquid media.

[g]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no real difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. Two possible sources are www.fishersci.com and www.vwr.com, but there are many others.

FIG. RHY-4. ***Rhynchostylis retusa* plantlet produced from root explant. (Vij, 1993.)**

Micropropagation of *Rhynchostylis retusa* from Dermal Peels of Leaves

A method developed at the Botany Department, Panjab University, for plantlet production of *Rhynchostylis retusa* foliar peels (Vij and Kaur, 1992) was refined and presented at the Nagoya International Orchid Show (Vij, 1994). The rationale behind using dermal peels as explants was the observation that root and leaf explants produce new tissues from their dermal cells (Sood and Vij, 1986; Vij and Pathak, 1990).

Plant Material. Dermal peels, 5 mm long (width not indicated) and 1–4 cell layers thick (Fig. Rhy-5A, B) were taken from juvenile leaves (presumably of seedlings) in axenic cultures.

Surface Sterilization. Explants taken from axenic plants in vitro do not require surface sterilization.

Culture Vessels. Culture tubes, Erlenmeyer flasks, and other containers are suitable.

Culture Conditions. Cultures should be maintained under 25 ± 2°C under 12-h photoperiods or standard culture room conditions.

Culture Media. Several combinations of the Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976), activated charcoal, and hormones were tested in the original research (Table Rhy-11). The best results were obtained when the medium was supplemented only with 2 mg kinetin l^{-1} (bold in Table Rhy-11).

Procedure. Explants are placed on medium (Table Rhy-12) and cultured until PLBs form and increase in size. PLBs are subcultured on the same medium for plantlet development.

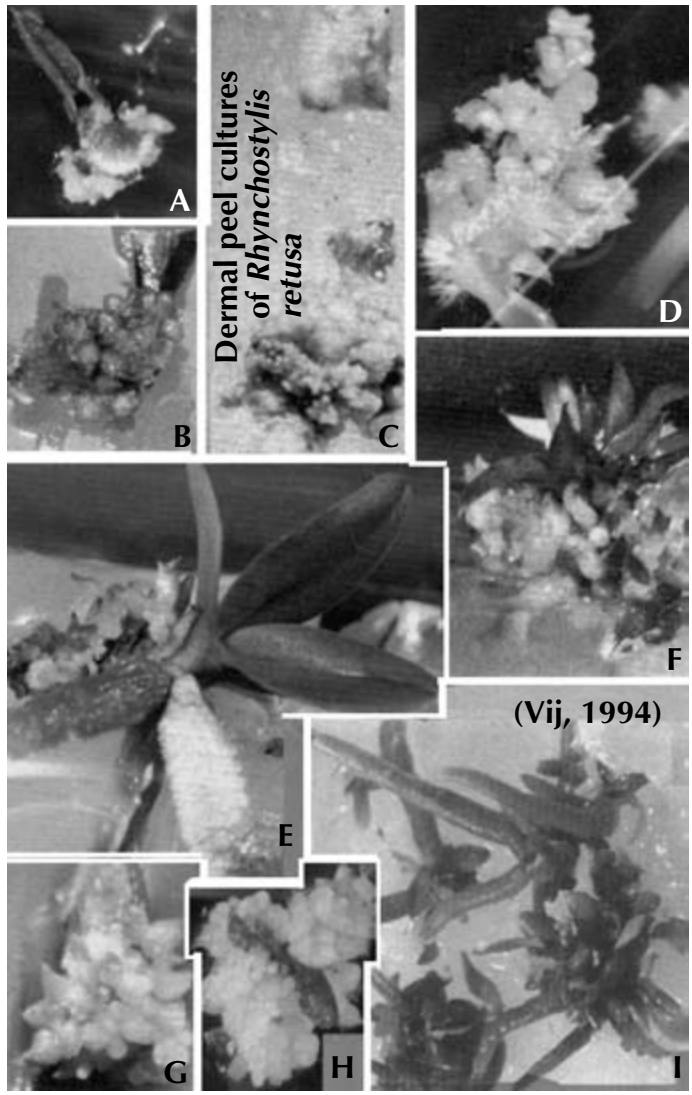


FIG. RHY-5. **Culture of dermal peels of *Rhynchostylis retusa*. A, B. Protocorm-like body development on NAA-containing medium. C, D. Callus-mediated PLB formation on 2,4-D-containing substrate. E. Plantlet. F. Plantlet differentiation on kinetin- and NAA-containing medium. G. PLB formation on solution containing kinetin (2 mg l^{-1}). H. PLB multiplication on medium containing kinetin (3 mg l^{-1}). I. Long-term cultures showing suppressed shoot growth and accelerated root development. (Vij, 1994.)**

TABLE RHY-11. **Hormone effects on dermal peels of *Rhynchostylis retusa* (Vij, 1994)**

Additives[a]	Amount, mg l^{-1}	Proportion responding, percent	PLBs per explant	Time required for development, weeks			
				PLB	Callus	Plantlets	Remarks
NAA	1	25	20	7		14	
NAA+AC	1+2	25	8	17		28	
2,4-D+AC	1+2	75	22	8	5	14	
Kinetin	**2**	**75**	**25**	**4**		**14**	
Kinetin	3	25	35	3		16	
NAA+kinetin	1+1	75	24	3		9	Deformed shoots
NAA+kinetin+AC	1+1+1	50	18	6		13	
IBA+kinetin	1+1	50		7	6		
IAA	1	0					
IBA	1	0					

[a]2,4-D, 2,4-dichlorophenoxyacetic acid; AC, activated charcoal; IAA, indoleacetic acid; IBA, indolebutyric acid; NAA, naphthaleneacetic acid; PLBs, protocorm-like bodies. The most effective treatment is in bold face.

Developmental Sequence. An average of 25 PLBs formed on 75% of the explants after 4 weeks of culture. Plantlets developed after 10 weeks after that.

General Comments. This protocol can be useful for propagating seedlings in cases when only a few seeds are available. It can also be useful for the propagation of tissue-culture-derived plantlets. If it could be adapted to young leaves of mature plants this method will also become useful for the propagation of selected cultivars of *Rhynchostylis retusa* and some of its intra- and intergeneric hybrids.

TABLE RHY-12. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Rhynchostylis retusa* leaf peels (Vij and Kaur, 1992)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Cytokinin				
14	Kinetin	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1 ml	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve add a few drops of 0.1 N HCl.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

Micropropagation of *Rhynchostylis retusa* through the Culture of Floral Buds

Floral buds attracted attention as explants because they are abundant and their removal does not endanger the donor plant. They were cultured at the Department of Botany, Panjab University (Kaur and Vij, 1995).

Plant Material. Inflorescences were excised "at different developmental stages" (Table Rhy-13) and cut into sections, 4–6 mm long.

Surface Sterilization. The sections should be washed with running water and Teepol (www.sigmaaldrich.com or other sources) or a mild liquid detergent. They should then be dipped in 70% ethanol (73–74 ml 95% ethyl alcohol diluted to 100 ml with distilled water) for 10 s, soaked in 0.01% streptomycin (www.sigmaaldrich.com, www.fishersci.com, www.vwr.com, and other sources), submerged in saturated calcium hypochlorite (7 g calcium hypochlorite in 100 ml distilled water stirred vigorously, allowed to stand until a precipitate forms, stirred again, and allowed to settle before the liquid is decanted and used) for 15 min, and rinsed three times with sterile distilled water.

Culture Vessels. Culture tubes, Erlenmeyer flasks, and other containers can be used.

Culture Conditions. Explant cultures should be maintained at $25 \pm 2°C$ under 12-h photoperiods of 3500 lx or standard culture room conditions.

Culture Media. The response of the explants seems to depend on the developmental stage of the flower bud and to be affected by the medium used (Table Rhy-13). To induce PLBs, floral buds from the upper two-thirds part of the inflorescence should be cultured on MPR medium (Mitra et al., 1976) supplemented with NAA and darkened with activated charcoal (Table Rhy-14).

Procedure. The inflorescences are removed from the plant and sectioned. After surface sterilization, the sections are placed in culture. When PLBs develop they should be transferred to fresh medium of the same composition (Table Rhy-14) for plantlet formation.

Developmental Sequence. Explants from the upper two-thirds portion of inflorescences form PLBs which develop into plantlets (Table Rhy-13).

General Comments. This method makes possible the propagation of selected clones. Only buds from the upper part of the inflorescence form PLBs and eventually plantlets (Table Rhy-13). Explants from the lower two-thirds of the inflorescence produce roots but no shoots on MPR medium darkened with activated charcoal or supplemented with NAA (Table Rhy-13). It may be possible to induce shoot formation on these root-bearing explants by moving them to a medium that contains 1 mg BAP l^{-1} but no NAA or charcoal. To produce flowers in a flask (mostly

TABLE RHY-13. **Response of *Rhynchostylis retusa* floral buds to different media**[a]

Medium	Differentiated floral buds	Undifferentiated floral buds	
		Upper $^2/_3$ part of inflorescence	Lower $^2/_3$ part of inflorescence
MPR + NAA			Proximal end of explants rooted in four weeks. No shoot formation
MPR + NAA + AC		PLB formed in 5 weeks and proliferated to a count of 20. Plantlets formed in 12 weeks	Proximal end of explants rooted in four weeks. No shoot formation
MPR + NAA + CW	Flowers developed		
MPR + NAA + CW + AC	Flowers developed		
MPR + CW	Flowers developed		
MPR + CW + AC	Flowers developed		
VW + NAA + BAP		Vegetative shoots develop in six weeks. Plantlets form in 13 weeks	
VW + NAA + CW	Flowers developed		
VW + NAA + CW + AC	Flowers developed		
VW + CW	Flowers developed		
VW + CW + AC	Flowers developed		

[a]AC, activated charcoal, 2 g l^{-1}; BAP, benzylaminopurine, 1 mg l^{-1}; CW, coconut water, 150 ml l^{-1}; MPR, Mitra, Prasad, and Roychowdhury medium; NAA, naphthaleneacetic acid, 1 mg l^{-1}; VW, Vacin and Went medium. Note: Flower development requires CW, seems not to be affected one way or another by MRP, VW, NAA and AC, but is inhibited by BAP. PLB formation occurs only on MRP in the presence of NAA and AC. Vegetative shoots develop only on VW supplemented with NAA and BAP. Rooting occurs only on MPR which contains NAA; AC is not required. Response differs with the stage of development of the floral buds.

as a stunt), differentiated buds should be cultured on MPR medium supplemented with coconut water plus NAA or NAA and activated charcoal, but no BA (Table Rhy-13).

In Vitro Propagation of *Rhynchostylis retusa*

Rhynchostylis retusa was propagated in vitro on Vacin and Went medium (Vacin and Went, 1949) containing 400 mg casein hydrolysate l^{-1}, 20% (v/v) coconut water, 1 mg IAA l^{-1}, and 1 mg BA l^{-1} (Sarker and Roy, 1993).

Effects of pH and Micronutrients on Protocorm-like Bodies of *Rhynchostylis gigantea*

PLBs of *Rhynchostylis gigantea* multiply best in a medium designated as WS, which consists of Vacin and Went macronutrients (Vacin and Went, 1949), MS micronutrients (Murashige and Skoog, 1962), and 15% (v/v) coconut water, also containing 25–50 µmol iron, 25–100 µmol manganese, and 0.075 µmol copper with a presterilization pH of 4.5 and 5.0 (Wannakrairoj and Tanyasonti, 1996).

TABLE RHY-14. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of floral buds of *Rhynchostylis retusa* (Kaur and Vij, 1995)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	1.0	120 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	7.0 g	No stock	No stock	Weigh
	Darkening agent				
18	Activated charcoal (AC)[h]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin fails to dissolve add a few drops of 0.1 N KOH.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved add the charcoal slowly with vigorous stirring to insure even distribution. When the charcoal is completely dispersed pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

Rhynchostylis gigantea Plantlet Production from Thin Cell Layers

A monopodial orchid with considerable economic potential, *Rhynchostylis gigantea*, is endangered due to deforestation. A thin cell layer tissue culture system developed for tobacco was extended to this orchid (Le et al., 1999).

Plant Material. Transverse thin cell layers (tTCL), 0.3–0.5 mm thick, were excised from stem nodes and internodes, 3 mm in diameter, from the base to the tip of 1-year-old in-vitro-grown plantlets. It is not clear from the original papers if these were seedlings or plantlets obtained through tissue culture.

Surface Sterilization. Explants taken from plants growing in vitro do not require surface sterilization. If nodes and internodes are taken from mature plants they should be sterilized by the method used for *Rhynchostylis retusa* in the previous method before excising tTCL for culture.

Culture Vessels. The tTCL explants should be cultured in Petri dishes, 10 cm in diameter, containing 20 ml medium. Shoots and plantlets should be cultured in 250- or 500-ml Erlenmeyer flasks or other suitable containers filled with medium to 20–30% of their capacity.

Culture Conditions. Explants (tTCL) and shoots and plantlets in vitro should be maintained at 25 ± 1°C under 16-h photoperiods of 100 μmol m^{-2} s^{-1}. Standard culture room conditions may also prove to be suitable. Plantlets 3 cm tall with 2–3 roots should be grown in a greenhouse.

Culture Media. Experiments with different concentrations of BA, NAA, TDZ, and *N*-(2-chloro-4-pyridyl)-*N′*-phenylurea (forchlofenuron, CPPU) in MS medium (Murashige and Skoog, 1962) established that: (1) 3 μmol each of BA and TDZ in MS were optimal for bud induction (Fig. Rhy-6B, C; Table Rhy-15), and (2) roots were induced by 10 μmol of CPPU in MS with 10 g sucrose (Fig. Rhy-6A; Table Rhy-16).

Procedure. The thin layers are placed on the first medium (Table Rhy-15) and cultured until buds are formed (Fig. Rhy-6A, B, D, F) and start to develop (Fig. Rhy-6E). On transfer to the second medium (Table Rhy-16), the developed buds (Fig. Rhy-6E) form plantlets (Fig. Rhy-6C) which acclimatize on transfer to a greenhouse (Fig. Rhy-6G).

Developmental Sequence. PLBs, buds, and shoots are produced by the thin layers on the first medium (Table Rhy-15). The shoots form roots and develop into plantlets (Fig. Rhy-6C) on the second medium (Table Rhy-16). These plantlets mature in a greenhouse.

General Comments. If explants are taken from seedlings this method cannot be used to selected desirable cultivars. Explants taken from mature plants do not endanger the donor plant.

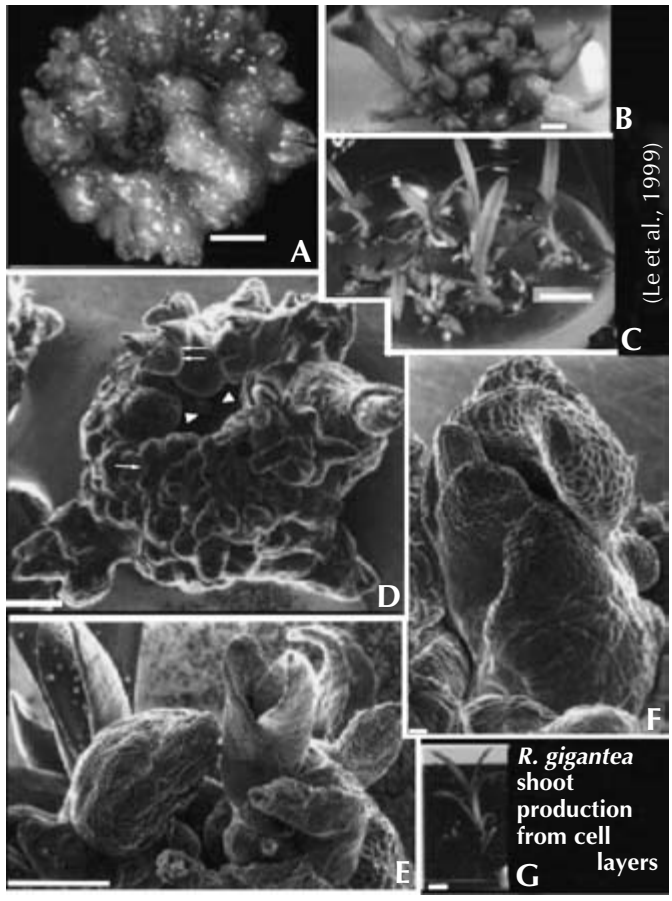


FIG. RHY-6. ***Rhynchostylis gigantea*** **plantlet production from thin cell layers. A. A transverse thin cell layer, 3 mm in diameter, forming PLBs and buds. B. Transverse thin cell layer with developed buds. C. Plantlets developing on the second medium. D. Bud at early (single arrow) and more advanced (double arrows) stages of development. E. Bud at advanced stage of development. F. Early stage of bud development. G. Plantlet in greenhouse. Bars are 1 mm in A–F and G; 0.1 mm in E. (Le et al., 1999.)**

TABLE RHY-15. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of thin cell layers of *Rhynchostylis retusa* (Le et al., 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Cytokinins				
8	N^6-benzyladenine (benzylaminopurine, BAP)	0.676	67.6 mg 100 ml^{-1} 95% ethanol[e,f]	1	
9	Thidiazuron (TDZ)	0.661	66.1 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If either or both cytokinins fail to dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–9 to 900 ml of distilled water (item 11), adjust pH to 5.7, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Hormones (items 8 and 9) are heat-labile and should be added after autoclaving to the hot solution under sterile conditions with sterilized pipettes, mixed well, and distributed to preautoclaved culture vessels. In this case the cytokinins are added before autoclaving. Agar is not added to liquid media. The original paper states that the explants were cultured on "agar (Difco)-solidified medium containing Murashige and Skoog (MS) macro/micro-elements." This implies that the amino acids, polyol, and vitamins which are usually incorporated in MS were excluded. If the explants fail to grow on the medium shown in this table these components (see below) should be added. This statement does not indicate whether sugar was added to the medium or not. The amount suggested in this table is the concentration usually used for orchids.

	Amino acid			
	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol			
	myo-inositol	100.0	No stock	No stock	Weigh
	Vitamins			
	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	

TABLE RHY-16. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the rooting of shoots produced by thin cell layers of *Rhynchostylis retusa* (Le et al., 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Cytokinin				
8	*N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (CPPU)	2.5 mg	250 mg 100 ml^{-1} 70% ethanol[e,f]	1	
	Sugar				
9	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[g]	To 1000 ml			
	Solidifier				
11	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]Dissolve in a minimal volume of water and bring to 100 ml with 95% ethanol.

[g]Add items 1–8 to 900 ml of distilled water (item 10), adjust pH to 5.7, add sugar (item 9), and raise volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Hormones (item 8) are heat-labile and should be added after autoclaving to the hot solution under sterile conditions with sterilized pipettes, mixed well, and distributed to preautoclaved culture vessels. In this case the cytokinin is added before autoclaving. Agar is not added to liquid media. The original paper states that the explants were cultured on "agar (Difco)-solidified medium containing Murashige and Skoog (MS) macro/micro-elements." This implies that the amino acids, polyol, and vitamins which are usually incorporated in MS were excluded. If the explants fail to grow on the medium shown in this table these components (see below) should be added.

	Amino acid				
	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
	myo-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	

Saccolabium

Once upon a time there was what must have been a fairly large, highly variable genus called *Saccolabium*. But the taxonomists started to work on it and when they were done not much was left. *Saccolabium* was divided into *Abdominea*, *Acampe*, *Aerides*, *Ascocentrum*, *Ascochilopsis*, *Ascoglossum*, *Cleisostoma*, *Gastrochilus*, *Malelola*, *Micropera, Pennilabium*, *Pomatocalpa*, *Renanthera*, *Rhynchogyna*, *Rhynchostylis*, *Robiquetia*, *Schoenorchis*, *Smitinandia*, *Trichoglottis*, and perhaps additional genera. However, despite the nomenclatural gymnastics and classification changes by the taxonomists, horticulturists still refer to some former *Saccolabium* genera as *Saccolabium*. Therefore the nomenclature used here is the one employed in the original reports. References to the procedures below are also included under *Gastrochilus* for *Gastrochilus* (*Saccolabium*) *calceolare* and *Acampe* for *Acampe papillosa* (*Saccolabium papillosum*).

Isolation of *Saccolabium calceolare* (*Gastrochilus calceolare*) Protoplasts

The method developed for *Acampe praemorsa* was used to isolate 6.1×10^4 protoplasts from leaves of *Saccolabium calceolare* (*Gastrochilus calceolare*; Seeni and Abraham, 1986).

Mass Rapid Clonal Propagation of *Saccolabium calceolare* (*Gastrochilus calceolare*)

Research designed to "test the morphogenetic response of *Saccolabium calceolare* inflorescence axes *in vitro*" resulted in a method for mass rapid clonal propagation (Vij et al., 1986). *Saccolabium calceolare* is an epiphytic species from the outer Himalayan ranges that flowers during May and June.

Plant Material. "Inflorescence axes (30–40 mm) with or without differentiated buds" are cut into 10–20-mm sections following surface sterilization, "and those with intact apices [are] used as explants."

Surface Sterilization. The inflorescences are surface-sterilized first by immersing them in 95 or 70% ethanol for 1–2 min and then by placing them in 0.5% mercuric chloride solution (0.5 g $HgCl_2$ per 100 ml distilled water) with Teepol as a wetting agent (Tween 20 can also be used) for 6–7 min. (Mercuric chloride is a dangerous chemical and should be handled with care.) A rinse with sterile distilled water is advisable before the inflorescences are sectioned.

Culture Vessels. Test tubes or Erlenmeyer flasks can be used.

Culture Conditions. Cultures should be maintained under 12-h photoperiods of 3500 lx (provided by fluorescent tubes) at 25–27°C.

Culture Media. Several media were used in the original research, but the one that may prove to be the most practical is a modification of the Mitra et al. (1976) medium (Table Sccm-1). Plantlets can be subcultured on the same medium or on any of the standard culture media of seeds, protocorms, seedlings, and PLBs (see Tables Aranda-7 and Cym-11).

Procedure. Place the explants on the first medium, and allow them to form plantlets, which are excised and subcultured.

Developmental Sequence. Apices "grew into vegetative shoots, [and] floral buds slowly developed into shoot buds." Roots in this medium (Table Sccm-1) are smaller than those cultured on a solution containing (per liter) 25 mg urea and 2 mg IAA. However, flower buds form shoots only on the IAA-free medium (Table Sccm-1). Shoots form within 6–8 weeks, and roots appear after 70 days.

General Comments. "The ability of the buds to grow into a vegetative or a reproductive shoot seems to be correlated with their physiological age." Young buds form vegetative structures, whereas well-differentiated ones flower in vitro.

Acampe, from the Greek ἀκαμπή (*akampes*) meaning rigid, refers to the "small, brittle flowers, in which there is no flexibility" (Schultes and Pease, 1963). *Saccolabium* comes from the Latin *saccus* for bag and *labium*, which means lip; the combination of the two words refers to the bag-like shape of the labellum or lip (the median petal).

TABLE SCCM-1. **Modified Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) for the culture of explants from inflorescence axes of *Saccolabium calceolare* (Vij et al., 1986)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100	10 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	200	20 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180	18 g l^{-1}	10	
5	Monosodium phosphate, NaH_2PO_4	150	15 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40 mg l^{-1}	10	One solution
(e)	Potassium iodide, Kl	0.03	3 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Organic additive				
14	Urea	50	No stock	No stock	Weigh
	Sugar				
15	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[f]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original recipe used 3 ml $Na_2FeEDTA$, a preparation that may not be readily available. The substitute listed here is common in orchid tissue culture media. Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium.

[e]Keep refrigerated or frozen.

[f]Add items 1–14 to 900 ml distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense solution into culture vessels, and autoclave. Omit agar for liquid medium. Media that contain hormones and vitamins should not be autoclaved without prior experimentation to determine that this will have no deleterious effects. This is such a case (Mitra, 1971; Mitra et al., 1976; Vij et al., 1986), so the medium may be autoclaved.

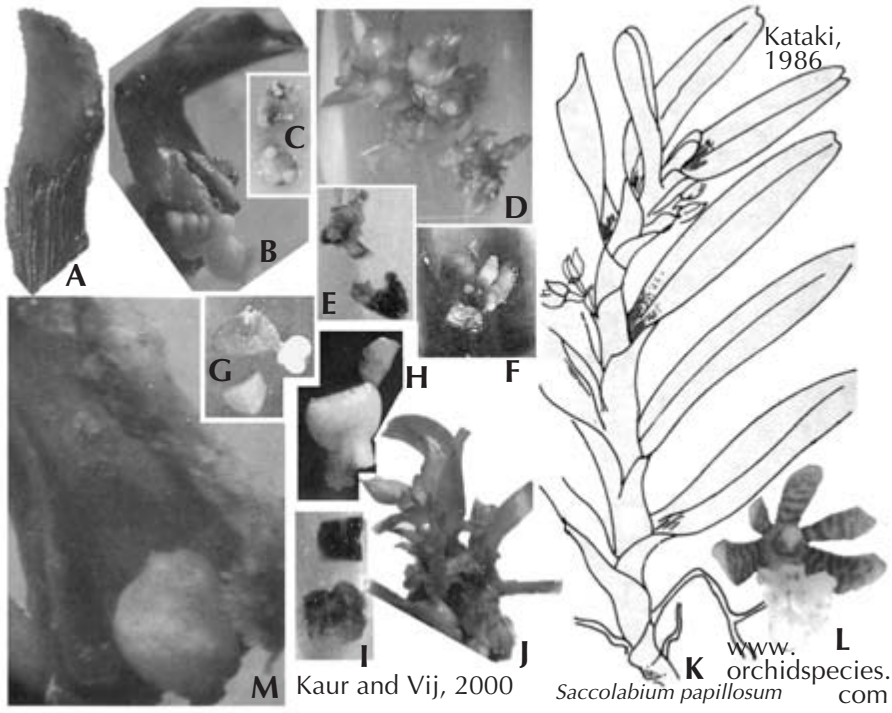


FIG. SCCM-1. ***Saccolabium papillosum* (*Acampe papillosa*). A, M. Start of proliferation at cut end and along the veins of leaf explants from greenhouse-grown plants. B. PLB on an explant from a greenhouse-grown plant. C. Production of PLBs on an explant from a plant grown in vitro. D. Shoot development from a leaf of an in vitro plant. E. Callus-mediated PLB production from an in vitro plant explant. F. Fasciated roots produced on explants from a plant grown in vitro. G. PLB production on an explant from a plant grown in vitro. H. Callus production on an explant from an in vitro plant. I. Unorganized cell proliferations on explants from a greenhouse-grown plant. J. Plantlet with leaves and roots produced from an explant of a plant grown in vitro. K. Mature plant with inflorescence. L. Flower. (Sources: A–J, M, Kaur and Vij, 2000; K, Kataki, 1986; L, www.orchidspecies.com.)**

Micropropagation of *Saccolabium papillosum* (*Acampe papillosa*) through the Culture of Leaf Segments

Saccolabium papillosum Lindl. (*Acampe papillosa* Lindl.; Fig. Sccm-1K, L) is an epiphytic species found in tropical valleys (up to an elevation of 800 m) from Kumaon to Arunacal Pradesh in India. It is used therapeutically. Leaf segment capacity to produce plantlets was investigated with a view to the development of a micropropagation protocol (Kaur and Vij, 2000). The original report refers to this orchid as *Saccolabium papillosum* (Kaur and Vij, 2000). Another source lists the species as *Acampe papillosa* (Kataki, 1986). The name used here is the one preferred by the authors of the tissue culture protocol (Kaur and Vij, 2000). However, a reference to this procedure is included in the *Acampe* section.

Plant Material. The most suitable explants are leaves, either: (1) 1 cm long or less from plants growing in vitro (the original paper does not state if these were seedlings or plantlets produced through tissue culture, but the protocol could probably be used for both), or (2) 2 cm long or less from plants growing in a greenhouse.

Surface Sterilization. Leaves taken from plants growing in vitro do not require surface sterilization. However, they should be washed several times with sterile distilled water to remove medium residues. Leaves from greenhouse (and field) grown plants should first be washed with running water and a mild detergent (0.2% of Teepol, available from www.sigmaaldrich.com, was used in the original research, but a mild household detergent or baby shampoo are also suitable) and a soft brush (a used toothbrush is very suitable). After that they should be dipped in 70% ethanol (73–74 ml 95% ethanol brought to 100 ml with distilled water) three times (30 s per dip), rinsed thoroughly several times with sterile distilled water, immersed in 0.1% mercuric chloride (which should be handled with care because it is toxic) containing 1–2 drops Teepol (or a mild household detergent or baby shampoo) for 5 min, washed well with sterile distilled water several times, soaked in 0.02% streptomycin (www.sigmaaldrich.com) for 5 min, rinsed with sterile distilled water three times, submerged in 0.01% bavistin (a product of BASF) for 5 min, and finally washed very well with sterile distilled water three times.

Culture Vessels. Culture tubes or other containers are suitable. They should be filled with medium to 25–30% of their capacity.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx (the illumination source is not described in the original paper) or standard culture room conditions.

Culture Media. Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) supplemented with BA should be used for leaves from greenhouse (or field) grown plants (Table Sccm-2). MPR containing BA and NAA is suitable for leaves from plants growing in vitro (Table Sccm-3). The original report does not indicate if these or other media should be used to grow plantlets to the community pot stage. If these media (Tables Sccm-2 and Sccm-3) are not suitable, plantlets can be cultured on one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C medium that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. The leaves are put in culture after surface sterilization, but the original report does not indicate if they should be sectioned or cultured whole and if the latter whether it is preferable to insert them into the medium or place them

TABLE SCCM-2. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Saccolabium papillosum* (*Acampe papillosa*) leaves from greenhouse-grown plants (Kaur and Vij, 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Cytokinin				
8	6-Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1.0	
	Vitamins				
9	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
14	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Agar[f]	9.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

TABLE SCCM-3. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Saccolabium papillosum* (*Acampe papillosa*) leaves from in-vitro-grown plants (Kaur and Vij, 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Auxin				
8	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1.0	
	Cytokinin				
9	6-Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1.0	
	Vitamins				
10	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
14	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
15	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[f]	To 1000 ml			
	Solidifier				
18	Agar[f]	9.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.5, add sugar (item 16), and bring volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

horizontally on it (and if so, whether their upper or lower surface should be in contact with the substrate). PLBs and plantlets produced by the explants can be cultured on one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, and V-4) with or without banana homogenate or activated charcoal. If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C medium that does contain this darkening agent (see Table Pln-1, footnote *f*).

Developmental Sequence. The leaves produce PLBs on their adaxial surface (Fig. Sccm-1). Plantlets with 2–3 leaves and 1–2 roots develop within 24 weeks. When cultured on an appropriate medium, these plantlets grow to a stage which makes them suitable for planting in community pots.

General Comments. The use of leaf explants does not endanger donor plants. Because of this, they are preferable to the utilization of shoot-tip explants. Also, mature plants have many leaves which means that explants are plentiful. Outstanding cultivars can be propagated through the use of leaf section. Leaf explants taken from seedlings grown in vitro or in a greenhouse cannot be used to propagate selected cultivars simply because their quality is not known.

Professor S. P. Vij and his associates have taken a lead in the identification and use of explants other than shoot tips. They should be congratulated for taking this initiative and for their successes. Professor Vij will probably be retired by the time this is published.

Sarcanthus

Several hundred species were included in the genus *Sarcanthus* at one point. However, many species were removed from this genus and placed in 25–30 existing or newly created genera.

In Vitro Growth of *Sarcanthus scolopendrifolius* (*Cleisostoma scolopendrifolium*)

A Korean orchid *Sarcanthus scolopendrifolius* was renamed *Cleisostoma scolopendrifolium*, but is listed here under the name used in the original report. The original paper (Lee et al., 1999) is in Korean with an English summary from which it is not clear whether the experiments were carried out with seedlings or tissue culture-derived plantlets. Table captions and one sentence in the abstract suggest that the work was carried out with seedlings, but a shortened version of the abstract is presented here just in case tissue cultured plants were also involved.

> The effective medium for shoot differentiation and seedling growth of the orchid [*S. scolopendrifolius* or *C. scolopendrifolium*] was Murashige and Skoog's basal medium supplemented with 5% sucrose and 0.7% agar, and the medium was adjusted to pH 4.0. Number of shoot and fresh weight were enhanced in the medium . . . with 500 mg L^{-1} activated charcoal, but root growth . . . decreased. Seedlings . . . died in . . . medium . . . with 1.0 mg L^{-1} NAA. However, shoot number, shoot length, leaf number, root number, root length and fresh weight . . . markedly increased in MS medium containing 10.0 ml·L^{-1} BA with 0.1 mg·L^{-1} NAA. (Lee et al., 1999)

Satyrium

As presently constituted, the genus *Satyrium* is found in Africa (tropical and southern), Australia, China, India, Indonesia, and Madagascar. The plants are assumed to have aphrodisiac properties.

Micropropagation of *Satyrium nepalense* through the Culture of Leaf Explants

A method for the micropropagation of *Satyrium nepalense* was developed at the Department of Botany, Panjab University by Professor Suraj P. Vij and his associate Promila Pathak as part of an extended research program on the micropropagation of orchids through leaf segments (Fig. Satm-1; Vij and Pathak, 1990).

Plant Material. Basal sections, 5–10 mm long, with leaf bases intact and taken from leaves up to 2 cm long of 16–40-week-old seedlings in vitro, are cultured.

Surface Sterilization. Explants taken from seedlings in vitro do not require surface sterilization.

Culture Vessels. Culture tubes or other containers can be used.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx.

Culture Media. Explants should be cultured initially on Knudson C medium (Knudson, 1946) supplemented with BA, NAA, and yeast extract (Table Satm-1). No medium is suggested for the culture of plantlets to the community pot stage. Plantlets can be cultured on one of the versions of Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C medium that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. Leaves are taken from seedlings and their basal parts are sectioned and placed in culture on the first medium (Table Satm-1). If necessary, plantlets can be grown in one of the other media.

Developmental Sequence. Only 33.3% of the explants produce PLBs. PLBs are first formed 4 weeks after the start of culture. Explants produce an average of 28 PLBs. The first leaf appears after 8 weeks of culture. Roots are formed after 13 weeks.

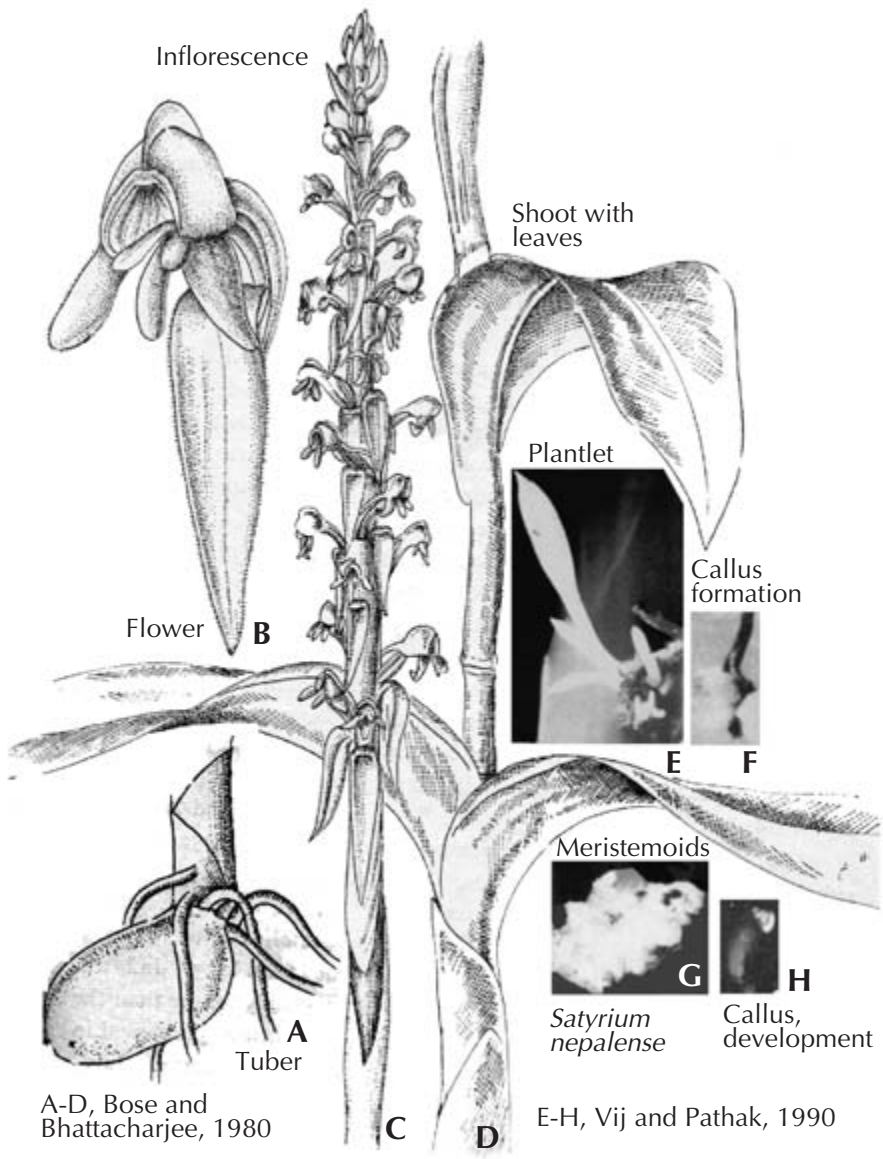


FIG. SATM-1. ***Satyrium nepalense*** **and its micropropagation.**

TABLE SATM-1. **Knudson C (KC) medium (Knudson, 1946) modified for the culture of *Satyrium nepalense* leaf explants**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
	Microelement[d]				
6	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
7	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
8	6-Benzylaminopurine	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
9	Yeast extract (YE)[g]	1.0 g	No stock	No stock	Weigh
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[h]	To 1000 ml			
	Solidifier				
12	Agar[h]	9.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. Knudson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4{\cdot}7H_2O$ l^{-1}, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored.

[d]There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If incorporation of additional microelements becomes necessary, those used in the Murashige–Skoog (MS) medium are suitable.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[g]Available from www.phytotechlab.com or www.sigmaaldrich.com

[h]Add items 1–9 to 900 ml of distilled water (item 11), adjust pH to 5.5, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If the incorporation of additional microelements is necessary, those used in the Murashige–Skoog (MS) medium are suitable.

General Comments. This method seems to be simple and effective, but cannot be used for the propagation of selected clones because it uses explants from seedling leaves.

Satyrium comes from *satyrion* (σατύριν), sylvan demigods (sometimes thought to be half goat, half man) of Greek mythology noted for their lasciviousness. This name was applied to the orchid because of its presumed aphrodisiac properties. The illustration is of a hurdy-gurdy-playing Satyr. (Detail from an engraving by an Italian master ca 1515; source: www.metmuseum.org.)

Schomburgkia

The method for *Cattleya* is also applicable to *Schomburgkia superbiens* (Scully, 1967).

Serapias

A genus of 19 species, *Serapias* is distributed from the Azores and Canary Islands eastward to the Caucasus, south to North Africa, and north to Britanny (Pridgeon et al., 2003). The name commemorates *Serapis*, an Egyptian god associated with licentiousness.

Vegetative Multiplication in Vitro of *Serapias olbia* and *Serapias pseudocordigera*

A method for propagating these two European orchids was developed at the Station de Botanique et de Pathologie Végétale, Antibes, France (Ponchet et al., 1985). The report is in French with an English summary which is abbreviated below.

> Meristems . . . [are] grown on a . . . medium rich in organic substances [un]till they . . . reach . . . 5 mm size (optimum . . . for further . . . propagation). Only one culture medium containing Knudson [macroelements], Murashige and Skoog microelements, B and C vitamins, sucrose and . . . benzyladenine (2 to 4 mg/ml) [this is probably a typographical error, because 2–4 mg ml^{-1} amounts to 2–4 g l^{-1}, an extremely high and probably toxic concentration; the meaning is probably 2–4 mg l^{-1}], was used to obtain . . . propagation [through buds] and . . . stoloniferous plantlets. These plantlets, after their separation from the buds, were kept on a third medium where benzyladenine was replaced with IAA (1 mg/l) [this implies that IAA and BA belong to the same group of hormones, which they do not; IAA is an auxin whereas BA is a cytokinin] to [bring about] complete development of the tubers. The transfer from in vitro conditions was carried out on . . . vermiculite under water saturated air, before the final planting in [the] ground. The tuberized plantlets were able to grow normally [like] "natural" plants. No symbiotic fungus was used during *in vitro* and *in vivo* culture.

Comments. This is an interesting paper, but being in French, will be read by few scientists and growers.

Sophrolaeliocattleya

Procedures that are suitable for *Cattleya* (particularly Kako, 1969; Ishii et al., 1976) can also be used for this genus.

Spathoglottis

A genus consisting of approximately 40 species, *Spathoglottis* is found in South East Asia, Australia, China, Indonesia, New Guinea, and the Pacific Islands. It was first described by C. L. Blume in 1825.

Tissue Culture of *Spathoglottis*

A method for *Spathgolottis* is mentioned in a paper on *Phalaenopsis* (Intuwong and Sagawa, 1974).

Culture of *Spathoglottis* Flower Buds

Flower buds of *Spathoglottis*, 5–10 mm in size, can be cultured by a method used for *Mokara* (Lim-Ho et al., 1984). The initiation of PLBs and the rate of growth were faster for *Spathoglottis* than for *Mokara*.

Root Formation in *Spathoglottis* Cultures

An attempt was made to induce callus through the culture of "excised rhizomatous portions of young seedlings of the orchid *Spathoglottis plicata*" (Bapat and Narayanaswami, 1977).

Plant Material. "Leaves and secondary roots of 6-week old seedlings were excised and discarded and the rhizomatous stock alone bearing the stem disc was cultured . . ." (Bapat and Narayanaswami, 1977).

Culture Vessels. Test tubes and Erlenmeyer flasks can be used.

Culture Conditions. Cultures should be maintained under constant illumination (sources and intensity not given) at a temperature of 25 ± 2°C and a relative humidity of 50–60%.

Culture Media. Callus forms on a modified MS medium (Table Spa-1) and is maintained on a second modification (Table Spa-2). Roots form on a third modification of the medium (Table Spa-3).

Procedure. Culture the explants on the first medium (Table Spa-1) to initiate callus, which is maintained on the second solution (Table Spa-2). Then move to the third medium (Table Spa-3) for root induction.

Developmental Sequence. The explants develop callus, which forms roots, but do not develop leaves or plantlets.

TABLE SPA-1. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for callus induction from *Spathoglottis* explants (Bapat and Narayanaswami, 1977)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	5.0	500 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
11	Kinetin	1.0	400 mg 100 ml^{-1} 95% ethanol[f,g]	0.25	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
15	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]If auxins and cytokinins do not dissolve, add a few drops of dilute KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–7, 9, and 15 to 700 ml distilled water (item 17), adjust pH to 5.5, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour the solution into a 2-l flask and autoclave. Add amino acid, hormones, and vitamins (items 8, and 10–14) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

TABLE SPA-2. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for the maintenance of callus derived from *Spathoglottis* explants (Bapat and Narayanaswami, 1977)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxins				
10	Naphthaleneacetic acid (NAA)	6.0	600 mg 100 ml^{-1} 95% ethanol[f,g]	1	
11	2,4-Dichlorophenoxyacetic acid (2,4-D)	2.0	800 mg 100 ml^{-1} 95% ethanol[f,g]	0.25	
	Cytokinin				
12	Kinetin	2.0	800 mg 100 ml^{-1} 95% ethanol[f,g]	0.25	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
16	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
17	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[h]	To 1000 ml			
	Solidifier				
19	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]If auxins or cytokinin do not dissolve, add a few drops of dilute KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–7, 9, and 16 to 700 ml distilled water (item 18), adjust pH to 5.5, add sugar (item 17), and adjust volume to 1000 ml with distilled water (item 18). Bring solution to a gentle boil, and add agar (item 19) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour the solution into a 2-l flask and autoclave. Add amino acid, hormones, and vitamins (items 8, and 10–15) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

TABLE SPA-3. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for root induction on callus derived from *Spathoglottis* explants (Bapat and Narayanaswami, 1977)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	6.0	600 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
11	Kinetin	2.0	80 mg 100 ml^{-1} 95% ethanol[f,g]	0.25	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
15	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin do not dissolve, add a few drops of dilute KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–7, 9, and 15 to 700 ml distilled water (item 17), adjust pH to 5.5, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour the solution into a 2-l flask and autoclave. Add amino acid, hormones, and vitamins (items 8, and 10–14) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar for liquid medium.

General Comments. This method can serve as a starting point for further research and may be applicable to *Habenaria*.

Isolation of Protoplasts from *Spathoglottis plicata*

The method developed for *Acampe praemorsa* was used to isolate 2.4×10^4 protoplasts from leaves of *Spathoglottis plicata* (Seeni and Abraham, 1986).

Micropropagation of *Spathoglottis plicata* through the Culture of Leaf Bases

Spathoglottis plicata is found from Sumatra to the Philippines. Many of its forms are cultivated. Hybrids also exist. A method for the culture of leaf bases was developed at the Post-Graduate Department of Botany, Utkal University, Bhubaneswar, Orissa, India (Nayak et al., 1988).

Plant Material. Leaf bases were taken from seedlings, 5 cm high and 120 days old, grown on MS medium (Murashige and Skoog, 1962) supplemented with 2 mg NAA l^{-1} and 0.5 mg BA l^{-1} and maintained at 25 ± 1°C under continuous illumination of 2500 lx provided by cool white fluorescent lamps.

Surface Sterilization. Explants taken from seedlings growing in vitro do not require surface sterilization.

Culture Vessels. "Conical . . . Borosil" (i.e., Pyrex or equivalent Erlenmeyer) flasks of 150-ml capacity were used in the original research. Other containers are also suitable.

Culture Conditions. Cultures should be maintained at 25 ± 1°C under 18-h photoperiods of 2500 lx provided by cool white fluorescent lamps or standard culture room conditions.

Culture Media. Explants should be cultured first on MS medium (Murashige and Skoog, 1962) containing 10 mg BA l^{-1} and 2.5 mg NAA l^{-1} (Table Spa-4). Shoots produced on this medium develop roots on MS containing 2 mg NAA l^{-1} (Table Spa-5). No medium is suggested for the culture of plantlets to maturation. Plantlets can be cultured on one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

TABLE SPA-4. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Spathoglottis plicata* leaf bases (Nayak et al., 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	2.5	250 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.8, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media.

TABLE SPA-5. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the rooting of shoots produced by leaf bases of *Spathoglottis plicata* (Nayak et al., 1999)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.8, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. Agar is not added to liquid media.

Procedure. Leaves are taken from seedlings and their bases are excised and placed on the first medium (Table Spa-4). Shoots which form on this medium are transferred to the second solution (Table Spa-5) for root induction. Plantlets from the second or a third medium are hardened and established in a garden or greenhouse.

Developmental Sequence. Vegetative buds and eventually shoots develop from the cut ends of the leaf bases within 5 weeks of culture on the first medium (Table Spa-4). Root initiation becomes apparent 2 weeks after the shoots are moved to the second medium (Table Spa-5). The shoots form 2–4 well developed roots after 4 weeks on the second medium (Table Spa-5) and become plantlets. For further growth the plantlets can be moved to one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, Cym-1 to Cym-3, Cym-5, Cym-24, C-19, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, and V-4) with or without banana homogenate or activated charcoal. If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*). These plantlets become established and hardened on being moved to a greenhouse or garden.

General Comments. This procedure is efficient and useful, but it cannot be used to select outstanding cultivars because the quality of seedlings is not known.

Micropropagation of *Spathoglottis plicata* through the Culture of Nodal and Leaf Explants

Hybrids of *Spathoglottis plicata* can be found throughout the world. Many of them have attractive flowers. Propagation by conventional methods is slow. Therefore, a micropropagation method was developed at the Hong Kong Institute of Biotechnology and the National Pingtung Polytechnic Institute in Taiwan (Teng et al., 1997).

Plant Material. Internode sections consisting of one-half of the internodal portion above and below the node, and leaves (2–3 mm explants) were taken from 8-month-old seedlings, growing in pots. Sections of PLBs produced through this method can also be cultured.

Surface Sterilization. The surface sterilization procedure used as part of this protocol is unusual and overly complex. It is quoted here verbatim with comments in brackets:

> Eight-months-old pot-grown seedlings *S. plicata*, native to Taiwan were washed thoroughly in water, sonicated in 1% NaOCl [this is 18 or 17 ml of household bleach which contains 5.25% or 6% NaOCl, respectively, diluted to 100 ml with distilled water] for 20 min and soaked in a solution of 1000 mg/liter of both penicillin-G and streptomycin for 3 h. The antibiotic solution was prepared by adding 1 g pencillin-G and 1 g streptomycin [both are available from www.sigmaaldrich.com and other

> sources] to 20 ml of distilled water, which was then filtered through a 0.22-μm Millipore filter [in effect sterilizing the solution]. The sterile antibiotic solution was diluted to 1 l and ten drops of sterile Tween 20 [available from www.sigmaaldrich.com and other sources] were added into the solution (i.e., one drop per 100 ml of solution). The final concentration of each antibiotic was 1000 mg/l [that is 1 g l^{-1}]. The antibiotic solution was removed from the seedlings using a 400-μm polyester screen filter [plain filter paper or cheese cloth or even a paper towel used as filter paper are also suitable]. The seedlings were then rinsed thoroughly with sterile distilled water. To remove any residuals of antibiotic, the seedlings were left in constant stirring water for 5 min and then filtered to remove the water . . . [This process was repeated three times]. (Teng et al., 1997)

PLBs taken from in vitro cultures do not require surface sterilization.

Culture Vessels. Erlenmeyer flasks, culture tubes, and other containers can be used.

Culture Conditions. Explants should be cultured at 25 ± 2°C under continuous illumination of 20 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps.

Culture Media. To generate PLBs, explants should be cultured on modified MS medium (Murashige and Skoog, 1962) supplemented with 5.37 μmol NAA l^{-1} and 0.44 μmol BA l^{-1} (Table Spa-6). PLBs should be cultured on MS with 2.69–10.74 μmol NAA l^{-1} and 8.88 μmol BA l^{-1} (Table Spa-7) for plantlet development. Sections of PLBs produced on the first medium should be cultured on the same solution (Table Spa-6) for further proliferation. And PLBs produced from the sections should be cultured on the second medium (Table Spa-7) to produce plantlets.

Procedure. Surface-sterilized nodal and leaf explants are cultured on the first medium (Table Spa-6) to produce PLBs (Fig. Spa-1). These PLBs can be sectioned and the sections cultured on the same medium (Table Spa-6) to produce even more PLBs. Regardless of whether they are produced from the original explants or sections, PLBs are cultured on the second medium (Table Spa-7) for plantlet development.

Developmental Sequence. Explants and PLB sections produce PLBs on the first medium (Table Spa-6). Plantlets are produced by the PLBs on the second medium (Table Spa-7).

General Comments. The reason for developing this procedure seems to have been *Spathoglottis* hybrids, which produce beautiful flowers, but whose propagation by division is very slow. However, it is not at all certain that a procedure developed with explants taken from 8-month-old seedlings will be applicable to mature plants.

TABLE SPA-6. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Spathoglottis plicata* leaf and nodal explants and PLB sections (Teng et al., 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh
	Darkening agent				
18	Activated charcoal[h]	2.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved add the charcoal (item 18) with constant stirring to insure complete dispersion through the medium, pour the solution into culture vessels, and autoclave. Agar is not added to liquid media.

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

TABLE SPA-7. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for plantlet formation from protocorm-like bodies of *Spathoglottis plicata* (Teng et al., 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.25	125 mg 100 ml^{-1} 95% ethanol[e,f]	1	0.5–2 mg can be used
	Cytokinin				
11	Benzyladenine (BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh
	Darkening agent				
18	Activated charcoal[h]	2.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved add the charcoal with constant stirring to insure complete dispersion through the medium, pour the solution into culture vessels and autoclave. Agar is not added to liquid media.

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

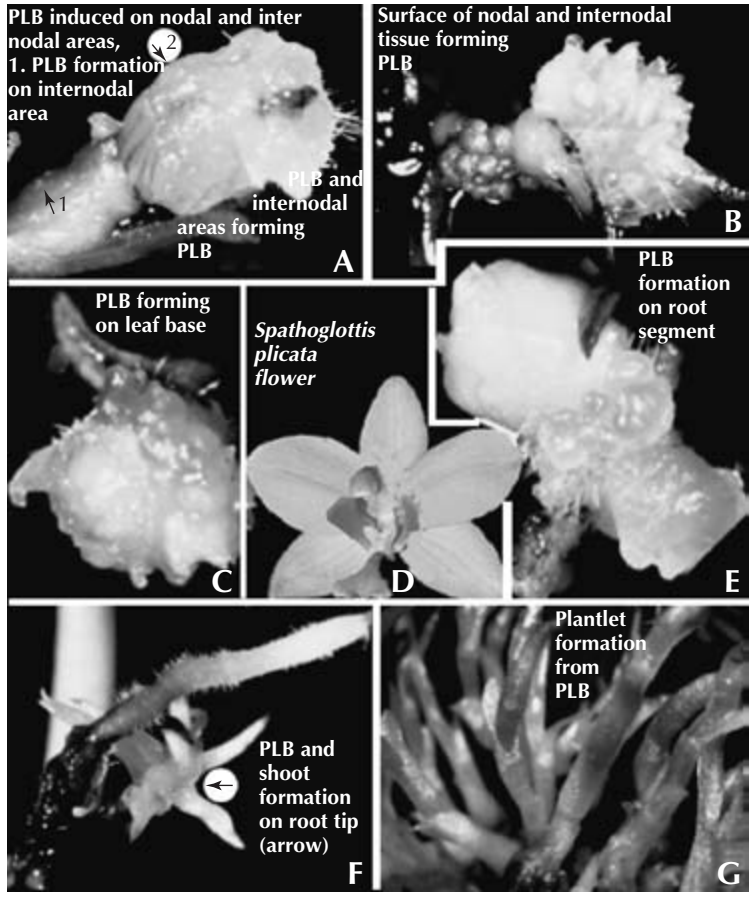


FIG. SPA-1. **PLB and plantlet formation from *Spathoglottis plicata* explants. (A–C, E–G, Teng et al., 1997; D, Joseph Arditti.)**

In Vitro Multiplication of *Spathoglottis plicata* through Tissue and Organ Culture

Several explants were used in an attempt to develop in vitro propagation procedures for *Spathoglottis plicata* because it is a very attractive terrestrial orchid "distributed in Tropical Asia and Indonesia . . . [and] also occur[ring] in Meghalaya hills and in the foot-hills of Arunachal Pradesh" (Mazumder and Bhowmik, 1993).

Plant Material. A number of explants from seedlings in vitro were cultured (Table Spa-8).

Surface Sterilization. Explants taken from seedlings in vitro do not require surface sterilization.

TABLE SPA-8. **Explants of *Spathoglottis plicata* cultured and their response**

Explant	Nature of response	Medium	Percent of explants responding
Leaf			
Apex	No response		
Base	Callus and protocorm-like body formation	Table Spa-9	95
Split longitudinally	No response		
Petiole	No response		
Whole	No response		
Pseudobulb			
Whole	Formation of protocorm-like bodies	Table Spa-9	100
Stem			
Base	Formation of protocorm-like bodies	Table Spa-10	100
Root			
Segment	Formation, proliferation and differentiation of protocorm-like bodies	Table Spa-11	100

Culture Vessels. Culture tubes, Erlenmeyer flasks, or other containers can be used.

Culture Conditions. Standard laboratory conditions are suitable.

Culture Media. Leaf bases and pseudobulbs should be cultured on MS medium (Murashige and Skoog, 1962) containing 0.5 mg IAA l^{-1} and 100 mg casein hydrolysate l^{-1} (Table Spa-9). MS supplemented with 1.0 mg 2,4-D l^{-1} and 100 mg casein hydrolysate l^{-1} (Table Spa-10) is suitable for stem bases. Root segments proliferate and produce PLBs on MS with 0.5 mg NAA l^{-1} and 100 mg casein hydrolysate l^{-1} (Table Spa-11).

Procedure. Explants are placed on the appropriate medium and kept on it until they produce PLBs and/or callus. These can be subcultured onto the initial medium for further proliferation, the production of additional PLBs, and the start of plantlet formation (Fig. Spa-2). PLBs, shoots, and/or small plantlets can be cultured to community pot size with one or two transfers to one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C medium that contains this darkening agent (see Table Pln-1, footnote *f*).

Developmental Sequence. The explants form PLBs and/or callus on the initial medium (see Table Spa-8 for explant–medium combinations) within 8–10 weeks. PLBs and callus also proliferate on these media and may perhaps form shoots and/or plantlets on them. Further growth can occur on one of the media listed under the procedure above.

TABLE SPA-9. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of leaf bases and pseudobulbs of *Spathoglottis plicata* seedlings (Mazumder and Bhowmik, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indoleacetic acid (IAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
14	Casein hydrolysate[g]	100.0	No stock	No stock	Weigh
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar[h]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Not all casein hydrolysates are the same. Their compositions and analyses vary depending on the methods by which they were produced. A product sold by Merck (www.merck.de) as casein hydrolysate (CH) is obtained by degrading casein with hydrochloric acid; vitamins and other growth-promoting compounds are largely destroyed in this process. The CH sold by Sigma-Aldrich (www.sigmaaldrich.com) is pancreatic hydrolysate of casein. There is also a CH which is hydrolysed by enzymes. Becton, Dickinson and Company (www.bd.com) sells Difco products including a number of different casamino acid products, all of which are casein hydrolysates. Therefore if a method specifies a source for the CH in its culture medium it is best to obtain the additive from the same vendor. If a source is not listed it is best to purchase CH which is described as being suitable as a supplement for plant culture media. Failing that, CH should be purchased from a source which specializes in tissue culture and micropropagation supplies. One such source is www.caissonlabs.com. They list casein rather than CH as Stock #: SPCA205. Another source for CH is www.duchefa.com (item 1301). Additional sources include www.fishersci.com, www.thomassci.com, and others.

[h]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. As a rule, but not in this medium, amino acid (item 8), hormone (item 10), and vitamins (items 11–13) are added to a still warm and liquid autoclaved medium under sterile conditions with sterilized pipettes. The medium is mixed well and distributed to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE SPA-10. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of stem bases of *Spathoglottis plicata* seedlings (Mazumder and Bhowmik, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	2,4-Dichlorophenoxyacetic acid (2,4-D)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
14	Casein hydrolysate[g]	100.0	No stock	No stock	Weigh
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar[h]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Not all casein hydrolysates are the same. Their compositions and analyses vary depending on the methods by which they were produced. A product sold by Merck (www.merck.de) as casein hydrolysate (CH) is obtained by degrading casein with hydrochloric acid; vitamins and other growth-promoting compounds are largely destroyed in this process. The CH sold by Sigma-Aldrich (www.sigmaaldrich.com) is pancreatic hydrolysate of casein. There is also a CH which is hydrolysed by enzymes. Becton, Dickinson and Company (www.bd.com) sells Difco products including a number of different casamino acid products, all of which are casein hydrolysates. Therefore if a method specifies a source for the CH in its culture medium it is best to obtain the additive from the same vendor. If a source is not listed it is best to purchase CH which is described as being suitable as a supplement for culture media. Failing that, CH should be purchased from a source which specializes in tissue culture and micropropagation supplies. One such source is www.caissonlabs.com. They list casein rather than CH as Stock #: SPCA205. Another source for CH is www.duchefa.com (item 1301). Additional sources include www.fishersci.com, www.thomassci.com, and others.

[h]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. As a rule, but not in this medium, amino acid (item 8), hormone (item 10), and vitamins (items 11–13) are added to a still warm and liquid autoclaved medium under sterile conditions with sterilized pipettes. The medium is mixed well and distributed to preautoclaved culture vessels. Agar is not added to liquid media.

TABLE SPA-11. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of root segments of *Spathoglottis plicata* seedlings (Mazumder and Bhowmik, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
14	Casein hydrolysate[g]	100.0	No stock	No stock	Weigh
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar[h]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Not all casein hydrolysates are the same. Their compositions and analyses vary depending on the methods by which they were produced. A product sold by Merck (www.merck.de) as casein hydrolysate (CH) is obtained by degrading casein with hydrochloric acid; vitamins and other growth-promoting compounds are largely destroyed in this process. The CH sold by Sigma-Aldrich (www.sigmaaldrich.com) is pancreatic hydrolysate of casein. There is also a CH which is hydrolysed by enzymes. Becton, Dickinson and Company (www.bd.com) sells Difco products including a number of different casamino acid products, all of which are casein hydrolysates. Therefore if a method specifies a source for the CH in its culture medium it is best to obtain the additive from the same vendor. If a source is not listed it is best to purchase CH which is described as being suitable as a supplement for culture media. Failing that, CH should be purchased from a source which specializes in tissue culture and micropropagation supplies. One such source is www.caissonlabs.com. They list casein rather than CH as Stock #: SPCA205. Another source for CH is www.duchefa.com (item 1301). Additional sources include www.fishersci.com, www.thomassci.com and others.

[h]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. As a rule, but not in this medium, amino acid (item 8), hormone (item 10), and vitamins (items 11–13) are added to a still warm and liquid autoclaved medium under sterile conditions with sterilized pipettes. The medium is mixed well and distributed to preautoclaved culture vessels. Agar is not added to liquid media.

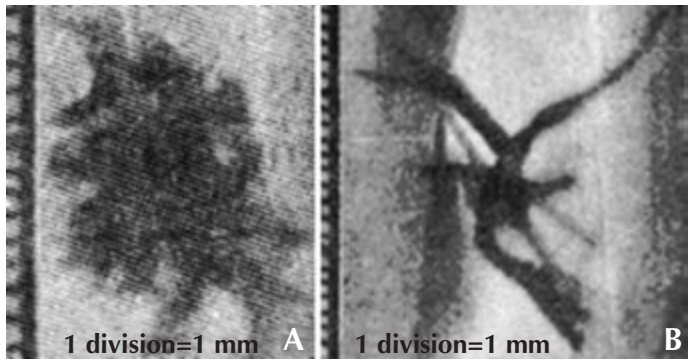


FIG. SPA-2. **In vitro culture of *Spathoglottis plicata*. A. Formation of PLBs from a stem base. B. Plantlet development from a pseudobulb. (The original illustrations are fuzzy; Mazumder and Bhowmik, 1993.)**

General Comments. This method can be used for rapid propagation, but not for the selection of outstanding cultivars.

Micropropagation of *Spathoglottis plicata* through the Culture of Seedling Shoots

Spathoglottis plicata is appreciated and thought of as having great potential as an outdoor and garden ornamental plant. It is cultivated throughout the tropics, including Venezuela where a micropropagation method was developed using seedling shoots as explants (Perdomo and Páez de Cásares, 2000).

Plant Material. Seeds were germinated on half-strength MS medium (Murashige and Skoog, 1962). Shoots, 6–10 mm long, taken from 5-month-old seedlings are preferable as explants. However, 3–5-mm-long shoots can also be used.

Surface Sterilization. Explants taken from seedlings in vitro do not require surface sterilization.

Culture Vessels. Culture tubes, Erlenmeyer flasks, and other containers are suitable.

Culture Conditions. Cultures should be maintained at 25 ± 2°C, under 16-h photoperiods of 35 mE m^{-2} s^{-1} and 40–80% relative humidity or standard culture room conditions.

Culture Media. Explants should be cultured on quarter-strength MS with 15 g sucrose l^{-1} (Table Spa-12). Since no plant hormones are mentioned it is reasonable and perhaps even necessary to assume that none are needed. Therefore the medium suggested here (Table Spa-12) is speculative to some extent. No medium is suggested

TABLE SPA-12. **Quarter-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of shoots of *Spathoglottis plicata* seedlings (Perdomo and Páez de Cásares, 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	412.5	41.25 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	110.0	11.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	92.5	9.25 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	475.0	47.5 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	42.5	4.25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	15.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[f]	To 1000 ml			
	Solidifier				
15	Agar[f]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

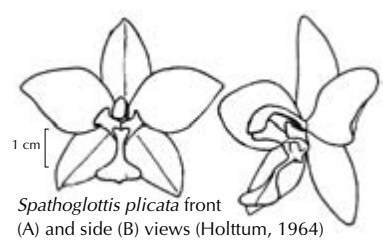
[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH to 5.5, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave.



Spathoglottis plicata front (A) and side (B) views (Holttum, 1964)

for the culture of plantlets to maturation. Plantlets can be cultured on one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. Explants are placed on the first medium (Table Spa-12) and allowed to develop new shoots. These shoots should be allowed to grow and form plantlets on the first medium (Table Spa-12) or moved to one of the other media mentioned above. When the plantlets are large enough they should be moved to community pots.

Developmental Sequence. The explants form shoots which grow, and produce roots and then plantlets.

General Comments. Not enough information is given in the original abstract to allow for a complete presentation here. Also, the procedure does not seem to offer a real advantage over propagation by seeds because it simply multiplies seedlings. Seed capsules usually contain many seeds which can produce numerous seedlings. And, since the quality of seedlings is not known, clonal propagation using them as explant sources offers no horticultural advantages (i.e., selection of superior clones is not possible).

There are several critical steps in the development of a micropropagation procedure. One is surface sterilization, which may damage the explant to the point of rendering it incapable of growth in vitro. Bypassing the surface sterilization step for the purpose of selecting a medium without having to deal with possible damage by a sterilant is fully justified. This is not such a case because there is ample literature on micropropagation and in vitro culture of *Spathoglottis* explants (Intuwong and Sagawa, 1974; Bapat and Narayanaswami, 1977; Lim-Ho et al., 1984; Arditti and Ernst, 1993; Mazumder and Bhowmik, 1993; Teng et al., 1997; Nayak et al., 1998*a*, 1999). Several media and surface sterilization procedures are known to work and could have been used to develop yet another method.

This method is presented here simply because it was published and the intent is to include in the current edition as many methods as could be found in the literature. The hope is also that the presentation of the method and this discussion will encourage future workers to: (1) develop micropropagation methods for mature plants, and (2) be more careful and detailed in their presentations (there is no indication in the abstracts whether some/all/any of the organics which are part of the MS formulation were used in this case; if one-quarter MS refers to all components or to the macroelements only; and what is the pH of the medium) and spelling (in the original abstract the generic name is consistently misspelled as *Spathoglotis* instead of the correct *Spathoglottis*).

Encapsulation in Alginate of Protocorm-like Bodies of *Spathoglottis plicata* and Subsequent Plantlet Production

The encapsulation of PLBs or somatic embryos (Hew and Yong, 2004) results in production propagules, which have been referred to as "synthetic seeds." Their use can simplify micropropagation. A protocol for large-scale propagation of *Spathoglottis plicata* using encapsulated PLBs was formulated at the Cytogenetics Laboratory, Post-Graduate Department of Botany, Utkal University, Bhubaneswar, Orissa, India (Nayak et al., 1998*b*).

Plant Material. PLBs were produced by culturing shoots taken from seedlings on MS medium (Murashige and Skoog, 1962) containing 1 mg NAA l^{-1} and 2 mg BA l^{-1} (Table Spa-13) at 25 ± 1°C under 16-h photoperiods of 35 μE m^{-2} s^{-1} provided by Philips cool white fluorescent tubes. About 85% of the explants produced an average of 7.6 PLBs per explant within 30–35 days of culture. These PLBs reached a size of 3–4 mm in 40–45 days of culture and were used for encapsulation.

Culture Vessels. Erlenmeyer flasks, 150-ml capacity, should be used for the culture of explants. Screw-cap glass jars, 200-ml capacity, or Petri dishes are suitable for in vitro germination of encapsulated PLBs. The same containers and pots can be used for horticultural germination. Developed plantlets should be planted in pots.

Culture Conditions. Suitable conditions for the germination of encapsulated PLBs are the same as those used to culture explants for the production of plant material (see above). Well-developed plantlets should be hardened in a plant growth chamber at 25 ± 1°C, 85% relative humidity, and a light intensity of 50 μE m^{-2} s^{-1} for 12–16-h photoperiods.

Low Temperature Storage. Encapsulated PLBs can be stored for up to 45 days in a refrigerator (the temperature of household refrigerators is approximately 4°C).

Alginate and Calcium Chloride Solutions. Sodium alginate solution, 4% (apparently manufactured by Reidel de Haen in Germany and purchased from CDH, Bombay) was dissolved in MS containing 2 mg NAA l^{-1} and 0.5 mg BA l^{-1} (Table Spa-14). Alginate from another source did not produce good results. Therefore it may be advisable to purchase the sodium alginate from CDH in Bombay. Sodium alginate is also available from several sources (including www.sigmaaldrich.com, www.fishersci.com, www.vwr.com, and others), but tests may be necessary to determine if sodium alginate obtained from them is suitable. If the encapsulated PLBs are to be germinated on a soil mixture, a bactericide (rose bengal at 0.2 mg l^{-1}; www.sigmaaldrich.com) and a fungicide (bavistin at 5 mg l^{-1} which is a preparation consisting of 50% carbendazim made by BASF; www.BASF.com) must be incorporated in the alginate solution (Table Spa-15). Calcium chloride ($CaCl_2 \cdot 2H_2O$), 75 mmol l^{-1}, should be dissolved in distilled water (Table Spa-16).

TABLE SPA-13. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of shoots taken from *Spathoglottis plicata* seedlings (Nayak et al., 1998*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (IAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.8, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. In general it is preferable to add the amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. All components of this medium are added before autoclaving. Agar is not added to liquid media.

TABLE SPA-14. **Sodium alginate solution in modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the encapsulation of protocorm-like bodies of *Spathoglottis plicata* which will eventually be germinated in vitro (Nayak et al., 1998*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (IAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Alginate				
15	Sodium alginate	4.9	No stock	No stock	Weigh; see text for comment
	Sugar				
16	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.8, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). The original paper does not indicate whether the medium should contain agar, but it seems logical to exclude it. In general it is preferable to add the amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. All components of this medium are added before autoclaving. Agar is not added to liquid media.

TABLE SPA-15. **Sodium alginate solution in modified Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the encapsulation of protocorm-like bodies of *Spathoglottis plicata* which will eventually be germinated under horticultural conditions (Nayak et al., 1998*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (IAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Alginate				
15	Sodium alginate	4.9	No stock	No stock	Weigh; see text for comments
	Bactericide				
16	Rose bengal	0.2	20 mg 100 ml distilled water[e]	1	See text for comments
	Fungicide				
17	Bavistin	5.0	500 mg 100 ml distilled water[e]	1	See text for comments
	Sugar				
18	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–17 to 900 ml of distilled water (item 19), adjust pH to 5.8, add sugar (item 18), and raise volume to 1000 ml with distilled water (item 19). The original paper does not indicate whether the medium should contain agar, but it seems logical to exclude it. In general it is preferable to add the amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. All components of this medium are added before autoclaving. Agar is not added to liquid media.

TABLE SPA-16. **Calcium chloride solution for encapsulating *Spathoglottis plicata* in alginate**

Component	Weight or volume	Remarks
Calcium chloride, $CaCl_2 \cdot 2H_2O$	11.03 g	75 mmol, formula weight is 147.02
Distilled water	To 1000 ml	Solution is 75 m*M* in respect to calcium choride

Media for the Germination of Encapsulated PLBs. MS containing NAA and BA (Table Spa-17) should be used for in vitro germination of encapsulated PLBs. Soilrite (Chougule Industries, Bangalore, India) moistened with sucrose and inositol-free MS (Table Spa-18) or a good garden soil moistened with the same solution (Table Spa-18) are appropriate for germination under horticultural conditions.

Media for Plantlet Growth. No media are suggested for the culture of plantlets to maturation. Plantlets can be cultured on one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

Potting Mix. A good orchid potting mix should be used to pot well-developed plantlets.

Encapsulation. PLBs, 45 days old and 4–5 mm in size, are mixed with the sodium alginate solution (Tables Spa-14 or Spa-15) and dropped into the calcium chloride solution (Table Spa-16). Small white beads are formed after 25 min. They are collected by decanting the solution in which they were formed.

Storage. See Low Temperature Storage section above. According to another report on encapsulation of PLBs, the alginate capsules may stick to each other. Dusting them with talcum powder may prevent this from happening. Capsules which will be germinated in vitro must be coated with sterilized talcum and stored under sterile conditions. Those that will be germinated horticulturally do not require sterility.

Germination in Vitro. Encapsulated PLBs should be prepared (using the alginate solution in Table Spa-14), stored, and germinated under sterile conditions on autoclaved medium (Table Spa-17). They should be allowed to germinate and develop shoots or plantlets which should be moved to one of the plant culture media once they become large enough.

Germination under Horticultural Conditions. PLBs should be encapsulated in a medium that contains a bactericide and a fungicide (Table Spa-15) and germinated on Soilrite mixture, a good garden soil, or a potting mix moistened with sucrose and

TABLE SPA-17. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the germination in vitro of protocorm-like bodies of *Spathoglottis plicata* encapsulated in alginate (Nayak et al., 1998)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (IAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.8, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave.

TABLE SPA-18. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for moistening the soil mix for horticultural germination of alginate-encapsulated protocorm-like bodies of *Spathoglottis plicata* (Nayak et al., 1998*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Auxin				
9	Naphthaleneacetic acid (IAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
10	Benzylaminopurine (BAP)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Solvent				
14	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively. Since there is no indication in the original repport regarding the use of plant hormones the amounts suggested here are the same as in Table Spa-17.

[g]Add items 1–13 to 900 ml of distilled water (item 14), adjust pH to 5.8, and raise volume to 1000 ml with distilled water (item 14).

The very first artificial (i.e., human-made) orchid hybrid produced in Singapore was *Spathoglottis* Primrose (*Spathoglottis aurea* × *Spathoglottis plicata*). The cross was made in 1932 at the Singapore Botanic Gardens by Professor R. E. Holttum, who was the director at the time.

inositol-free MS (Table Spa-18). Once the plantlets are large enough they should be moved to an appropriate potting mix (see above).

Procedure. Explants are cultured on the first medium (Table Spa-13). When PLBs reach a size of 4–5 mm they should be encapsulated (Tables Spa-14, Spa-15, and Spa-17) and stored for up to 45 days. These encapsulated PLBs are germinated in vitro. The ones encapsulated with the solution in Table Spa-14 should be germinated on the medium in Table Spa-17 or under horticultural conditions (those encapsulated with the solution in Table Spa-15 should be placed on a mix wetted with the MS in Table Spa-18). Shoots or plantlets that develop in vitro (Fig. Spa-3A) are eventually moved to one of the growth media (see Tables Aranda-6 to Aranda-8,

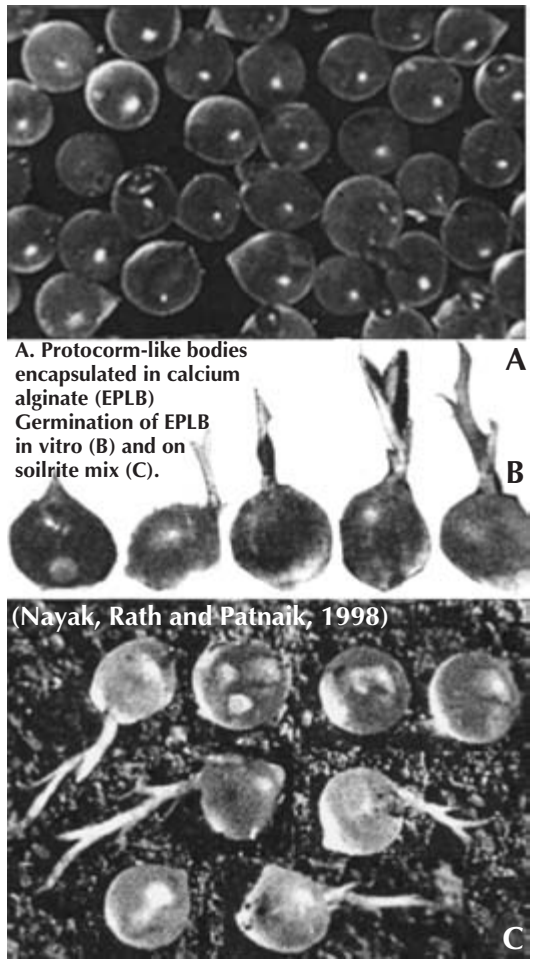


FIG. SPA-3. **Encapsulated PLBs and their germination in vitro (A) and in soil mix (B). (Modified from Nayak et al., 1998*b*.)**

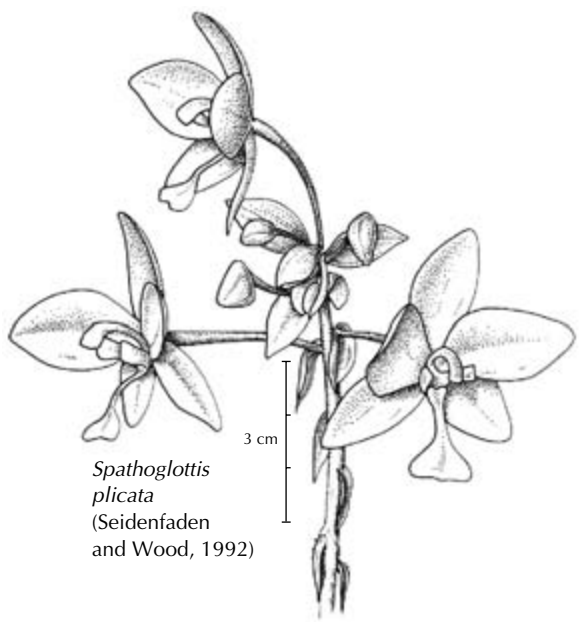
Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, or V-4). When plantlets on one of these media become large enough they are potted in pots in a potting mix. Plantlets that develop under horticultural conditions (Fig. Spa-3B) are allowed to grow to appropriate size before being potted.

Developmental Sequence. When placed on the appropriate medium (Table Spa-17) in vitro, 92–95% of the encapsulated PLBs (formed in the solutions in Tables Spa-14 and Spa-16) will germinate in 10–12 days. About 80–85% of these PLBs will multiply extensively and produce an average of 12–14 plantlets within 60 days. The plantlets can be expected to have 4–6 leaves and 3–4 roots.

When germinated under horticultural conditions, only 45–51% of the encapsulated PLBs (formed in the solutions in Tables Spa-15 and Spa-16) form plantlets.

General Comments. Encapsulation of PLBs in alginate is a new technology which may eventually play a major role in micropropagation (for a more extensive discussion of encapsulation and an excellent color photograph see Hew and Yong, 2004).

Spathoglottis is derived from the Greek *spathe* (σπάθη) which means spathe and *glotta* (γλῶττα) for tongue. The allusion is to the very broad mid lobe of the labellum (Schultes and Pease, 1963; Holttum, 1964).



Spathoglottis plicata (Seidenfaden and Wood, 1992)

Spiranthes

A terrestrial genus, *Spiranthes* consists of approximately 50 species most of which are found in the Americas. A few species occur in Europe and Asia. One is of much wider distribution. The name of the genus is derived from the Greek *speira* (σπειρα), which means coil, and *anthos* (ἄνθος) for flower and alludes to the coiled inflorescence.

Micropropagation of *Spiranthes sinensis*

Spiranthes sinensis is distributed from Sakhalin and Siberia, eastern Russia, in the north to India, the Malay Archipelago, Australia and the southwestern Pacific Islands. It grows in sunny, open, grassy, or disturbed areas up to elevations of 2000 m. A micropropagation method for this species was developed at the Laboratory of Plant Chromosomes and Gene Stock, Faculty of Science, Hiroshima University, Japan (Sato et al., 1987; Tanaka, Kondo and Sato, 1997).

Plant Material. "Shoot primordia" taken from diploid and colchicine-induced tetraploid protocorms were cultured. The protocorms were produced by germinating "10- to 13-day-old" immature seeds on a medium consisting of 2.5 g Hyponex l^{-1}, 35 g sucrose l^{-1}, 2 g peptone l^{-1}, and 9 g agar l^{-1} at 22°C under 500 lx of "full illumination" (perhaps meaning 24-h photoperiods).

Surface Sterilization. Explants taken from protocorms growing in vitro do not require surface sterilization.

Culture Vessels. Culture tubes, 30 × 200 mm, containing 25 ml medium were used in the original research. Other containers are also suitable.

Culture Conditions. Protocorms that will be the source of the explants should be cultured in liquid medium in culture tubes or other containers placed on a rotating shaker, 1 m in diameter, rotating at 2 rpm. In the original research the cultures were maintained under 2000–10,000 lx of "full [perhaps meaning continuous or 24-h] illumination." Shoot primordia should be cultured under the same conditions. When cultured under the same conditions, PLBs produced by the primordia multiplied and produced plantlets.

Culture Media. Shoot primordia (presumably the reference is actually to shoot initials or shoot tips) should be cultured on B5 medium (Gamborg et al., 1968) supplemented with 2 mg BA l^{-1} (Table Spi-1). PLBs produced in this medium "differentiated and multiplied PLBs and later regenerated plantlets in B5 liquid medium lacking growth substance" (Table Spi-2).

Procedure. Shoot primordia (initials or tips) from seedlings are cultured on the first medium (Table Spi-1) and subcultured on the same solution every 3 weeks. PLBs should be grown on the second medium (Table Spi-2) to produce plantlets.

TABLE SPI-1. **B5 medium (Gamborg et al., 1968) modified for the culture of *Spiranthes sinensis* shoot primordia and protocorm-like bodies (Tanaka et al., 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	134.0	13.4 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	150.0	15.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	2500.0	250.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.0	300.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	10.0	1.0 g l^{-1}		
(e)	Potassium iodide, KI	0.75	75.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.0	200.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
9	Benzyladenine (BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
10	Niacin (nicotinic acid)	1.0	10 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–8 to 900 ml of distilled water (item 14), adjust pH to 5.7, add sugar (item 13), and adjust volume to 1000 ml with distilled water (item 14). Pour the solution into culture vessels and autoclave. Vitamins (items 10–12) and hormones (item 9) are generally not heat-stable and it is preferable to add them to culture media after autoclaving. This is not done with the present medium.

TABLE SPI-2. **B5 medium (Gamborg et al., 1968) modified for plantlet production from protocorm-like bodies of *Spiranthes sinensis* (Tanaka et al., 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	134.0	13.4 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	150.0	15.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	2500.0	250.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.0	300.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	10.0	1.0 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.75	75.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.0	200.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
9	Niacin (nicotinic acid)	1.0	10 mg 100 ml^{-1} 95% ethanol[e]	1	
10	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Thiamine (vitamin B_1)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
12	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
13	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Add items 1–8 to 900 ml of distilled water (item 13), adjust pH to 5.7, add sugar (item 12), and adjust volume to 1000 ml with distilled water (item 13). Pour the solution into culture vessels and autoclave. Vitamins (items 9–11) are generally not heat-stable and it is preferable to add them to culture media after autoclaving. This is not done with the present medium.

Developmental Sequence. Explants produce PLBs on the first medium (Table Spi-1). These PLBs "differentiate and multiply" on the same solution (Table Spi-1). Plantlets are produced on the second medium (Table Spi-2).

General Comments. Seeds of terrestrial orchids from temperate climates are more difficult to germinate than those of tropical species. Explants of temperate climate orchids are also more difficult to culture in vitro. Therefore this protocol is of special interest.

Thelymitra

The method developed for *Diuris longifolia* (Collins and Dixon, 1992) is also suitable for the genus *Thelymitra* (Kingsley W. Dixon, pers. comm., Kings Park and Botanical Garden, West Perth, Australia, Kingsley.Dixon@mail.bigpond.com).

Thunia

One of the most beautiful orchids from the northeastern Himalayas, *Thunia alba* has white, mildly scented flowers. This species is slow growing and on the verge of extinction. A single shoot is produced every year, and as a result propagation is also slow. The method described here accelerates propagation without damaging or endangering an existing plant (Singh and Prakash, 1984).

Plant Material. Sections 4–6 cm long of flower stalks are removed from mature plants.

Surface Sterilization. Flower stalks should be wiped clean with cheesecloth soaked in 95% ethanol and then cut into sections. The sections should be washed with a detergent (Teepol was used in the original research, other detergents are also suitable). After that the sheaths that cover the nodes are removed, and the sections are surface-sterilized in 4% sodium hypochlorite (80 ml Clorox or another household bleach that contains 5% sodium hypochlorite diluted to 100 ml with distilled water) to which a drop of detergent has been added. The sections should be rinsed with sterile distilled water after that.

Culture Vessels. Test tubes, Erlenmeyer flasks (250-ml capacity containing 100 ml liquid medium), or other containers can be used.

Culture Conditions. Cultures should be maintained under 16-h photoperiods of 1.5 W m^{-2} (sources not described) at 25 ± 2°C. Liquid cultures are placed on a rotary shaker at 120 rpm.

Culture Media. Sections are first cultured on a modified Vacin and Went medium (Table Thu-1). After 35 days on this medium the sections should be moved to a second modification (Table Thu-2). Multiple plantlets and PLBs are induced in a liquid medium (Table Thu-3). PLBs are moved to a fourth medium (Table Thu-4) for differentiation.

Procedure. Place sections on the first (Table Thu-1) and then second (Table Thu-2) media for the production of single plantlets. If multiple plants are desired, culture the sections in the liquid medium (Table Thu-3) and move the resulting PLBs to the differentiation solution (Table Thu-4).

Developmental Sequence. Buds start to grow after 17 days and produce shoots within 35 days. Rooted plantlets are obtained 67 days following transfer to the

TABLE THU-1. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of flower-stalk buds of *Thunia alba* (Singh and Prakash, 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Antiauxin				
8	*trans*-Cinnamic acid	100	No stock	No stock	Weigh
	Cytokinin				
9	Benzyl adenine (BA)	10	No stock	No stock	Weigh
	Complex additive				
10	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
11	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[e]	To 1000 ml			
	Solidifier				
13	Agar[e]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Add items 1 and 3–10 to the 500 ml distilled water (item 12) that contains item 2; adjust pH to 5.2, add sugar (item 11), and adjust volume to 1000 ml with distilled water (item 12). Bring solution to a gentle boil, and add agar (item 13) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar for liquid medium. Media that contain hormones and other labile organic additives should not be autoclaved unless experimental evidence has determined that this will not have a deleterious effect. This is such a case.

second medium (Table Thu-2). PLBs form after 21–27 days in liquid solution (Table Thu-3). Multiple plantlets are obtained 41–47 days after transfer to the fourth formulation (Table Thu-4). In the original research 95 plants (out of 153, or 62.1%) survived the transfer to pots.

General Comments. Dormant flower-stalk buds "remain dormant under normal conditions. . . . [and] rarely develop into a secondary shoot. . . . [they are] induced to form multiple plantlet[s]" through this method without endangering the existing plant. These features make this a very useful, effective, and safe method for the propagation of a rare, endangered, and desirable species.

TABLE THU-2. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of growing flower-stalk buds of *Thunia alba* (Singh and Prakash, 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxin				
8	Indolebutyric acid (IBA)	5.0	500 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Keep refrigerated or frozen between uses. If the auxin fails to dissolve add a few drops of 0.1 N KOH.

[f]Add items 1 and 3–8 to the 500 ml distilled water (item 10) that contains item 2; adjust pH as required, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar for liquid medium. Media that contains hormones and other labile organic additives should not be autoclaved unless experimental evidence has determined that this will not have a deleterious effect. This is such a case.

TABLE THU-3. **Modified Vacin and Went medium (Vacin and Went, 1949) for the induction of multiple plantlets from flower-stalk buds of *Thunia alba* (Singh and Prakash, 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxin				
8	Indolebutyric acid (IBA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
9	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
10	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
11	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
12	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Keep refrigerated or frozen between uses.

[f]Add items 1 and 3–10 to the 500 ml distilled water (item 12) that contains item 2; adjust pH to 5.2, add sugar (item 11), and adjust volume to 1000 ml with distilled water (item 12). Dispense medium into culture vessels, and autoclave. Omit agar for liquid medium. Media that contain hormones and other labile organic additives should not be autoclaved unless prior experimental evidence indicates that this will not have a deleterious effect. This is such a case.

TABLE THU-4. **Modified Vacin and Went medium (Vacin and Went, 1949) for plantlet differentiation from protocorm-like bodies derived from flower-stalk buds of *Thunia alba* (Singh and Prakash, 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxin				
8	Indolebutyric acid (IBA)	5.0	500 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
9	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
10	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Agar[f]	9 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate the preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Keep refrigerated or frozen between uses. If the auxin fails to dissolve add a few drops of 0.1 N KOH.

[f]Add items 1 and 3–9 to the 500 ml distilled water (item 11) that contains item 2; adjust pH as required, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Bring solution to a gentle boil, and add agar (item 12) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar for liquid medium. Media that contain hormones and other labile organic additives should not be autoclaved unless experimental evidence indicates this will not have a deleterious effect. This is such a case.

Trudelia

See *Vanda*.

Vanda

In Hawaii and other areas, *Vanda* blossoms are of considerable importance as cut flowers for local use and export. Furthermore, many clones are much sought after by hobby growers. *Vanda* Miss Joaquim is the national flower of Singapore; as a consequence this orchid is grown very widely in that island-city republic. For these reasons methods for mass rapid clonal propagation were needed for a long time, but the genus proved difficult to culture for a while (Morel, 1964*a*, 1965*a*, 1965*b*). Air layering was used for some *Vanda* clones (Watkins, 1942), but these methods are slow. The first experiments in *Vanda* tissue culture were carried out at the University of Singapore with callus masses derived from seedlings (Rao, 1963) and a medium that contains 2,4-D and tomato juice (Rao, 1967). In later work *Vanda* explants grew slowly, produced callus, and eventually formed PLBs (Morel, 1970). When tissue culture procedures were developed, some were used to induce polyploidy in plantlets produced by these methods (Sagawa and Sehgal, 1967; Kunisaki et al., 1972; Sanguthai and Sagawa, 1973; Teo et al., 1973).

Shoot-tip Culture of Terete-leaf *Vanda*

The culture of *Vanda* shoot tips was difficult if not impossible until a sugar-free medium was formulated (Kunisaki et al., 1972).

Plant Material. Stems of *Vanda* (Miss Joaquim in the original experiments) are removed at the node of the fifth visible leaf. Tissue above this node is still soft and succulent, and axillary buds are easy to excise.

Surface Sterilization. Before removal of the sheaths, 10% Clorox is used for 10 min, followed by 5% Chlorox for 5 min.

Culture Vessels. The vessels employed for *Cymbidium* are used (Sagawa et al., 1966).

Culture Conditions. Liquid cultures are placed on a rotator (a New Brunswick Model V shaker operated at 160 rpm was used in the original research). Solid ones are maintained on a shelf or bench. Both are kept under 200 ft-c of continuous illumination provided by General Electric Power Groove white fluorescent lamps (Sylvania Grow Lux could also prove satisfactory). The temperature should be 26 ± 3°C.

Culture Media. Explants are placed initially on solid modified Vacin and Went medium (Table V-1). The best proliferation is obtained in a sugar-free liquid modification of the same solution (Table V-2). Differentiation occurs on a third modification (Table V-3).

TABLE V-1. **Modified Vacin and Went medium (Vacin and Went, 1949) for the culture of shoot tips of terete-leaf *Vanda* (Kunisaki et al., 1972)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier				
11	Agar[e]	8 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Add items 1 and 3–8 to the 500 ml distilled water (item 10) that contains item 2, adjust pH to 5.2–5.5, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). For solid medium bring the solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar if preparing liquid medium.

Procedure. Remove leaf portions beyond the sheath, and cut stems at internodes into smaller sections. Surface-sterilize these sections in 10% Clorox for 10 min. Remove the sheaths, sterilize with 5% (v/v) Clorox for 3 min, and place on culture medium. After 45 days on solid modified medium (Table V-1) approximately 63% of the explants will form semispherical structures. At this stage, the leaves and apex should be removed from each swollen bud; otherwise proliferation will not occur and only one plantlet will be formed. Proliferation will begin approximately 60 days after the second excision. For rapid and vigorous proliferation, transfer tissue to the liquid medium (Table V-2). When plantlets are desired, the proliferating bodies are transferred to the third modification (Table V-3).

Developmental Sequence. Initial growth and proliferation occur on the first medium, increased proliferation takes place on the second, and plantlets differentiate on the third.

TABLE V-2. **Modified Vacin and Went medium (Vacin and Went, 1949) for the proliferation of shoot-tip explants of terete-leaf *Vanda* (Kunisaki et al., 1972)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water	150 ml	No stock	No stock	Measure
	Solvent				
9	Water, distilled[e]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.
[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.
[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of the medium after that.
[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.
[e]Add items 1 and 3–8 to the 500 ml of distilled water (item 9) that contains item 2, set pH to 5.2–5.5, and adjust volume to 1000 ml with distilled water (item 9), dispense medium into culture vessels and autoclave.

TABLE V-3. **Modified Vacin and Went medium (Vacin and Went, 1949) for the differentiation of shoot-tip explants of terete-leaf *Vanda* (Kunisaki et al., 1972)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	10 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[e]	To 1000 ml			
	Solidifier				
11	Agar[e]	8 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.
[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.
[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of the medium after that.
[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.
[e]Add items 1 and 3–8 to the 500 ml of distilled water (item 10) that contain item 2, adjust pH to 5.2–5.5, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring solution to a gentle boil, and add agar (item 11) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar if preparing liquid medium.

General Comments. This is a rapid method for clonal propagation of terete *Vanda* that requires the sacrifice of a shoot, two excisions, and several tissue transfers. Because of that it may seem a bit complicated. However, with limited practice it should be an easy procedure to master.

Stem Culture of Terete-leaf *Vanda*

Until 1967, *Vanda* clones were not being propagated fast enough to suit the needs of Hawaiian growers. In the search for faster methods, a successful attempt was made to develop methods for aseptic stem propagation of *Vanda* Miss Joaquim (Sagawa and Sehgal, 1967).

Plant Material. Clean stems cut into 45–50-mm sections are used.

Surface Sterilization. Use 10% (v/v) Clorox for 20–30 min (10 ml Clorox and 90 ml distilled water).

Culture Vessels. Employ 25 × 100 mm vials containing culture medium to a height of 30–35 mm. As plants become larger, transfer to 125- or 250-ml Erlenmeyer flasks containing 25 or 50 ml medium, respectively.

Culture Conditions. Maintain cultures under conditions appropriate for orchid seed germination (Arditti, 1967; Arditti et al., 1982).

Culture Media. Vacin and Went medium modified by the use of 8 g agar (Table V-4) is used.

Procedure. Sections should be 45–50 mm long with 12–15 mm above and 35–40 mm below the node. Using a sterile razor blade, remove the leaf, exposing the bud. Surface-sterilize with 10% Clorox for 20–30 min. On a sterile surface, using a sterilized razor blade or scalpel, remove 3–4 mm from each end (these are usually discolored portions). Then insert the sections in the medium at a slight angle with approximately 10 mm of the part below the bud in the agar. Place vials under the appropriate culture conditions. Generally, treat sections and the resulting plantlets like seedlings.

Developmental Sequence. Shoot growth may occur first, but within 2–3 months cuttings are are well rooted and ready for planting in a greenhouse.

General Comments. Up to 80% of vegetative stem cuttings produce plantlets with this method. Sections from flower stalks may swell, but the results are not as good.

TABLE V-4. **Modified Vacin and Went medium (Vacin and Went, 1949) for stem culture of terete-leaf *Vanda* (Sagawa and Seghal, 1967)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Sugar				
8	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	8 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Add items 1 and 3–7 to the 500 ml distilled water (item 9) that contain item 2, adjust pH as required, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring solution to a gentle boil, and add agar (item 10) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar if preparing liquid medium.

Propagation of Strap-leaf *Vanda* by Shoot-tip Culture

Even as late as 1973, limited literature was available on shoot-tip or other tissue culture methods for the clonal propagation of monopodial orchids. Because of that, publication of a new method for the propagation of strap-leaf *Vanda* (*Vanda insignis* × *Vanda tessellata*) was of considerable interest (Teo et al., 1973).

Plant Material. Apical and axillary buds from seedlings of strap-leaf *Vanda* that had 8–12 leaves were used in the original experiments.

Surface Sterilization. The procedures used for *Cymbidium* (Sagawa et al., 1966) are suitable.

Culture Vessels. Vessels employed for *Cymbidium* (Sagawa et al., 1966) can be used.

Culture Conditions. Maintain cultures at 26 ± 3°C under continuous illumination of approximately 200 ft-c. In the original experiment the light sources were General

TABLE V-5. **Modified Vacin and Went medium (Vacin and Went, 1949) for culture of shoot-tip explants from strap-leaf *Vanda* (Kunisaki et al., 1972)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water	150 ml	No stock	No stock	Measure
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[e]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.
[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.
[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of the medium after that.
[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.
[e]Add items 1 and 3–8 to the 500 ml distilled water (item 10) that contains item 2, adjust pH to 5.2–5.5, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Dispense medium into culture vessels and autoclave.

Electric Power Groove fluorescent lamps, but others (e.g., Sylvania Gro Lux) should also prove satisfactory. Liquid cultures should be agitated (a New Brunswick Model V shaker operated at 160 rpm was used by Teo et al., 1973).

Culture Media. Modified liquid Vacin and Went medium (Table V-5) is used for initial culture. Proliferation occurs in a second modification of this solution (Table V-6). Differentiation occurs on solid Vacin and Went medium (see Table Aranda-6).

Procedure. Excise and sterilize buds according to the procedure used for the clonal propagation of *Cymbidium* through shoot-tip meristem culture (Sagawa et al., 1966). Place the explants in the initial medium (Table V-5). After 1 month, excise the leaf primordia that cover the explants. When growth and proliferation start, transfer the tissues to the second modification (Table V-6). PLBs will form and separate from each other due to the agitation. These should be moved to solid Vacin and Went medium (see Table Aranda-6) for plantlet formation.

TABLE V-6. **Modified Vacin and Went medium (Vacin and Went, 1949) for the proliferation of shoot-tip explants from strap-leaf *Vanda* (Kunisaki et al., 1972)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
	Iron[d]				
6	Ferric tartrate, $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	28	No stock	No stock	
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Complex additive				
8	Coconut water	150 ml	No stock	No stock	Measure
	Solvent				
9	Water, distilled[e]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[e]Add items 1 and 3–8 to the 500 ml distilled water (item 9) that contains item 2, adjust pH to 5.2–5.5, and adjust volume to 1000 ml with distilled water (item 9). Dispense medium into culture vessels and autoclave. For a similar medium with sugar see Table V-5.

Developmental Sequence. Approximately 25% of all cultures can be expected to proliferate. The explants swell initially and then become spherical owing to the development of leaf primordia. If these are not removed, there will be no further proliferation. When they are excised, the tissues proliferate very well on Vacin and Went with coconut water but no sucrose, forming PLBs that are separated by the agitation. Some tissues in proliferating cultures produce PLBs continuously. Others continue to proliferate but also form plantlets. A third group may form clumps of plantlets with only leaves. On transfer to solid Vacin and Went medium, plantlets differentiate from the PLBs.

General Comments. When the buds are visible (i.e., not covered by leaf sheaths), culture contamination rates may be high. This is probably because debris accumulates in the leaf axils, facilitated by the strap leaves. To minimize contamination, it is best to obtain explants from buds that are still tightly enclosed within their sheaths. Owing to the need to perform a second excision and to transfer the tissues at least twice, the procedure may require some practice.

In Vitro Culture of *Vanda* Miss Joaquim

One of the most common and popular hybrids is *Vanda* Miss Joaquim (*Vanda teres* × *Vanda hookeriana*), the national flower of Singapore. It is not surprising, therefore, that a number of workers have used it in efforts to develop tissue culture methods for *Vanda*. One method was developed at the Botany Department of the University of Singapore (Goh, 1970).

Plant Material. Shoot tips, axillary buds, and roots excised from 15-month-old seedlings are used.

Surface Sterilization. Since the tissues are obtained from seedlings growing under sterile conditions, surface decontamination should not be needed. When necessary, tissues can be sterilized for 5 min with 5% (w/v) calcium hypochlorite (5 g calcium hypochlorite mixed with enough distilled water to bring the total volume up to 100 ml) and rinsed with sterile distilled water before being placed into culture.

Culture Vessels. Use 100- or 125-ml Erlenmeyer flasks containing 20 or 25 ml medium, respectively.

Culture Conditions. Shoot cultures should be maintained under 12-h photoperiods (light intensity and type of illumination not given) at 27°C. Root tips were kept in the dark during the initial experiments.

Culture Media. A modification of White's medium (Table V-7) is used for growing the seedlings that serve as explant sources. Shoot and root tips are cultured on another modification of this medium (Table V-8). Excised roots can also be cultured in two additional modifications (Table V-9, V-10).

Vanda Miss Joaquim: a Foundling which Rose to Prominence

Vanda Miss Joaquim is a natural hybrid between *Vanda hookeriana* and *Vanda teres*. It is not known which of them is the seed or pollen parent, but it is reasonably safe to assume that the pollinating agent was a carpenter (*Xylocopa*) bee. The original plant was discovered in 1893 in a clump of bamboo by Miss Agnes Joaquim. The hybrid became a sensation and quickly spread to many countries. *Vanda* Miss Joaquim was a mainstay of the Hawaiian orchid industry for many years, but was eventually replaced by other varieties. With time it was almost forgotten. In 1981 *Vanda* Miss Joaquim was selected to become the National Flower of Singapore and gained prominence again. At present *Vanda* Miss Joaquim can be seen in gardens and parks throughout Singapore. Its image is also on stamps, decorations, currency, jewelry, garments and even refrigerator magnets.

TABLE V-7. **Modified White's medium (White, 1943) for the culture of seedlings of *Vanda* Miss Joaquim (Goh, 1970)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[c]	200	20.0 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4$	360	36 g l^{-1}	10	
3	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	16.5	1.65 g l^{-1}	10	
4	Potassium chloride, KCl	65	6.5 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	80	8 g l^{-1}	10	
6	Sodium sulfate, Na_2SO_4	200	20.0 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	3.4	344 mg l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	4.66	466 mg l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3[f]	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$[f]	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$[f]	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	6.65	665 mg l^{-1}		
(e)	Potassium iodide, KI	0.75	75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$[f]	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.67	267 g l^{-1}		
	Amino acid				
9	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
13	Coconut water	200 ml	No stock	No stock	Measure
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[h]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]There are many inconsistencies and contradictions in the literature regarding White's medium, some of them in his own papers. This table is based on an excellent recent study, reevaluation, and modernization of the medium (Singh and Krikorian, 1981). As a result the microelements and iron vary slightly from the one used by Goh (1970).

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]To prepare chelated iron add chelating agent (item 7a) and iron salt (item 7b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, stir and/or heat until dissolved. Dispense volume indicated per liter of culture medium.

[f]Not part of White's recipe but advisable to add.

[g]Keep refrigerated or frozen between uses.

[h]Add items 1–8 and 13 to 700 ml distilled water (item 15), adjust pH to 5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add items 9–12 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE V-8. **Modified White's medium (White, 1943) for the culture of shoot tips and axillary buds of *Vanda* Miss Joaquim (Goh, 1970)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[c]	200	20.0 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4$	360	36 g l^{-1}	10	
3	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	16.5	1.65 g l^{-1}	10	
4	Potassium chloride, KCl	65	6.5 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	80	8 g l^{-1}	10	
6	Sodium sulfate, Na_2SO_4	200	20.0 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	3.4	344 mg l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	4.66	466 mg l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3[f]	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$[f]	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$[f]	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	6.65	665 mg l^{-1}		
(e)	Potassium iodide, KI	0.75	75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$[f]	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.67	267 g l^{-1}		
	Amino acid				
9	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Auxin				
13	2,4-Dichlorophenoxyacetic acid (2,4-D)	2	200 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[h]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]There are many inconsistencies and contradictions in the literature regarding White's medium, some of them in his own papers. This table is based on an excellent recent study, reevaluation, and modernization of the medium (Singh and Krikorian, 1981). As a result the microelements and iron vary slightly from the one used by Goh (1970).

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]To prepare chelated iron add chelating agent (item 7a) and iron salt (item 7b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, stir and/or heat until dissolved. Dispense volume indicated per liter of culture medium.

[f]Not part of White's recipe but advisable to add.

[g]Keep refrigerated or frozen between uses.

[h]Add items 1–8 to 700 ml distilled water (item 15), adjust pH to 5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add items 9–13 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE V-9. **Modified White's medium (White, 1943) for the culture of root tips of *Vanda* Miss Joaquim (Goh, 1970)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[c]	200	20.0 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4$	360	36 g l^{-1}	10	
3	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	16.5	1.65 g l^{-1}	10	
4	Potassium chloride, KCl	65	6.5 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	80	8 g l^{-1}	10	
6	Sodium sulfate, Na_2SO_4	200	20.0 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	3.4	344 mg l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	4.66	466 mg l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3[f]	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$[f]	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$[f]	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	6.65	665 mg l^{-1}		
(e)	Potassium iodide, KI	0.75	75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$[f]	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.67	267 g l^{-1}		
	Amino acid				
9	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
13	Tomato juice[h]	100 ml	No stock	No stock	Measure
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[i]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]There are many inconsistencies and contradictions in the literature regarding White's medium, some of them in his own papers. This table is based on an excellent recent study, reevaluation, and modernization of the medium (Singh and Krikorian, 1981). As a result the microelements and iron vary slightly from the one used by Goh (1970).

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]To prepare chelated iron add chelating agent (item 7a) and iron salt (item 7b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, stir and/or heat until dissolved. Dispense volume indicated per liter of culture medium.

[f]Not part of White's recipe but advisable to add.

[g]Keep refrigerated or frozen between uses.

[h]Commercial tomato juice, even so-called "natural" brands, may contain preservatives that can inhibit growth. Therefore it may be advisable to squeeze fresh ripe tomatoes, filter the juice to remove all seeds (which may also contain inhibitors), and use the filtrate. Store unused portions in a freezer.

[i]Add items 1–8 and 13 to 700 ml distilled water (item 15), adjust pH to 5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add items 9–12 to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE V-10. **Modified White's medium (White, 1943) for the culture of root tips of *Vanda* Miss Joaquim (Goh, 1970)**[a]

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[b]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[c]	200	20.0 g l^{-1}	10	
2	Magnesium sulfate, $MgSO_4$	360	36 g l^{-1}	10	
3	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	16.5	1.65 g l^{-1}	10	
4	Potassium chloride, KCl	65	6.5 g l^{-1}	10	
5	Potassium nitrate, KNO_3[c]	80	8 g l^{-1}	10	
6	Sodium sulfate, Na_2SO_4	200	20.0 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	3.4	344 mg l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	4.66	466 mg l^{-1}		
8	**Microelements**[e]				
(a)	Boric acid, H_3BO_3[f]	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$[f]	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$[f]	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	6.65	665 mg l^{-1}		
(e)	Potassium iodide, KI	0.75	75 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$[f]	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.67	267 g l^{-1}		
	Amino acid				
9	Glycine	3	300 mg 100 ml^{-1} 95% ethanol[g]	1	
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
11	Pyridoxine (vitamin B_6)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Auxin				
13	Indoleacetic acid (IAA)	100	No stock	No stock	Weigh
	Sugar				
14	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[h]	To 1000 ml			
	Solidifier				
16	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]There are many inconsistencies and contradictions in the literature regarding White's medium, some of them in his own papers. This table is based on an excellent recent study, reevaluation, and modernization of the medium (Singh and Krikorian, 1981). As a result the microelements and iron vary slightly from the one used by Goh (1970).

[b]Amounts are given in mg unless indicated otherwise.

[c]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[d]To prepare chelated iron add chelating agent (item 7a) and iron salt (item 7b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[e]Add all microelements to the same 1 l of distilled water, stir and/or heat until dissolved. Dispense volume indicated per liter of culture medium.

[f]Not part of White's recipe but advisable to add.

[g]Keep refrigerated or frozen between uses.

[h]Add items 1–8 to 700 ml distilled water (item 15), adjust pH to 5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add 100 mg auxin (item 13) to combined items 9–12 in a small vial, shake well several times at 5-min intervals and allow mixture to stand before adding to hot solution under sterile conditions with sterilized pipettes. Mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

Procedure. Place explants into culture and allow them to develop. Remove plantlets, when formed, and culture individually.

Developmental Sequence. Rapid cell division takes place in shoot-tip explants, forming new growth centers. Some of them become organized into growing apices from which young shoots develop within about 3 months. When excised and subcultured, these shoots produce one or more plantlets.

Axillary buds give rise to side shoots when cultured on a solid medium. No roots are produced, however; therefore, plantlets are not formed.

Roots elongate in culture and turn white. Their anatomical features are the same as those of normal roots.

General Comments. Shoot-tip explants can be used for the clonal propagation of *Vanda* Miss Joaquim. On an appropriate medium, roots should develop on the shoots that arise from axillary buds. Perhaps the medium used to induce root formation on *Dendrobium* shoots (see Table Den-20) could be used.

Mericloning of *Vanda* through the Culture of Axillary Buds

The procedures employed for the culture of *Aranda*, *Dendrobium*, *Mokara*, and *Oncidium* (Khaw et al., 1978*a*, 1978*b*) can be used to culture apical and axillary buds of *Vanda*.

In Vitro Clonal Multiplication of *Vanda*

The propagation of *Vanda* from seeds and through tissue culture was studied extensively at the Plant Morphogenesis and Tissue Culture Section, Bio-Organic Division, Bhaba Atomic Research Centre, Bombay, India (Mathews and Rao, 1980, 1985*a*) leading to a tissue culture method (Mathews and Rao, 1985*b*).

Plant Material. In the original research, shoot tips 0.2–0.3 mm and 0.5 mm long and young leaves 5–15 mm in length were excised from seedlings of *Vanda* TMA × *Vanda* Miss Joaquim.

Surface Sterilization. There is no need to surface-sterilize explants taken from plants that are growing in vitro.

Culture Vessels. Test tubes and Erlenmeyer flasks can be used.

Culture Conditions. Cultures should be kept under continuous illumination of 900 lx (sources not listed) at 25 ± 2°C and 55–60% relative humidity.

Culture Media. An effective medium for the culture of shoot-tip explants and young leaves contains 2-isopentenyl adenosine (Table V-11). Other media tested during the original research also supported growth.

TABLE V-11. **Medium for the culture of *Vanda* explants (Mathews and Rao, 1980, 1985*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	500	50 g l^{-1}	10	
2	Ammonium sulfate, $(NH_4)_2SO_2$	400	50 g l^{-1}	10	
3	Calcium sulfate, $CaSO_4 \cdot 2H_2O$	80	8 g l^{-1}	10	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	180	18 g l^{-1}	10	
6	Potassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
7	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot H_2O$	0.05	5 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5 mg l^{-1}		
	Cytokinin				
9	2-Isopentenyl adenosine (2ip)	8	No stock	No stock	Weigh
	Vitamins				
10	Biotin	0.05	10 mg 100 ml^{-1} 95% ethanol[e]	0.5	
11	Folic acid	0.3	60 mg 100 ml^{-1} 95% ethanol	0.5	
12	Niacin (nicotinic acid)	1.25	250 mg 100 ml^{-1} 95% ethanol[e]	0.5	
13	Pyridoxine (vitamin B_6)	0.3	60 mg 100 ml^{-1} 95% ethanol[e]	0.5	
14	Riboflavine	0.05	10 mg 100 ml^{-1} 95% ethanol[e]	0.5	
15	Thiamine (vitamin B_1)	0.3	60 mg 100 ml^{-1} 95% ethanol[e]	0.5	
	Sugar				
16	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[f]	To 1000 ml			
	Solidifier				
18	Agar, Difco Bacto	8 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 7a) and iron salt (item 7b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep refrigerated or frozen between uses.

[f]Add items 1–15 to 900 ml distilled water (item 17), adjust pH to 5.6–5.6, add sugar (item 16), and adjust volume to 1000 ml with distilled water (17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar if preparing liquid medium. Media that contain hormones and vitamins should not be autoclaved unless prior experimentation has determined that the effects will not be deleterious. This is such a case.

Procedure. Explants are placed on the medium and allowed to form plantlets and/or PLBs. These can be moved to the same medium (Table V-11), to Knudson C solution (see Table Aranda-7), or to Vacin and Went formulation (see Table Aranda-6) for further differentiation and growth.

Developmental Sequence. Both the shoot-tip explants and young leaves produced PLBs. The main region of PLB production by leaves is the basal meristem.

General Comments. Like other procedures based on seedlings this method may or may not prove to be suitable for mature plants.

Mericloning of *Vanda* through the Culture of Root and Leaf Tips

Mass rapid clonal propagation of monopodial orchids

> "by shoot meristem culture has proved too impracticable. More often than not, the prized individual is killed as a result of excision of the only shoot meristem of the plant, and the explant also does not survive because of infection. It is, therefore, necessary to employ meristematic tissues of other organs, *viz.*, tips of leaf and root." (Chaturvedi and Sharma, 1986)

Plant Material. In the original research, root and leaf tips 2–8 mm long were excised from "aseptically established plants" (Chaturvedi and Sharma, 1986), which could refer to either seedlings or clonally propagated plantlets or both.

Surface Sterilization. There is no need to surface-sterilize plants that are growing under aseptic conditions.

Culture Vessels. Culture tubes and Erlenmeyer flasks can be used.

Culture Conditions. Cultures should be maintained at 27 ± 1°C under 14-h photoperiods of 3000 lx provided by fluorescent tubes.

Culture Media. A modified Vacin and Went medium (Table V-12) served as the basal solution. Formation of PLBs occurs on a modification of this medium (Table V-13). Plantlet development takes place on a third version (Table V-14). Unmodified Vacin and Went medium (see Table Aranda-6) can be used for growth of these plantlets.

Procedure. Place explants on the initial medium (Table V-12), and allow them to establish themselves. To form PLBs move the explants to the second modification (Table V-13). When small plants are visible, move them to the third medium (Table V-14) for development. Larger plants can be moved to unmodified Vacin and Went medium (see Table Aranda-6).

Developmental Sequence. Explants first increase in size and root tips turn green. After approximately 15 days, PLBs start to form. Bases of leaf-tip explants swell and start to form PLBs within 10 days. Explants on the second medium "showed

TABLE V-12. **Modified Vacin and Went medium (Vacin and Went, 1949) used as a basal solution for the culture of *Vanda* explants (Chaturvedi and Sharma, 1986)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	500	50 g l^{-1}	10	
2	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
3	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[e]				
(a)	Aluminum chloride, $AlCl_3$	0.03	3 mg l^{-1}		
(b)	Boric acid, H_3BO_3	1	100 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.03	3 mg l^{-1}		
(d)	Ferric chloride, $FeCl_3 \cdot 6H_2O$	1	100 mg l^{-1}	10	One solution
(e)	Manganese sulfate, $MnSO_4 \cdot H_2O$	0.1	10 mg l^{-1}		
(f)	Nickel chloride, $NiCl_2 \cdot 6H_2O$	0.03	3 mg l^{-1}		
(g)	Potassium iodide, KI	0.01	1 mg l^{-1}		
(h)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1	100 mg l^{-1}		
	Vitamins				
9	Niacin	0.1	20 mg 100 ml^{-1} 95% ethanol[f]	0.5	
10	Pyridoxine	0.1	20 mg 100 ml^{-1} 95% ethanol[f]	0.5	
11	Thiamine	0.1	20 mg 100 ml^{-1} 95% ethanol[f]	0.5	
	Sugar				
12	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
13	Water, distilled[e]	To 1000 ml			
	Solidifier				
14	Agar[g]	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of the medium after that.

[d]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Add all microelements to the same 1 l of distilled water, stir and/or heat until dissolved.

[f]Keep refrigerated or frozen between uses.

[g]Add items 1 and 3–8 to the 500 ml distilled water (item 13) that contain item 2, adjust pH to 5.2–5.5, add sugar (item 12), and adjust volume to 1000 ml with distilled water (item 13). Bring solution to a gentle boil, and add agar (item 14) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into a 2-l flask and autoclave. Add vitamins (items 9–11) under sterile conditions, swirl flask several times to insure complete mixing, and dispense medium into preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE V-13. **Modified Vacin and Went medium (Vacin and Went, 1949) for the induction of protocorm-like bodies from *Vanda* explants (Chaturvedi and Sharma, 1986)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	500	50 g l^{-1}	10	
2	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
3	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[e]				
(a)	Aluminum chloride, $AlCl_3$	0.03	3 mg l^{-1}		
(b)	Boric acid, H_3BO_3	1	100 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.03	3 mg l^{-1}		
(d)	Ferric chloride, $FeCl_3 \cdot 6H_2O$	1	100 mg l^{-1}	10	One solution
(e)	Manganese sulfate, $MnSO_4 \cdot H_2O$	0.1	10 mg l^{-1}		
(f)	Nickel chloride, $NiCl_2 \cdot 6H_2O$	0.03	3 mg l^{-1}		
(g)	Potassium iodide, KI	0.01	1 mg l^{-1}		
(h)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1	100 mg l^{-1}		
	Vitamins				
9	Niacin	0.1	20 mg 100 ml^{-1} 95% ethanol[f]	0.5	
10	Pyridoxine	0.1	20 mg 100 ml^{-1} 95% ethanol[f]	0.5	
11	Thiamine	0.1	20 mg 100 ml^{-1} 95% ethanol[f]	0.5	
	Auxins				
12	Indoleacetic acid (IAA)	1.0	200 mg 100 ml^{-1} 95% ethanol[f,g]	0.5	
13	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.1	20 mg 100 ml^{-1} 95% ethanol[f,g]	0.5	
	Cytokinin				
14	Benzylaminopurine (BAP)	0.1	20 mg 100 ml^{-1} 95% ethanol[f,g]	0.5	
	Complex additive				
15	Casein hydrolysate	200	No stock	No stock	Weigh
	Sugar				
16	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar[h]	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of the medium after that.

[d]The iron salt used in the original recipe for this medium is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Add all microelements to the same 1 l of distilled water, stir and/or heat until dissolved.

[f]Keep refrigerated or frozen between uses.

[g]If auxins or cytokinin do not dissolve, add a few drops of KOH or HCl, respectively.

[h]Add items 1, 3–8, and 15 to the 500 ml distilled water (item 17) that contains item 2, adjust pH to 5.2–5.5, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l Erlenmeyer flask and autoclave. Add vitamins (items 9–11) and hormones (items 12–14) under sterile conditions, swirl flask several times to insure complete mixing, and dispense medium into preautoclaved culture vessels. Omit agar if preparing liquid medium.

TABLE V-14. **Modified Vacin and Went medium (Vacin and Went, 1949) for the development of *Vanda* plantlets (Chaturvedi and Sharma, 1986)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	500	50 g l^{-1}	10	
2	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
3	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200	No stock	No stock	
4	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
6	Potassium nitrate, KNO_3[b]	525	52.5 g l^{-1}	10	
7	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
8	**Microelements**[e]				
(a)	Aluminum chloride, $AlCl_3$	0.03	3 mg l^{-1}		
(b)	Boric acid, H_3BO_3	1	100 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.03	3 mg l^{-1}		
(d)	Ferric chloride, $FeCl_3 \cdot 6H_2O$	1	100 mg l^{-1}	10	One solution
(e)	Manganese sulfate, $MnSO_4 \cdot H_2O$	0.1	10 mg l^{-1}		
(f)	Nickel chloride, $NiCl_2 \cdot 6H_2O$	0.03	3 mg l^{-1}		
(g)	Potassium iodide, KI	0.01	1 mg l^{-1}		
(h)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	1	100 mg l^{-1}		
	Vitamins				
9	Niacin	0.1	20 mg 100 ml^{-1} 95% ethanol[f]	0.5	
10	Pyridoxine	0.1	20 mg 100 ml^{-1} 95% ethanol[f]	0.5	
11	Thiamine	0.1	20 mg 100 ml^{-1} 95% ethanol[f]	0.5	
	Auxin				
12	Indoleacetic acid (IAA)	0.1	20 mg 100 ml^{-1} 95% ethanol[f]	0.5	
	Complex additive				
13	Casein hydrolysate	200	No stock	No stock	Weigh
	Sugar				
14	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	16 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]This salt is hard to dissolve, and it is not possible to prepare a stock solution. To facilitate preparation of medium, place 200 mg of the salt in 500 ml water, and stir and/or heat until it dissolves. Add other components of the medium after that.

[d]The iron salt used in the original recipe for this medium is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to the culture medium.

[e]Add all microelements to the same 1 l of distilled water, and stir and/or heat until dissolved.

[f]Keep refrigerated or frozen between uses.

[g]Add items 1, 3–8, and 13 to the 500 ml distilled water (item 15) that contains item 2, adjust pH to 5.2–5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring solution to a gentle boil, and add agar (item 16) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add vitamins (items 9–11) and hormone (item 12) under sterile conditions, swirl flask several times to insure complete mixing, and dispense medium into preautoclaved culture vessels. Omit agar if preparing liquid medium.

enormous proliferation of plb's and within a period of 30 days hundreds of them were formed." The PLBs develop leaves and form tiny plantlets, which become larger on the third medium.

General Comments. Like other procedures based on seedlings this method may or may not prove to be suitable for mature plants. Those who attempt to culture root tips from mature plants must select explants that do not contain fungi. It is possible, but not certain, that the tips of very young leaves of mature plants may respond to culture like leaf explants from seedlings.

Clonal Propagation of *Vanda* through the Culture of Flower Stalks and Buds

Flower buds have been used to culture a number of orchids and other plants. Research at the University of the Philippines, Los Baños has extended this approach to *Vanda* (Valmayor et al., 1986).

Plant Material. Inflorescence tips with flower buds attached are cultured. The bracts that cover or subtend each bud are removed.

Surface Sterilization. Cut the inflorescences, swab them with alcohol, surface-sterilize by immersing them in 1% calcium hypochlorite (1 g calcium hypochlorite in 100 ml distilled water stirred several times at 3–5-min intervals and filtered or decanted) for 30–40 min, and rinse three times with sterile distilled water. After that remove the bracts, immerse the explants for 30–60 s in 0.5% calcium hypochlorite (50 ml of the previous solution diluted to 100 ml with distilled water), and rinse again with sterile distilled water.

Culture Vessels. Erlenmeyer flasks are used for liquid or solid medium. Culture tubes or Erlenmeyer flasks can be employed for agar media.

Culture Conditions. Illumination and temperature regimes employed with other procedures for the culture of *Vanda* explants are suitable.

Culture Media. Explants should be cultured initially on a liquid modified Knudson C medium (Table V-15). PLBs formed on this medium differentiate well on unmodified Vacin and Went solution (see Table Aranda-6).

Procedure. Place explants in liquid medium (Table V-15; presumably on a shaker), and culture until PLBs form. These can be subcultured on the same medium (Table V-15) or moved to Vacin and Went solution (see Table Aranda-6) for differentiation and growth. The PLBs can be cultured in the initial medium (Table V-15) without subculture for 1–2 months. However, proliferation is more rapid if the PLBs are subcultured often by being moved to fresh medium (Table V-15). PLBs and plantlets can be moved to the second medium (see Table Aranda-6) containing banana homogenate after 2 months. If moved earlier they may die.

TABLE V-15. **Modified Knudson C medium (Knudson, 1946) for the culture of *Vanda* explants (Valmayor et al., 1986)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
	Iron[c]				
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Cytokinins				
7	Benzyladenine (BA)	1	20 mg 100 ml^{-1} 95% ethanol[d,e]	0.5	
8	Kinetin	1	20 mg 100 ml^{-1} 95% ethanol[d,e]	0.5	
	Complex additive				
9	Coconut water	150 ml	No stock	No stock	Weigh
	Sugar				
10	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[d]Keep refrigerated between uses.

[e]If cytokinins fail to dissolve, add a few drops of dilute HCl.

[f]Add items 1–6 and 9 to 700 ml distilled water (item 11), adjust pH to 5.0–5.5, add sugar (item 10), and adjust volume to 1000 ml with distilled water (item 11). Pour solution into a 2-l flask, and autoclave. Add cytokinins (items 7 and 8) under sterile conditions, swirl well to insure complete mixing, and dispense into preautoclaved culture vessels. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in an autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl), to lower pH if necessary.

Developmental Sequence. Formation of PLBs and plantlet growth is rapid, and plantlets that can be moved to community pots grow within a relatively short period.

General Comments. This system should serve as a model since it is effective, rapid, applicable to mature plants, and does not endanger the plant that serves as a source for explants.

Micropropagation of the Blue Vanda, *Vanda coerulea*

Sometimes called the queen of the vandas, *Vanda coerulea* was discovered in the Khasi Hills of Assam, India, in 1857. Its flowers are blue and/or purple, large, numerous and long-lasting. Some plants may bloom several times a year. This beautiful orchid is a "victim to its own beauty" and was threatened with extinction due to overcollecting. One attempt at conservation through micropropagation was made at the Tropical Botanic Garden and Research Institute, Palode Trivandrum, India (Anonymous, 1987).

Plant Material. Leaf bases (0.5–0.8 cm) and intact leaves with meristematic bases, both taken from seedlings, were cultured in the original research.

Surface Sterilization. There is no need to surface-sterilize explants taken from seedlings that grow under aseptic conditions.

Culture Vessels. Culture tubes and Erlenmeyer flasks can be used.

Culture Conditions. The cultures should be maintained under 12-h photoperiods of 4000 lx provided by fluorescent tubes at 25 ± 2°C.

Culture Media. A medium consisting of 800 mg Gaviota-63 l^{-1}, 30% coconut water, 500 mg peptone l^{-1}, 1 mg NAA l^{-1}, 1 mg BA l^{-1} and 7 g agar l^{-1} gave the best results. Satisfactory results can also be obtained on a modified Mitra, Prasad, and Roychowdhury medium (Table V-16). The addition to 70 g banana l^{-1} increases plantlet production.

Procedure. Place explants on the culture medium and allow to develop. To increase proliferation section the callus with the PLBs and transfer the segments to a medium containing 70 g banana l^{-1}. When the plantlets are large enough, they should be moved to community pots.

Developmental Sequence. Leaf bases (whether the leaves have been excised or are intact) swell and produce callus masses within 3 weeks. Shoots can form directly from the callus or from PLBs. Growth of shoots continues for 12 weeks, at which time roots appear. At the end of 14 weeks it is possible to harvest up to 70 plantlets from a single leaf base. The number can be doubled on banana-containing medium.

General Comments. If enough seeds are available, a clonal propagation procedure for seedlings is more valuable as a starting point for future research and attempts to culture explants from mature plants. When seeds are not available, such a method can be used to increase the number of rare and/or valuable seedlings.

TABLE V-16. **Modified Mitra, Prassad, and Roychowdhury medium (Mitra et al., 1976) for the culture of *Vanda coerulea* explants**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100	10 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	200	20 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180	18 g l^{-1}	10	
5	Monosodium phosphate, NaH_2PO_4	150	15 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	1	400 mg 100 ml^{-1} 95% ethanol[e,f]	0.25	
	Cytokinin				
15	Benzylaminopurine (BAP)	1	400 mg 100 ml^{-1} 95% ethanol[e,f]	0.25	
	Complex additive				
16	Peptone	2 g	No stock	No stock	Weigh
	Sugar				
17	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Agar, Difco Bacto[g]	7 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]The original recipe used 3 ml $Na_2FeEDTA$, which may not be readily available. The substitute listed here is very common in orchid tissue culture media. Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, stir and/or heat until dissolved. Dispense volume indicated per liter of culture medium.

[e]Keep refrigerated or frozen between uses.

[f]If auxin or cytokinin do not dissolve, add a few drops of KOH or HCl, respectively.

[g]Add items 1–16 to 900 ml distilled water (item 18), adjust pH to 5.6, add sugar (item 17), and adjust volume to 1000 ml with distilled water (item 18). Bring solution to a gentle boil, and add agar (item 19) slowly while stirring. Agar can also be added to the cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, dispense medium into culture vessels and autoclave. Omit agar if preparing liquid medium. Media that contain hormones and vitamins should not be autoclaved unless prior experiments have determined that this will have no deleterious effects. This is such a case (Mitra, 1971; Mitra et al., 1976), so medium may be autoclaved.

Development of Colchicine-treated *Vanda*

Some PLBs of *Vanda* Louise de Waldner (*Vanda* Waikiki × *Vanda teres*) treated with 0.05–0.15% colchicine for 5–20 days developed "leaves with curly tips and maroon coloration. . . . A few developed flower-like structures. . . . When potted out these variant plants" remained stunted, produced only a few roots, and died before reaching a height of 3 cm (Lim-Ho et al., 1988)

Isolation of *Vanda* Protoplasts

The method developed for *Acampe praemorsa* was used to isolate 14.1×10^4 and 5.5×10^4 protoplasts from leaves and roots, respectively, of *Vanda teres* (Seeni and Abraham, 1986).

Clonal Multiplication of *Vanda* through Root- and Leaf-tip Culture in Vitro

Root and leaf tips taken from "aseptically-grown plantlets" (probably seedlings) of *Vanda* TMA × *Vanda teres* formed PLBs on a modified Vacin and Went medium supplemented with 1 ppm BA, 1 ppm NAA, and 200 ppm casein hydrolysate (CH). The PLBs develop without callus formation. The plantlets proliferate with periodic subculture (every 2 months) on a medium containing 0.5 ppm BA, 1 ppm IAA, and 0.1 ppm 2,4-D. Plantlets develop well on a medium containing 0.1 ppm IAA and 200 ppm CH (Sharma and Chaturvedi, 1988).

Micropropagation of *Vanda coerulea* through the Culture of Leaf Bases

Leaf bases of *Vanda coerulea* seedlings formed PLBs and plantlets on "solid nutrient media in different factorial combinations of naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BA) . . . in modified Mitra . . . medium and also in the Gaviota-63 fertilizer supplemented with 35 g [l^{-1}] banana pulp" and 1 ppm BA plus 0.75 ppm NAA (Seeni, 1988).

Plantlet Regeneration from Root Tips of *Vanda testacea*

Root tips without apices of *Vanda testacea* taken from 32-week-old seedlings developed

> shoot buds or . . . PLBs at their proximal regions in certain selective combinations. BAP [BA] was obligatory for PLBs regeneration, whereas [kinetin] and GA_3 [gibberellic acid] in the medium promoted only shoot buds. Shoot buds and differentiating callus were simultaneously formed when only IAA was used in the medium and seedlings complete with leaves and roots were formed within 10 weeks in this combination. (Vij and Pathak, 1988*b*)

Clonal Propagation of *Vanda* through the Culture of Leaf Explants

PLBs form on explants from seedling leaves of *Vanda* cultured by a method developed for *Phalaenopsis* (Tanaka et al., 1974).

Plantlet Production from Root Explants of *Vanda bicolor*

William Griffith (1810–1845), who was Assistant-Surgeon in Madras and Superintendent of the Calcutta Botanic Garden, named this Himalayan species. Its sepals and petals are spatulate with undulate margins and brownish-purple in color with yellow markings (Fig. V-1A, B; Teuscher, 1977).

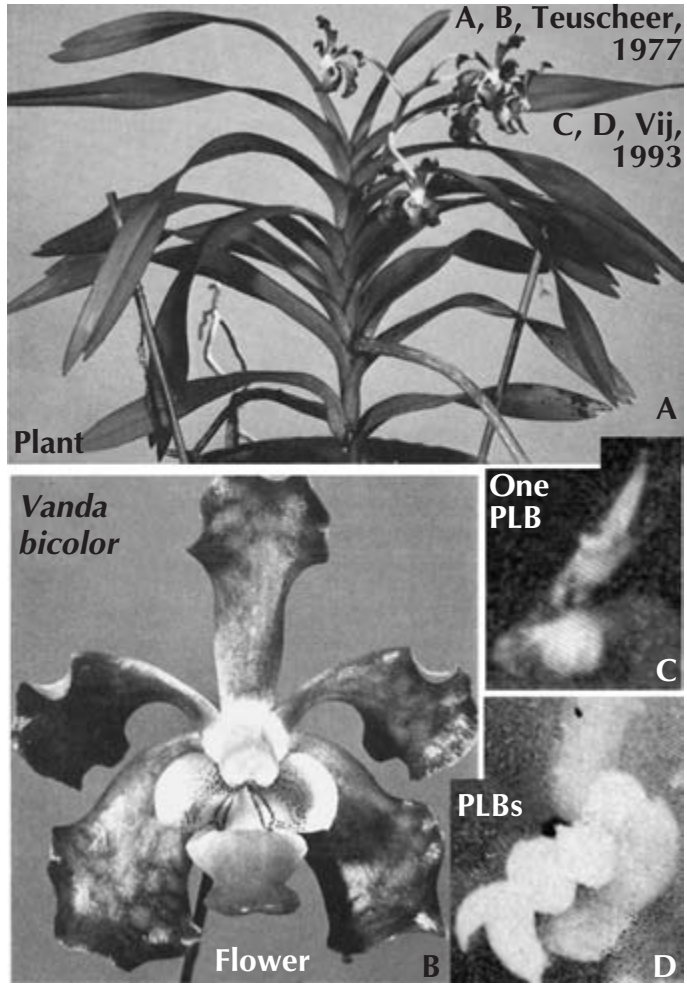


FIG. V-1. ***Vanda bicolor*** **and its micropropagation. A. The whole plant. B. Flower. C. PLBs forming on an explant. D. A proliferating PLB. (Sources: A, B, Teuscher, 1977; C, D, Vij, 1993.)**

Plant Material. Root explants (tips with and without well-developed caps) were taken from 16–30-week-old seedlings in vitro.

Surface Sterilization. Explants taken from seedlings in axenic culture do not require surface sterilization.

Culture Vessels. Culture tubes, Erlenmeyer flasks, or other containers are suitable.

Culture Conditions. The original research cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx.

Culture Media. To bring about the formation of PLBs, the explants should be cultured on Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) containing 1 mg kinetin l^{-1} and 1 mg NAA l^{-1} (Table V-17). PLBs multiply on MPR with 1 mg kinetin l^{-1} (Table V-18). Media for plantlet formation and growth are not suggested in the original report. No medium/media is/are suggested for plantlet formation and the culture of plantlets to maturation. Suitable media for this may be one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. The explants are placed on the first medium (Table V-17) and cultured on it until PLBs are formed. If the explants release exudates which darken the medium they should be moved to new culture vessels with fresh substrate. PLBs are moved to the second medium (Table V-18) for proliferation. Once there are enough PLBs, shoots, and plantlets they can be cultured on one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Rntda-1, V-4, and Pln-1).

Developmental Sequence. PLBs form on the explants (Fig. V-1C) on the first medium (Table V-17). They proliferate (Fig. V-1D) on the second medium (Table V-18) and may form shoots and plantlets on this solution or on one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Rntda-1, V-4, and Pln-1). Leaves appear after 5 weeks and roots form 3 weeks after that.

General Comments. The removal of root tips from a plant does not endanger it. Also plants produce many root tips. Therefore using root tips as explants is a clever idea. However, a method for the micropropagation of seedlings may prove to be useful mainly for the multiplication of seedlings when only a few are produced by a cross. As a means of propagating desirable clones, a method using explants from seedlings is not useful because the quality of the plants is not known.

TABLE V-17. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Vanda bicolor* root explants (Vij, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Cytokinin				
15	Kinetin	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or the cytokinin does not dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.5, add sugar (item 16), and bring volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

TABLE V-18. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the proliferation of protocorm-like bodies of *Vanda bicolor* derived from root explants (Vij, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Cytokinin				
14	Kinetin	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve add a few drops of 0.1 N HCl.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and bring volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

Micropropagation of *Vanda cristata* through the Culture of Root Explants

Found in Tibet, the Himalayas from Nepal to Bhutan, Khasia, and Bangladesh this species was discovered in Nepal by Nathaniel Wallich and named *Vanda cristata* by John Lindley. In 1986, Leslie A. Garay segregated *Trudelia* from the genus *Vanda* on the basis of floral structure and in 1988 Karheinz Senghas transferred *Vanda cristata* to the new genus as *Trudelia cristata* (Bechtel et al., 1992; Grove, 1995). A method for its micropropagation through the culture of root explants was developed at the Department of Botany, Panjab University (Vij, 1993).

Plant Material. Root explants (tips with and without well-developed caps) were taken from 16–30-week-old seedlings in vitro.

Surface Sterilization. Explants taken from seedlings in axenic culture do not require surface sterilization.

Culture Vessels. Culture tubes, Erlenmeyer flasks, or other containers are suitable.

Culture Conditions. The original research cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx.

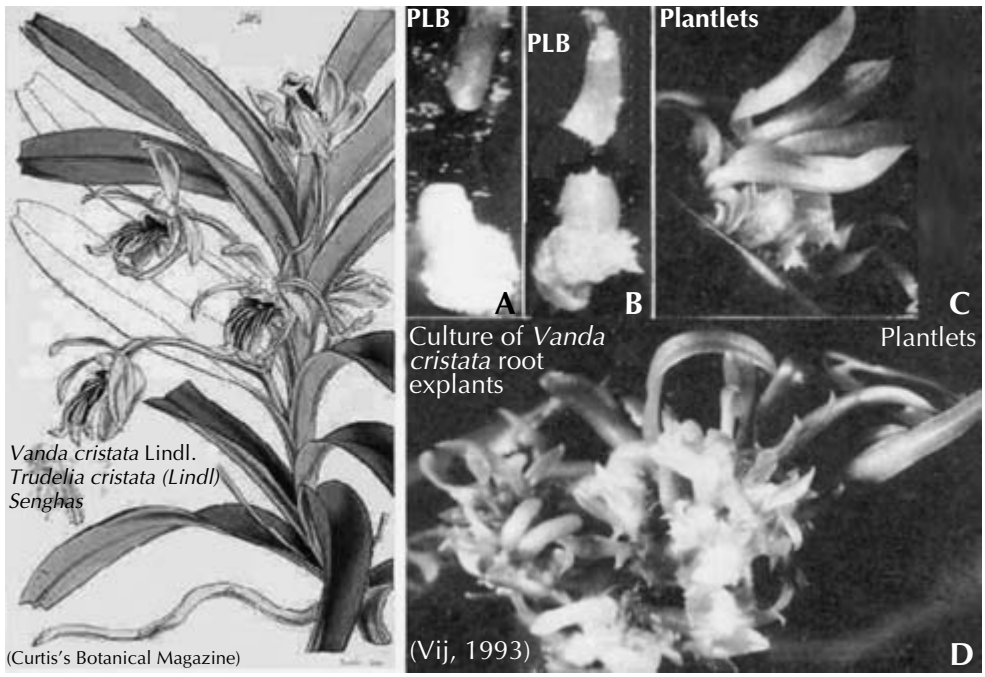


FIG. V-2. ***Vanda cristata*** **(left) and its micropropagation through the culture of root tips.**

Culture Media. To bring about the formation of PLBs, the explants should be cultured on Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) containing 1 g peptone l^{-1} and 1 mg gibberellic acid (GA_3) l^{-1}, and 0.2% activated charcoal (Table V-19). PLBs multiply and produce plantlets on the same medium. No medium/media is/are suggested for plantlet formation and the culture of plantlets to maturation. Suitable media for this may be one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. The explants are placed on the medium (Table V-19) and cultured on it until PLBs are formed. If the explants release exudates which darken the medium they should be moved to a new culture vessel with fresh substrate. PLBs are moved to fresh medium of the same composition (Table V-19) for proliferation and plantlet formation. Once there are enough PLBs, shoots, and plantlets they can be cultured on one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Rntda-1, V-4, and Pln-1).

Developmental Sequence. PLBs form (Fig. V-2A, B) on the explants after 4 weeks of culture. The first leaves appear after 5 weeks. Roots form 5 weeks after that (i.e. 10 weeks after the start of culture).

General Comments. The use of root tips as explants does not endanger the donor plant because plants produce many root tips. However, a method for micropropagation of seedlings cannot be used to propagate outstanding forms since the quality of seedlings is not known. Such a method can be of limited use in the multiplication of seedlings when only a few can be produced. This procedure is one of a few to use a GA_3-containing medium.

TABLE V-19. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Vanda cristata* root explants (Vij, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Gibberellin				
14	Gibberellic acid (GA_3)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Complex additive				
15	Peptone[f]	1.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	7.0 g	No stock	No stock	Weigh
	Darkening agent				
19	Activated charcoal[h]	2.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Peptones can vary. It is best to use a peptone which is described as being suitable or tested for plant tissue culture media.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.5, add sugar (item 16), and bring volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, add the charcoal (item 19) with vigorous stirring to insure equal distribution through the solution, pour the medium into culture vessels, and autoclave. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

Root Tip Culture of *Vanda* "Kasem"

The name Kasem, that of a famed Thai *Vanda* breeder, is in quotation marks above because there is no *Vanda* by that name. However there is a hybrid named Kasem's Delight which is probably the plant from which root tips were taken for culture (Vij, 1993).

Plant Material. Root explants (tips with and without well-developed caps) were taken from 16–30-week-old seedlings in vitro.

Surface Sterilization. Explants taken from seedlings in axenic culture do not require surface sterilization.

Culture Vessels. Culture tubes, Erlenmeyer flasks, or other containers are suitable.

Culture Conditions. The original research cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx.

Culture Media. To bring about the formation of PLBs, the explants should be cultured on Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) containing 1 mg IAA l^{-1} (Table V-20). PLBs multiply but there is no report that they produce plantlets on the same medium. No medium/media is/are suggested for plantlet formation. Suitable media for plantlet production and growth may be one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. The explants are placed on the medium (Table V-20) and cultured on it until PLBs are formed. If the explants release exudates which darken the medium they should be moved to a new culture vessel with fresh substrate. PLBs are moved to fresh medium of the same composition (Table V-20) for proliferation. Once there are enough PLBs, they can be cultured on one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Rntda-1, V-4, and Pln-1).

Developmental Sequence. PLBs (Fig. V-3A) will form on approximately 25% of the explants after 5 weeks of culture. There is no information about plantlet production.

General Comments. The use of root tips as explants does not endanger the donor plant because plants produce many roots tips. However, a method for the

TABLE V-20. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Vanda* Kasems's Delight root explants (Vij, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Indoleacetic acid (IAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve add a few drops of 0.1 N KOH.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and bring volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

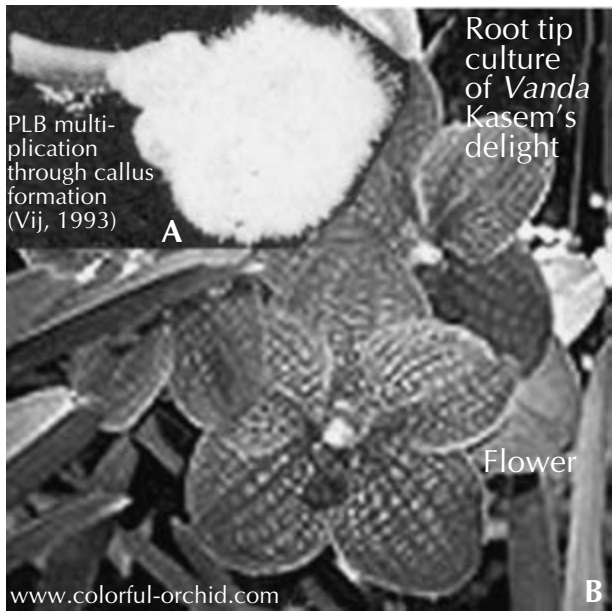


FIG. V-3. **Root-tip explant culture of *Vanda* Kasem's Delight. A. PLBs. B. Flower.**

micropropagation of seedlings cannot be used to propagate outstanding forms since the quality of seedlings is not known. Such a method can be of limited use in the multiplication of seedlings when only a few can be produced. It is possible that in this case the PLBs will not produce plantlets.

Plantlet Production of *Vanda teres* through the Culture of Root Tips

Vanda teres is distributed from the Himalayas to Burma, Laos, Thailand, and Vietnam (Hew et al., 2002). It is still grown in collections and gardens, but its main claim to fame is as a parent of the national flower of Singapore, *Vanda* Miss Joaquim (Hew et al., 2002). A method for plantlet production from its root tips was developed in India (Vij, 1993).

Plant Material. Root explants (tips with and without well-developed caps) were taken from 16–30-week-old seedlings in vitro.

Surface Sterilization. Explants taken from seedlings in axenic culture do not require surface sterilization.

Culture Vessels. Culture tubes, Erlenmeyer flasks, or other containers are suitable.

Culture Conditions. The original research cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx.

TABLE V-21. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for plantlet production from root explants of *Vanda teres* (Vij, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Complex additive				
14	Yeast extract (YE)[f]	1.0 g	No stock	No stock	Weigh
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	7.0 g	No stock	No stock	Weigh
	Darkening agent				
18	Activated charcoal[h]	2.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Yeast extracts can vary. It is best to use a peptone which is described as being suitable or tested for plant tissue culture media.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and bring volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, add the charcoal (item 18) with vigorous stirring to insure equal distribution through the solution, pour the medium into culture vessels and autoclave. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

Culture Media. To bring about the formation of PLB and plantlet production, the explants should be cultured on Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) containing 1 g yeast extract l^{-1} and 2 g activated charcoal l^{-1} (Table V-21). No medium/media is/are suggested for plantlet formation. Suitable media for plantlet production and growth may be one of the versions of Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. The explants are placed on the medium (Table V-21) and cultured on it until PLBs are formed. If the explants release exudates which darken the medium they should be moved to a new culture vessel with fresh substrate. PLBs are moved to fresh medium of the same composition (Table V-21) for proliferation. Once there are enough PLBs, they can be cultured on one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Rntda-1, V-4, and Pln-1).

Developmental Sequence. Approximately 12.5% of the explants will produce plantlets (Fig. V-4A) after 4 weeks of culture.

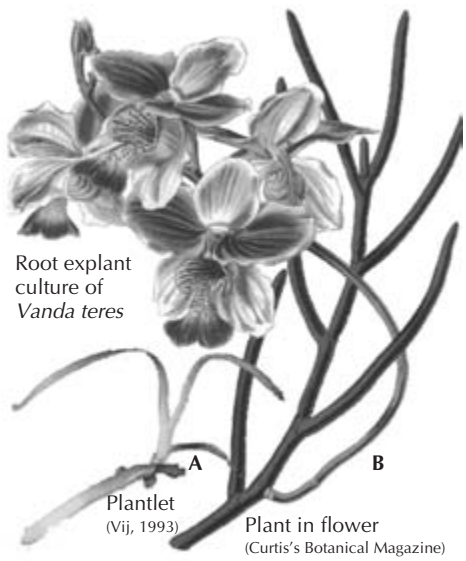


FIG. V-4. **Root explant culture of *Vanda teres*. A. Plantlet. B. Flower. (Vij, 1993.)**

General Comments. The use of root tips as explants does not endanger the donor plant because plants produce many roots tips. However, a method for the micropropagation of seedlings cannot be used to propagate outstanding forms since the quality of seedlings is not known. Such a method can be of limited use in the multiplication of seedlings when only a few can be produced. It is possible that in this case the PLBs will not produce plantlets. According to some taxonomists the correct name of this species is *Papilionanthe teres*.

Micropropagation of *Vanda testacea* through the Culture of Root Explants

Distributed in Assam, Burma, India, Nepal, Sikkim, Sri Lanka, and Thailand, *Vanda testacea* is also considered to be a synonym of *Vanda parviflora* (or vice versa). A micropropagation method using root tips was developed in India (Vij, 1993).

Plant Material. Root explants (tips with and without well-developed caps) were taken from 16–30-week-old seedlings in vitro.

Surface Sterilization. Explants taken from seedlings in axenic culture do not require surface sterilization.

Culture Vessels. Culture tubes, Erlenmeyer flasks, or other containers are suitable.

Culture Conditions. The original research cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx.

Culture Media. To bring about the formation of PLB and plantlet production, the explants should be cultured on Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) containing 1 g peptone l^{-1}, 1 mg BA l^{-1}, and 2 g activated charcoal l^{-1} (Table V-22). No medium/media is/are suggested for plantlet formation. Suitable media for plantlet production and growth may be one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. The explants are placed on the medium (Table V-22) and cultured on it until PLBs are formed. If the explants release exudates which darken the medium they should be moved to a new culture vessel with fresh substrate. PLBs are moved to fresh medium of the same composition (Table V-22) for proliferation. Once there are enough PLBs, they can be cultured on the same medium (Table V-22) or on one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19,

TABLE V-22. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the micropropagation of *Vanda teres* through the culture of root explants (Vij, 1993)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Cytokinin				
14	Benzylaminopurine	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1.0	
	Complex additive				
15	Peptone[f]	1.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	7.0 g	No stock	No stock	Weigh
	Darkening agent				
19	Activated charcoal[h]	2.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses. If the cytokinin does not dissolve add a few drops on 0.1 N HCl.

[f]Peptones can vary. It is best to use a peptone which is described as being suitable or tested for plant tissue culture media.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.5, add sugar (item 16), and bring volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, add the charcoal (item 19) with vigorous stirring to insure equal distribution through the solution, pour the medium into culture vessels and autoclave. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

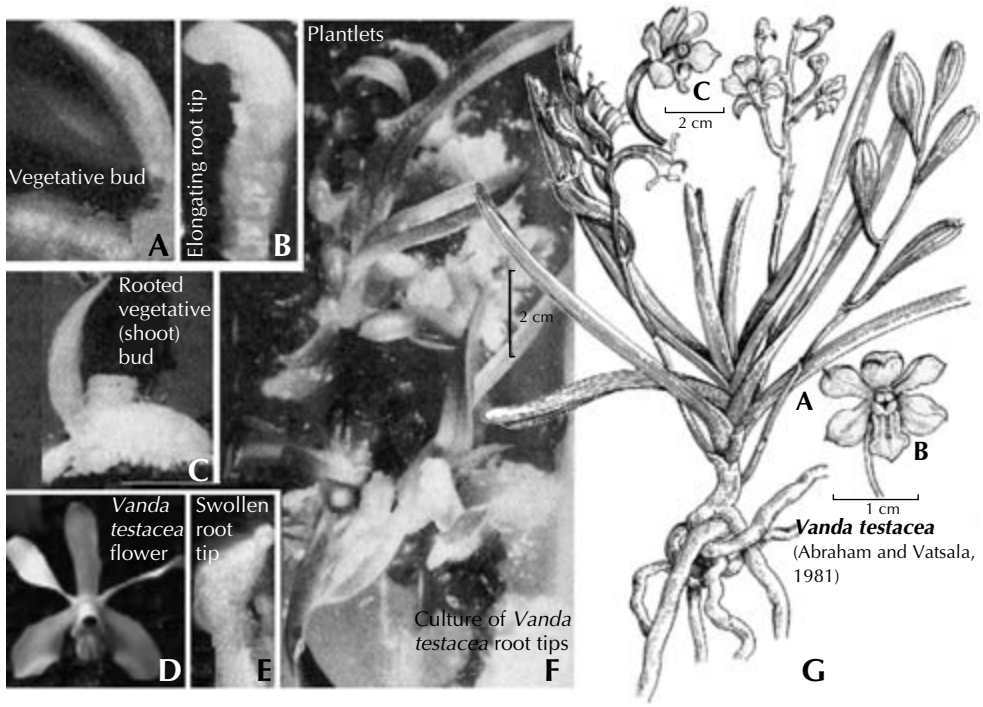


FIG. V-5. **Micropropagation of *Vanda testacea*. A. Vegetative bud. B. Elongating root tip. C. Rooted vegetative (shoot) bud. D. Flower. E. Swollen root tip. F. Plantlets. G. Botanical drawing. (Sources: A–C, E, F, Vij, 1993; D, photograph by Joseph Arditti; G, Abraham and Vatsala, 1981.)**

Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Rntda-1, V-4, and Pln-1).

Developmental Sequence. Approximately 25% of the explants respond by elongating (Fig. V-5B), swelling (Fig. V-5E), producing buds (Fig. V-5A), rooting (Fig. V-5C), and generating plantlets (Fig. V-5F) after 4–9 weeks of culture.

General Comments. The use of root tips as explants does not put the donor plant at risk because plants produce many roots tips. However, a method for the micropropagation of seedlings cannot be used to propagate outstanding forms since the quality of seedlings is not known. Such a method can only be of limited use in the multiplication of seedlings when only a few can be produced. It is possible that in this case the PLBs will not produce plantlets.

Production of *Vanda coerulea* Plantlets from Foliar Peels

Foliar peels are abundant, their removal does not endanger or even damage the donor plant, and they "are low in endogenous growth hormones . . . and are thus expected

to be more sensitive to even slightest changes [in] the quantity and quality of . . . growth regulators. . . .” Therefore, they are very suitable as explants for micropropagation of *Vanda coerulea* (Fig. V-6A) and other orchids (Vij and Kaur, 1992; Vij, 1994).

Plant Material. In the original research “peels, 1–4 layers in thickness and 0.5 mm in length were . . . [taken from] juvenile leaves [of] *in vitro* raised cultures.” It is not clear from this statement whether the “cultures” were of seedlings or plantlets produced through tissue culture. Also, photographs (see Figs Rhy-2A and V-6B, C) suggest that the length should be 0.5 cm.

Surface Sterilization. Explants from in vitro cultures do not require sterilization.

Culture Vessels. Culture tubes, Erlenmeyer flasks, or other containers can be used.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx or standard culture room conditions.

Culture Media. Several hormone combinations in the Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) were used in the original research. MPR with 1 mg l^{-1} each of NAA and BA (Table V-23) seems most suitable.

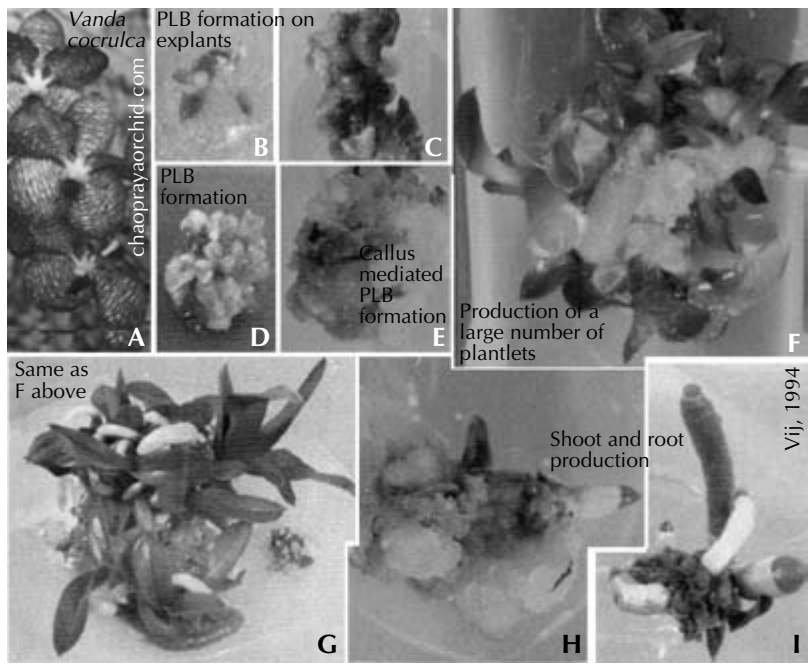


FIG. V-6. **Culture of dermal peels of *Vanda coerulea*.**

TABLE V-23. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Vanda coerulea* leaf peels (Vij and Kaur, 1992; Vij, 1994)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1 ml	
	Cytokinin				
15	Kinetin	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1 ml	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH or HCL, respectively.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.6, add sugar (item 16), and bring volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

Procedure. Peels are placed on the medium (Table V-23) where they form PLBs (Fig. V-6B–E) and eventually leaves, roots and plantlets (Fig. V-6F–I). If cultures become crowded, PLBs or plantlets should be subcultured on the same medium.

Developmental Sequence. PLBs are produced by 100% of explants after 3 weeks of culture. Plantlets (up to 25 per plant) are formed after 14 weeks.

General Comments. Explants from seedlings cannot be used to propagate specific cultivars, but this is less important for species than for hybrids.

Micropropagation of the Blue *Vanda*

> Protocorm-like bodies (plbs) [of *Vanda coerulea*, the Blue *Vanda*] were easily induced in axenic seedling-derived leaf base segments and cut basal ends of whole leaves cultured in Mitra et al. agar medium (1976) supplemented with organic additives (sucrose, coconut water, peptone and casein acid hydrolysate) and a combination of 1 mg/l BA and 0.75 mg/l NAA. While all the leaves of the seedlings were regenerative, approximately 40% of the leaves of the flowering plants produced callus to varying degrees but never formed plbs or shoots. Clonal propagation was, however, achieved with the successful production of plbs and shoots in shoot tip cultures. In liquid cultures, maximum proliferation of plbs was obtained with tender leaves measuring less than 8 mm. Proliferation of additional plbs with simultaneous differentiation of shoots from the first formed ones and accelerated growth of the shoots occurred if the tissues were subcultured to medium enriched with 70 g/l banana homogenate. Whole leaves produced a larger number of shoots than leaf base segment and shoot tip cultures. A maximum of 102, 62 and 68 shoots from the whole leaf, leaf base segment and shoot tip cultures, respectively, was obtained after 24 weeks of culture.
>
> Shoots of varying size with or without roots were separated and replanted in the medium to induce rooting and further growth of the shoots. Some of the shoots produced additional suckers during this period. Eight-months-old rooted plantlets were planted in 2″ clay pots containing a potting mixture of charcoal chips and broken tiles where the survival rate exceeded 95%. Root tip squashes prepared from 25 randomly collected samples showed a chromosome complement of 2n = 38. (Seeni, 1990)

Culture of *Vanda coerulea* Shoot Tips and Leaf Segments

Vanda coerulea was first collected in 1837 by William Griffith in the Jyntea and Khasia Hills in India, but it was described 10 years later by John Lidley in the *Botanical Register* and also in *Folia Orchidcea*. The species was rediscovered in 1850, also in the Khasia Hills, by Sir Joseph Hooker and Dr. T. Thompson. Thomas Lobb, a collector for Veitch and Sone, sent a large shipment to his employers in the same year and they introduced this remarkable *Vanda* to the trade. *V. coerulea* is also found in Thailand and Burma (Bechtel et al., 1992). Its blue flower made it popular when it was first introduced. It is still grown widely throughout the world and the South Pacific Island Republic Tuvalu (the former Ellice Islands) put it on one of its stamps. *V. coerulea* is also well liked in Japan, where a method for plant production from shoot tips and leaf explants was developed at Gifu University (Fukui et al., 2001).

Plant Material. The tops of shoots with three or four unexpanded leaves were taken from seedlings maintained under horticultural conditions. Tips, described as "0.8 mm" (this seems too small and may be a misspelled 0.8 cm), were cut from these shoots after surface sterilization, and cultured. Top leaves, 15 mm long, were taken from plantlets in vitro which had "four or five expanded leaves" and cut into three sections – tip, middle, and base. These sections were cultured.

Surface Sterilization. Shoot tips were surface-sterilized by immersing them for 10 min in 100 ml of 1% sodium hypochlorite solution (20 or 17 ml, respectively, of household bleach containing 5–5.25 or 6% sodium hypochlorite diluted to 100 ml with distilled water) containing 1 drop of Tween 20 (available from www.sigmaaldrich.com or other sources) and then washing them three times with sterile distilled water. Leaf explants were not surface-sterilized because they were taken from plantlets that were growing in vitro.

Culture Vessels. Culture tubes were used in the original research. Other containers can also be used.

Culture Conditions. The research cultures were maintained at 25°C under 16-h photoperiods of 3000 lx. Standard culture room conditions should also prove to be suitable.

Culture Media. Shoot tips produce callus on Knudson C (KC) medium supplemented with 1 μmol BA l^{-1} (Table V-24). The callus proliferates on the same medium. Leaf explants produce callus in the presence of 1 μmol BA l^{-1} and 0.5 μmol NAA l^{-1} in a medium which is not named, but it is reasonable to assume that it is KC (Table V-25). The leaf-derived callus proliferates well on a medium containing 0.5 μmol BA l^{-1} and 0.1 μmol NAA l^{-1} (Table V-26). The callus produces plantlets in the presence of 0.5 μmol BA l^{-1} and 0.5 μmol NAA l^{-1} (Table V-27). No medium is suggested for the culture of plantlets to community pot size. Plantlets can be cultured on one of the versions of the KC medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for KC that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. Shoot tips are taken, surface-sterilized, and washed. Tip explants are taken after that and placed in culture on the appropriate medium (Table V-24). When callus is formed it is subcultured on the same medium (Table V-24) for proliferation. The callus should be moved to another medium (Table V-27) for plantlet formation and growth. For further growth, the plantlets can be moved to one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, and V-4)

TABLE V-24. **Knudson C (KC) medium (Knudson, 1946) modified for the induction and culture of callus from shoot tips of *Vanda coerulea* (Fukui et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2{\cdot}4H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4{\cdot}7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4{\cdot}7H_2O$	27.8	2.78 g l^{-1}		
	Microelement[d]				
6	Manganese sulfate, $MnSO_4{\cdot}4H_2O$	7.5	750.0 mg l^{-1}	10	
	Cytokinin				
7	Benzylaminopurine (BAP)	0.225	22.5 mg 100 ml^{-1} 95% ethanol[e]	1 µmole	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[f]	To 1000 ml			
	Solidifier				
10	Agar[f]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. Kundson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4{\cdot}7H_2O$ l^{-1}, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored.

[d]There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If incorporation of additional microelements becomes necessary, those used in the Murashige–Skoog (MS) medium are suitable.

[e]If the cytokinin fails to dissolve add a few drops of 0.1 N HCl. Keep frozen between uses.

[f]Add items 1–7 to 900 ml of distilled water (item 9), adjust pH to 5–5.2, add sugar (item 8), and bring volume to 1000 ml with distilled water (item 9). Bring the solution to a gentle boil and add the agar (item 10) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave.

with or without banana homogenate or activated charcoal. If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to the medium selected for use as suggested for KC that contains this darkening agent (see Table Pln-1, footnote *f*).

Leaf explants are cut and placed on the callus-inducing medium (Table V-25). Callus produced on this medium is proliferated on another solution (Table V-26). To produce plantlets, this callus is moved to a plantlet-inducing medium (Table V-27). The plantlets can be grown on one of several media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, and V-4, with or without banana homogenate or activated charcoal).

TABLE V-25. **Knudson C (KC) medium (Knudson, 1946) modified for the induction and culture of callus from leaf explants of *Vanda coerulea* (Fukui et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement[d]				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
7	Naphthaleneacetic acid (NAA)	0.093	9.3 mg 100 ml^{-1} 95% ethanol[e]		0.5 µmole
	Cytokinin				
8	Benzylaminopurine (BAP)	0.225	22.5 mg 100 ml^{-1} 95% ethanol[e]		1 µmole
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. Knudson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$ l^{-1}, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored.

[d]There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If incorporation of additional microelements becomes necessary, those used in the Murashige–Skoog (MS) medium are suitable.

[e]If the auxin or cytokinin fail to dissolve, add a few drops of 0.1 N KOH or HCl, respectively. Keep frozen between uses.

[f]Add items 1–8 to 900 ml of distilled water (item 10), adjust pH to 5–5.2, add sugar (item 9), and bring volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave.

Developmental Sequence. Shoot tips produce callus which proliferates and eventually produces plantlets. Leaf explants produce callus, buds, and roots after 8 weeks in culture. Leaf segments produce callus as follows: 0% by tips, 5.5% by mid-leaf sections, and 38.9% by bases.

General Comments. Information about the sources of explants is not entirely clear. The opening sentence of the material and methods sections states that "seedling plants . . . were used as plant materials." These seedlings seem to have been older and not growing in vitro because the explants were surface-sterilized. The source leaf segments is described as "plantlets . . . regenerated from shoot tip culture." Since there is no mention of surface sterilization for the leaf explants it is possible that they were taken from plantlets in vitro produced from shoot tips taken from the seedlings mentioned above. If older seedlings were used as sources of shoot tips this method may prove to be suitable for similar explants from older plants.

TABLE V-26. **Knudson C (KC) medium (Knudson, 1946) modified for the proliferation of callus derived from leaf explants of *Vanda coerulea* (Fukui et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement[d]				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
7	Naphthaleneacetic acid (NAA)	0.019	1.9 mg 100 ml^{-1} 95% ethanol[e]		0.1 µmole
	Cytokinin				
8	Benzylaminopurine (BAP)	0.113	11.3 mg 100 ml^{-1} 95% ethanol[e]		0.5 µmole
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. Knudson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$ l^{-1}, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored.

[d]There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If incorporation of additional microelements becomes necessary, those used in the Murashige–Skoog (MS) medium are suitable.

[e]If the auxin or cytokinin fail to dissolve, add a few drops of 0.1 N KOH or HCl, respectively. Keep frozen between uses.

[f]Add items 1–8 to 900 ml of distilled water (item 10), adjust pH to 5–5.2, add sugar (item 9), and bring volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave.

Vanda coerulea (source: de Puydt, E. 1880. Les Orchidées. J. Rotschild, Éditeur, Paris)

TABLE V-27. **Knudson C (KC) medium (Knudson, 1946) modified for plantlet production from *Vanda coerulea* callus (Fukui et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1.0 g	100.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement[d]				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Auxin				
7	Naphthaleneacetic acid (NAA)	0.093	9.3 mg 100 ml^{-1} 95% ethanol[e]		0.5 µmole
	Cytokinin				
8	Benzylaminopurine (BAP)	0.113	11.3 mg 100 ml^{-1} 95% ethanol[e]		0.5 µmole
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 5a) and the iron salt (item 5b) to the same 1 l of distilled water and stir (heat if necessary) until both are dissolved. Knudson's original recipe for this medium calls for 25 mg ferrous sulfate, $FeSO_4 \cdot 7H_2O$ l^{-1}, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the original recipe and the amount suggested here is negligible and can be ignored.

[d]There are no microelements other than manganese (item 6) in the original recipe of the Knudson C medium (Knudson, 1946) and it is not clear whether the addition of such nutrients would be of benefit. If incorporation of additional microelements becomes necessary, those used in the Murashige–Skoog (MS) medium are suitable.

[e]If the auxin or cytokinin fail to dissolve add a few drops of 0.1 N KOH or HCl, respectively. Keep frozen between uses.

[f]Add items 1–8 to 900 ml of distilled water (item 10), adjust pH to 5–5.2, add sugar (item 9), and bring volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave.

Micropropagation of the Blue Vanda, *Vanda coerulea*

An epiphyte growing at elevations of 1000–1500 m, *Vanda coerulea*, the Blue Vanda of Asia, is threatened with extinction in India due to habitat destruction and overexploitation. A micropropagation procedure was used to establish clonally propagated plants in the southern forests of Western Ghats in India (Seeni and Latha, 2000).

Plant Material. Shoot tips, 5–8 mm in size, as well as the two youngest leaves, were excised from 6-month-old seedlings, and surface-sterilized top cuttings consisting of 4–5 nodes were taken from potted mature plants.

Surface Sterilization. The top cuttings were immersed in 1% (v/v) Teepol (www.sigmaaldrich.com and other sources) for 1–3 min, 70% ethanol (74 ml 95% ethanol diluted to 100 ml with distilled water) for 20 s, and 0.1% mercuric chloride ($HgCl_2$) for 3 min. After that they were washed with sterile distilled water three times. There is no need to sterilize seedlings that are growing in vitro.

Culture Vessels. Culture tubes, Erlenmeyer flasks, and other containers can be used.

Culture Conditions. Cultures should be maintained at 24 ± 2°C under 12-h photoperiods of 20–50 μmol m^{-2} s^{-1} provided by Philips daylight fluorescent tubes.

Culture Media. Shoot tips should be cultured initially in liquid Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) supplemented with 10% coconut water, 500 mg casein hydrolysate l^{-1}, 8.8 μmol BA l^{-1}, and 4.1 μmol NAA l^{-1} (Table V-28). Leaves should be cultured on the same medium in solid form (Table V-28). Explants which form buds or PLBs should be moved to MPR containing 35 g banana pulp l^{-1}, 30% coconut water, and 1.08 μmol NAA l^{-1} (Table V-29). Shoots should be moved to MPR with 35 g banana pulp l^{-1} and 1.08 μmol NAA l^{-1} (Table V-30). Liquid medium should be agitated.

Procedure. Shoot tips are cultured in agitated liquid MPR (Table V-28) until they produce buds and/or PLBs. Leaf explants are cultured on solid MPR (Table V-28) until they also produce PLBs and/or buds. Once PLBs and/or buds are produced, they or the explants which produced them are moved to a different modification of MPR (Table V-29). Shoots produced on this medium are rooted on yet another version of MPR (Table V-30). Rooted plants should be moved to pots.

Developmental Sequence. Approximately 75% of the shoot tips on the first medium (Table V-28) produce vegetative (shoot) buds (3–12 per tip with an average of 4.2) in 12 weeks. Leaf-base explants (64–88% of those cultured on the same medium, Table V-28) give rise to PLBs (3–8 per explant) in 4–8 weeks. When the explants (shoot tip and leaf) are moved to the second medium (Table V-29), "a maximum of 70 and 100 shoots of varied length were obtained from a single shoot tip/leaf base explant after 30 weeks of culture." Shoots (3.4 cm high) on the third medium (Table V-30) form roots within 3–4 weeks. The survival of rooted plants in pots is 95–100%.

General Comments. This method can be used to increase the number of seedlings and to propagate selected forms. Plantlets produced by this procedure are "cytologically stable ($2n = 38$)" and "free from morphological, growth and flowering abnormalities."

TABLE V-28. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of shoot tip and leaf explants of *Vanda coerulea* (Seeni and Latha, 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	0.764	76.4 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
15	Benzyladenine (BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additives				
16	Casein hydrolysate[g]	500.0	No stock	No stock	Weigh
17	Coconut water[h]	100.0	No stock	No stock	Measure
	Sugar				
18	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
19	Water, distilled[i]	To 1000 ml			
	Solidifier				
20	Agar[i]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. Shoot tips should be cultured in liquid (i.e., agar-free) medium, whereas leaf explants should be cultured on solid (i.e., agar-containing) substrate.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[g]Not all casein hydrolysates are the same. Their compositions and analyses vary depending on the methods by which they were produced. A product sold by Merck (www.merck.de) as casein hydrolysate (CH) is obtained by degrading casein with hydrochloric acid; vitamins and other growth-promoting compounds are largely destroyed in this process. The CH sold by Sigma-Aldrich (www.sigmaaldrich.com) is pancreatic hydrolysate of casein. There is also a CH which is hydrolysed by enzymes. Becton, Dickinson and Company (www.bd.com) sells Difco products including a number of different casamino acid products, all of which are casein hydrolysates. Therefore if a method specifies a source for the CH in its culture medium it is best to obtain the additive from the same vendor. If a source is not listed it is best to purchase CH which is described as being suitable as a supplement for culture media. Failing that, CH should be purchased from a source which specializes in tissue culture and micropropagation supplies. One such source is www.caissonlabs.com. They list casein rather than CH as Stock #: SPCA205. Another source for CH is www.duchefa.com (item 1301). Additional sources include www.fishersci.com, www.thomassci.com, and others.

[h]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[i]Add items 1–17 to 700 ml of distilled water (item 19), adjust pH to 5.6, add sugar (item 18), and bring volume to 1000 ml with distilled water (item 19). Bring the solution to a gentle boil and add the agar (item 20) slowly while stirring. The agar can also be added to the cold solution which is then brought to a boil and stirred. When the agar (item 20) is completely dissolved, pour the solution into culture vessels and autoclave. Agar should not be added if the medium is to be used to culture shoot tips. Liquid medium should be agitated. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

TABLE V-29. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for shoot production from shoot tips, leaf explants, and protocorm-like bodies of *Vanda coerulea* (Seeni and Latha, 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	0.200	20 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additives				
15	Banana homogenate[g]	35.0 g	No stock	No stock	Weight
16	Coconut water[h]	300.0	No stock	No stock	Measure
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[i]	To 1000 ml			
	Solidifier				
19	Agar[i]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Opinions differ regarding the use of banana in culture media. The predominant opinion is that only homogenized pulp of ripe banana should be used. Other views are that: (1) unripe (green) bananas are preferable, (2) the peel rather than the pulp should be used, (3) unpeeled banana homogenate should be incorporated in media, and (4) slices, ripe or unripe, peeled or unpeeled, rather than homogenate are most effective. Opinions also differ regarding which is the most effective banana variety (Gros Michel, Raja, Pisang Susu, and others). Since there is no experimental evidence to support any view the best approach is to follow the recommendations in the original reports and/or use what is suggested there.

[h]The correct term for the liquid endosperm of coconuts is coconut water, not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[i]Add items 1–16 to 500 ml of distilled water (item 18) in a homogenizer, homogenate until the banana pulp (item 15) is completely homogenized and thoroughly dispersed through the medium, raise volume to 900 ml if necessary, adjust pH to 5.6, add sugar (item 17), and bring volume to 1000 ml with distilled water (item 18). Bring the solution to a gentle boil and add the agar (item 19) slowly while stirring. The agar (item 19) can also be added to the cold solution which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

TABLE V-30. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the rooting of shoots produced by shoot tips, leaf explants, and protocorm-like bodies of *Vanda coerulea* (Seeni and Latha, 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	0.200	20 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additives				
15	Banana homogenate[g]	35.0 g	No stock	No stock	Weight
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar[h]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve add a few drops of 0.1 N KOH.

[g]Opinions differ regarding the use of banana in culture media. The predominant opinion is that only homogenized pulp of ripe banana should be used. Other views are that: (1) unripe (green) bananas are preferable, (2) the peel rather than the pulp should be used, (3) unpeeled banana homogenate should be incorporated in media, and (4) slices, ripe or unripe, peeled or unpeeled, rather than homogenate are most effective. Opinions also differ regarding which is the most effective banana variety (Gros Michel, Raja, Pisang Susu, and others). Since there is no experimental evidence to support any view the best approach is to follow the recommendations in the original reports and/or use what is suggested there.

[h]Add items 1–15 to 800 ml of distilled water (item 17) in a homogenizer, homogenate until the banana pulp (item 15) is completely homogenized and thoroughly dispersed through the medium, raise volume to 900 ml if necessary, adjust pH to 5.6, add sugar (item 16), and bring volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar (item 18) can also be added to the cold solution which is then brought to a boil and stirred. When the agar (item 18) is completely dissolved pour the solution into culture vessels and autoclave. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

Micropropagation of *Vanda cristata* through Leaf Segments

S. P. Vij, Professor of Botany at Panjab University and a noted Indian orchid expert, and his associates have devoted some of their efforts toward the identification of explants other than shoot meristems that are just as effective for micropropagation. They formulated methods for the culture of leaf segments of several orchids including *Vanda cristata* (Vij and Pathak, 1990).

Plant Material. Basal explants, 5–10 mm long, from leaves, approximately 2 cm long, of 16–40-week-old seedlings growing in vitro should be used. Apical segments do not produce PLBs. Explants from leaves of mature plants died in the original experiments.

Surface Sterilization. Explants taken from axenic seedlings growing in vitro do not require surface sterilization.

Culture Vessels. Erlenmeyer flasks, test tubes, and other containers filled with medium to 20–30% of their capacity are suitable.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx or standard culture room conditions.

Culture Media. Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) supplemented with BA and NAA (Table V-31) must be used for the culture of leaf segments. No medium is suggested for the culture of plantlets to maturation. Plantlets can be cultured on one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If banana homogenate is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal should be added to media as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. Leaves are taken from plantlets, and are sectioned and placed in culture on the initiation medium (Table V-31). Callus and PLBs should be subcultured on the same medium for the initiation of shoots and roots. Plantlets should be cultured on one of the Knudson C or Vacin and Went formulations suggested above, preferably one with banana homogenate and activated charcoal.

Developmental Sequence. Callus and PLBs form on the initiation medium (Table V-31) 4 weeks after the start of culture and can give rise to plantlets. When transferred to Knudson C or Vacin and Went formulation with banana homogenate and activated charcoal the plantlets should develop further.

TABLE V-31. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Vanda cristata leaf* segments (Vij and Pathak, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Auxin				
8	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]		
	Cytokinin				
9	Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]		
	Vitamins				
10	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[f]	0.25	
11	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[f]	0.25	
12	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[f]	0.25	
13	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[f]	0.25	
14	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[f]	0.25	
15	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[f]	0.25	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	9.0 g	No stock	No stock	Weigh
	Darkening agent				
19	Activated charcoal[h]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same that as in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]If the auxin or cytokinin does not dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[f]Keep frozen between uses.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.5, add sugar (item 16), and bring volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, add the charcoal with vigorous stirring to insure complete distribution through the medium, pour the solution into culture vessels, and autoclave. After autoclaving when the medium is cooling swirl the flasks to disperse the charcoal. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976; Vij and Pathak, 1990).

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

General Comments. The propagation of selected forms is not possible with this method because the explants are taken from seedlings. However, it may be possible to adapt the procedure to mature plants.

Micropropagation of *Vanda teres* through Leaf Segments

As part of a program to identify explants that are as effective for micropropagation as shoot meristems, Professor S. P. Vij and his associates at the Department of Botany, Panjab University formulated methods for the culture of leaf segments of several orchids including *Vanda teres* (Vij and Pathak, 1990).

Plant Material. Basal explants, 5–10 mm long, from leaves approximately 2 cm long, of 16–40-week-old seedlings growing in vitro should be used. Apical segments do not produce PLBs. Explants from leaves of mature plants died in the original experiments.

Surface Sterilization. Explants taken from axenic seedlings growing in vitro do not require surface sterilization.

Culture Vessels. Erlenmeyer flasks, test tubes, and other containers filled with medium to 20–30% of their capacity are suitable.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx or standard culture room conditions.

Culture Media. Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) supplemented with yeast extract and IBA (Table V-32) must be used for the culture of leaf segments. No medium is suggested for the culture of plantlets to maturation. Plantlets can be cultured on one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate (BH) or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal (AC) should be added to media as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. Leaves are taken from plantlets, and are sectioned and placed in culture on the initiation medium (Table V-32). PLBs should be subcultured on the same medium to bring about shoot and root production. Plantlets should be cultured on one of the Knudson C or Vacin and Went formulations suggested above, preferably one with BH and AC.

Developmental Sequence. PLBs form on the initiation medium (Table V-32) 2 weeks after the start of culture and can give rise to plantlets. When transferred to

TABLE V-32. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Vanda teres* leaf segments (Vij and Pathak, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Auxin				
8	Indolebutyric acid (IBA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]		
	Complex additive				
9	Yeast extract	1.0 g	No stock	No stock	Weigh
	Vitamins				
10	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[f]	0.25	
11	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[f]	0.25	
12	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[f]	0.25	
13	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[f]	0.25	
14	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[f]	0.25	
15	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[f]	0.25	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	9.0 g	No stock	No stock	Weigh
	Darkening agent				
19	Activated charcoal[h]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]If the auxin does not dissolve add a few drops of 0.1 N KOH.

[f]Keep frozen between uses.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.5, add sugar (item 16), and bring volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, add the charcoal with vigorous stirring to insure complete distribution through the medium, pour the solution into culture vessels, and autoclave. After autoclaving when the medium is cooling swirl the flasks to disperse the charcoal. Agar is not used in liquid media. As a rule media which contain vitamins, hormones, and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976; Vij and Pathak, 1990).

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

Knudson C or Vacin and Went formulation with BH and AC, the plantlets should develop further.

General Comments. Since explants are taken from seedlings, propagation of selected forms is not possible with this method. However, it may be possible to adapt the procedure to mature plants. This species was renamed *Papilionanthe teres* but it is listed here under *Vanda* because that is the name used in the original paper.

Micropropagation of *Vanda testacea* through the Culture of Leaf Segments

Plants have many leaves and the removal of a few does not endanger the donor plant. Therefore, explants are safe and practical. Explants from leaves of mature plants released brownish (phenolic?) exudates into the medium, lose their chlorophyll, and die within 2.5–5.5 months. However, explants from axenic seedlings proliferate along their cut ends (Vij and Pathak, 1990).

Plant Material. Young leaves, up to 2 cm in length from 3.5–9-month-old axenic seedlings, should be cut into 0.5–1.0-cm-long basal (with leaf base) and apical (with leaf tip) sections and used as explants.

Surface Sterilization. Explants from leaves growing in vitro do not require surface sterilization.

Culture Vessels. Culture tubes, Erlenmeyer flasks, and other containers are suitable.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx or standard culture room conditions.

Culture Media. When cultured on MS medium (Murashige and Skoog, 1962) supplemented with peptone, kinetin, and NAA, and darkened with active charcoal (Table V-33) 37.5% of both apical and basal explants will produce an average of 30 PLBs per explant (Fig. V-7). PLBs should be moved to MS supplemented with peptone, NAA, and BA (Table V-34) for plantlet production (Fig. V-7).

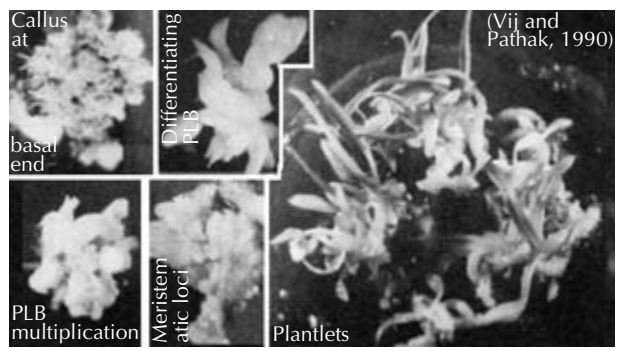


FIG. V-7. **Micropropagation of *Vanda testacea*.**

TABLE V-33. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Vanda testacea* leaf explants (Vij and Pathak, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Auxin				
13	Naphthaleneacetic acid (NAA)	1.0	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
14	Kinetin	1.0	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
15	Peptone[g]	1.0 g	No stock	No stock	Werigh
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar[h]	9.0 g	No stock	No stock	Weigh
	Darkening agent				
19	Activated charcoal (AC)[i]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin fails to dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[g]Peptones can vary. Therefore it is best to use one which is suitable for plant tissue culture. A possible source is www.sigmaaldrich.com.

[h]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.5, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, add the charcoal (item 19) with constant stirring to insure complete and even distribution, pour the solution into culture vessels, and autoclave.

[i]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. pH paper strips can be used for this measurement.

TABLE V-34. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for plantlet production from leaf-explant-derived protocorm-like bodies of *Vanda* testacea (Vij and Pathak, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Auxin				
13	Naphthaleneacetic acid (NAA)	1.0	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
14	Benzylaminopurine (BAP)	1.0	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
15	Peptone[g]	1.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar[h]	9.0 g	No stock	No stock	Weigh
	Darkening agent				
19	Activated charcoal (AC)[i]	2.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin fails to dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[g]Peptones can vary. Therefore it is best to use one which is suitable for plant tissue culture. Possible sources are www.sigmaaldrich.com.

[h]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.5, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved add the charcoal (item 19) with constant stirring to insure complete and even distribution, pour the solution into culture vessels, and autoclave.

[i]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. pH paper strips can be used for this measurement.

Procedure. The leaves are cut in half, placed on the medium, and cultured in the first medium (Table V-33). Once the PLBs produced by the explants are sufficient in number and are large enough they are transferred to the second solution (Table V-34). When the plantlets produced on the second solution are large enough they should be moved to community pots.

Developmental Sequence. The explants produce PLBs (Fig. V-7) on the first medium (Table V-33) 5 weeks after being placed in culture. The first leaf appears after 8 weeks. Roots appear 16 weeks after the cultures were initiated. Plantlets (Fig. V-7) develop well on the second substrate (Table V-34).

General Comments. This is an effective method which cannot be used to propagate specific clones because the quality of seedlings is not known.

Micropropagation of *Vanda cristata* through the Culture of Labella

The ideal explant for micropropagation is a tissue or organ which is plentiful, easily accessible, simple to handle, easy to surface-sterilize, and whose removal will neither damage nor endanger the plant from which it is taken and which requires relatively simple medium or media and culture conditions. Professor Suraj P. Vij and his associates at the Botany Department, Panjab University have devoted considerable attention to the use of such explants including the use of perianth segments (Vij and Sharma, 1996).

Plant Material. Young floral buds should be taken from greenhouse-grown plants (Fig. V-8G, I). In taking buds it is important to keep in mind that "the frequency with which the explants responded decline progressively with the increase in the level of bud maturity." In other words, it is better to take younger buds. Explants taken after anthesis (i.e., after the flowers opened) do not respond. The labellum (Fig. V-8A) should be excised from the buds after surface sterilization and cut into 5 mm sections (Fig. V-8C, D), all of which should be cultured.

Surface Sterilization. Buds should first be washed gently with running water and a mild detergent (this step is not part of the procedure in the original report). After the washing, the buds should be immersed in 0.1% streptomycin (available from www.sigmaaldrich.com and other sources) for 12 min, submerged in 4% sodium hypochlorite (80 or 66.7 ml, respectively, of household bleach containing 5–5.25 or 6% sodium hypochlorite diluted to 100 ml with distilled water) for 15 min, and dipped in 70% ethanol (74 ml of 95% ethanol diluted to 100 ml with distilled water) for 3 s and finally rinsed three times with sterile distilled water.

Culture Vessels. Erlenmeyer flasks, culture tubes, and other containers are suitable.

Culture Conditions. Cultures should be maintained at 25 ± 5°C under 12-h photoperiods of 3500 lx or standard culture room conditions.

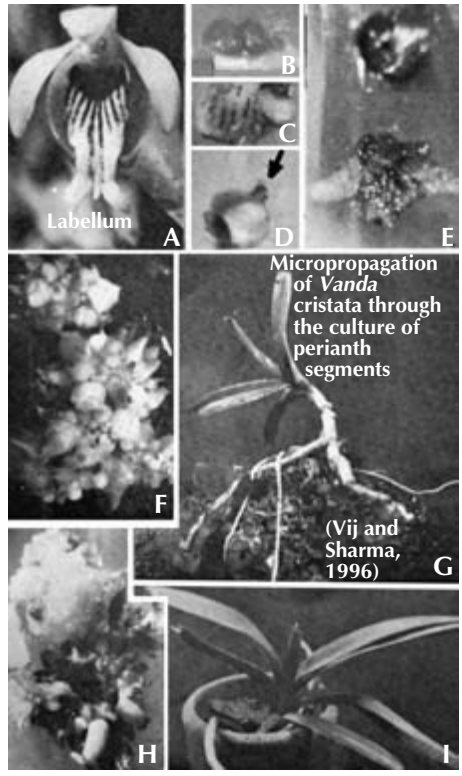


FIG. V-8. **Micropropagation of *Vanda cristata*. A. Flower. B. Compact spherical callus on an explant. C. Explant. D. Labellum explant showing hypodermal bulge which will form callus eventually. E. Callus emerging from an explant. F. PLB formation on callus. G. Three-month-old labellum-derived plant in a greenhouse. H. PLBs and plantlets on callus after 16 weeks of culture. I. Six-week-old plant in a greenhouse. (Vij and Sharma, 1996.)**

Culture Media. Explants should be cultured initially on Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) supplemented with 5 mg l^{-1} each of BA and NAA (Table V-35). The addition of 10 mg BA l^{-1} increases the percentage of explants which respond (from 37.5 to 50%) and the number of plantlets per explant (70 instead of 52), but high hormone levels can induce undesirable mutations and should be avoided. Callus or PLBs form on the first medium (Table V-35) and should be moved to a second version of MPR which contains 0.5 mg NAA l^{-1} and 500 mg sucrose l^{-1} (Table V-36). Plantlets will form on this medium.

Procedure. Explants are washed, surface-sterilized, and rinsed with sterile distilled water before their labella are removed and sectioned. The sections are cultured on the first medium (Table V-35) until callus and PLBs are formed. These are moved to the second medium (Table V-36) for plantlet formation. If necessary the plantlets can be cultured on one of the versions of the Knudson C medium (Knudson, 1946) with

TABLE V-35. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the initial culture of labella segments of *Vanda cristata* (Vij and Sharma, 1996)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	5.0	500 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Cytokinin				
15	Benzylaminopurine (BAP)	5.0	500 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[f]	To 1000 ml			
	Solidifier				
18	Agar[f]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin fails to dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.6, add sugar (item 16), and bring volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar (item 18) can also be added to the cold water which is then brought to a boil and stirred. When the agar (item 18) is completely dissolved, pour the solution into culture vessels and autoclave. Agar (item 18) is not used in liquid media. As a rule media which contain vitamins (items 8–13), hormones (items 14 and 15), and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

TABLE V-36. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for plantlet production from callus and protocorm-like bodies produced by labella segments of *Vanda cristata* (Vij and Sharma, 1996)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Sugar				
15	Sucrose	500.0	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[f]	To 1000 ml			
	Solidifier				
17	Agar[f]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin fails to dissolve add a few drops of 0.1 N KOH.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and bring volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar (item 17) can also be added to the cold water which is then brought to a boil and stirred. When the agar (item 17) is completely dissolved, pour the solution into culture vessels and autoclave. Agar (item 17) is not used in liquid media. As a rule media which contain vitamins (items 8–13), hormones (item 14), and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

(see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate (BH) or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal (AC) should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*). When plantlets are large enough they should be potted in a greenhouse.

Developmental Sequence. The explants (37.5%) produce callus and PLBs (Fig. V-8B, D-F) after 8 weeks in culture. The time required for plantlet production (on the medium in Table V-36) is 17 weeks.

General Comments. This seems to an effective procedure which utilizes a unique and abundant part of the plant.

Micropropagation of *Vanda cristata* through the Culture of Foliar Explants from Mature Plants

The use of shoot tips (usually and erroneously referred to as meristems) for micropropagation can be "detrimental to . . . the mother plant" especially in monopodial species. "Efforts have, therefore, been directed toward developing an equally effective multiplication system by activating adventitious meristems in organs whose excision does not endanger the survival of the source plant" (Sharma and Vij, 1997). Leaves of seedlings of a number of orchid species have been cultured (Vij and Pathak, 1990), but they do not make possible the multiplication of clones of known quality. Therefore a method was developed for the micropropagation of *Vanda cristata* by culturing explants taken from young leaves of mature plants (Sharma and Vij, 1997).

Plant Material. The youngest, still folded leaves (Fig. V-9E), up to 5 cm long, should be harvested from mature plants grown under horticultural conditions. After surface sterilization, the leaves should be cut into 5-mm sections (Fig. V-9A). Although all sections can be cultured, only the lower median section (Fig. V-9A, M_2) and the basal two (Fig. V9A, P_1 and P_2) produce callus (Fig. V-9B, C, F–H) and plantlets (Fig. V-9D, J, K).

Surface Sterilization. The leaves should first be washed with running water and a mild detergent [this step is not part of the procedure published by Sharma and Vij (1997)]. After that they should be submerged for 20 min in 0.1% streptomycin (available from www.sigmaaldrich.com), immersed in 0.1% mercuric chloride for 15 min, dipped in 70% ethanol (74 ml of 95% ethanol brought to 100 ml with distilled water) for 3 s and rinsed three times with sterile distilled water.

Culture Vessels. Erlenmeyer flasks, culture tubes, and other containers can be used.

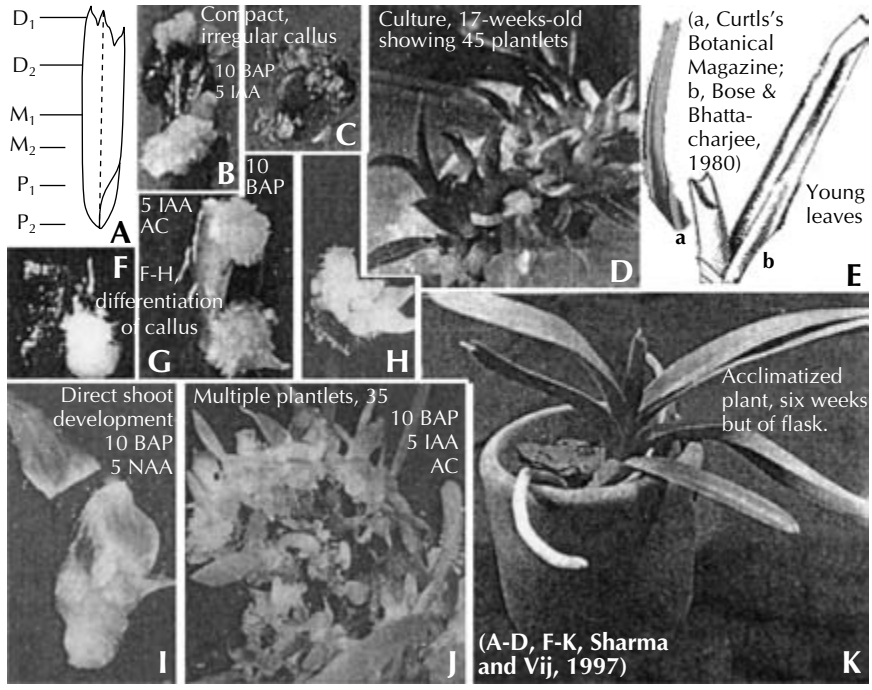


FIG. V-9. **Micropropagation of *Vanda cristata* through the culture of foliar explants. Captions are on the illustrations. AC, activated charcoal; BAP, benzylaminopurine; IAA, indoleacetic acid; the numbers associated with BA and NAA are in mg l^{-1}.**

Culture Conditions. The experimental cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx. Standard laboratory conditions should also prove suitable.

Culture Media. Explants should be cultured on Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) containing 10 mg BA l^{-1}, 5 mg IAA l^{-1}, and 2.2 mg copper sulfate l^{-1} with or without activated charcoal (Table V-37). Callus can be subcultured on the same medium. Plantlets, 3 cm tall, should be cultured on half-strength (micro- and macroelements) MPR without vitamins, hormones, or sugar (Table V-38) until they reach a height of 4–5 cm. On reaching this height the plantlets should be potted in a mixture of moss, pine bark, brick shards, and charcoal pieces (in the ratio of 1 : 1 : 1 : 1).

Procedure. The youngest leaves of mature plants are taken, washed, surface-sterilized, and sectioned. Sections are cultured on a modified MPR medium (Table V-37). Callus can be subcultured on the same medium (Table V-37). Plantlets should be maintained on this medium (Table V-37) until they reach a height of 3 cm, when they are moved to the second solution (Table V-38) and grown on each until they are 4–5 cm high. These plantlets should be removed from the flasks, washed with

TABLE V-37. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the initial culture of foliar explants of *Vanda cristata* (Sharma and Vij, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	2.2	220.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Indoleacetic acid (IAA)	5.0	500 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Cytokinin				
15	Benzylaminopurine (BAP)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin fails to dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.6, add sugar (item 16), and bring volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar (item 18) can also be added to the cold water which is then brought to a boil and stirred. When the agar (item 18) is completely dissolved pour the solution into culture vessels and autoclave. Agar (item 18) is not used in liquid media. As a rule media which contain vitamins (items 8–13), hormones (items 14 and 15), and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

TABLE V-38. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of plants produced by foliar explants of *Vanda cristata* (Sharma and Vij, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	50.0	5.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	100.0	10.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	125.0	12.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	90.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	75.0	7.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	18.7	1.87 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	13.9	1.30 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.3	30.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	1.1	110.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.2	20.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.015	1.5 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025	2.5 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.025	2.5 mg l^{-1}		
	Solvent				
8	Water, distilled[e]	To 1000 ml			
	Solidifier				
9	Agar[e]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The concentrations of macro- and microelements are half those in the original formulation.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Add items 1–7 to 900 ml of distilled water (item 8), adjust pH to 5.6, and bring volume to 1000 ml with distilled water (item 8). Bring the solution to a gentle boil and add the agar (item 9) slowly while stirring. The agar (item 9) can also be added to the cold water which is then brought to a boil and stirred. When the agar (item 9) is completely dissolved, pour the solution into culture vessels and autoclave. Agar is not used in liquid media.

lukewarm water to remove all agar, potted in the potting mix in small pots, and grown in 90% shade. To maintain humidity, the pots should be covered with polyethylene bags. Holes of increasing size should be cut in these bags over a period of 4 weeks at which point they should be removed and the pots should be moved to sunlight. The young plants should be sprayed with a fungicide [1% Bavistin was used by Sharma and Vij (1997)]. Approximately 80% can be expected to survive under these conditions.

General Comments. This is an ideal procedure in all aspects but one, which is the relatively high level of BA.

Micropropagation of *Vanda* Kasem's Delight 'Tom Boykin' through the Culture of Foliar Explants

Large colorful *Vanda* hybrids like *Vanda* Kasem's Delight are popular, expensive, and monopodial (i.e., they have only one shoot tip per plant). Therefore, a micropropagation method that uses foliar explants is of importance (Vij et al., 1994*b*).

Plant Material. In the original research, sections 5 mm long (Fig. V-10Aa, C, Ea) of young leaves (up to 2.5 cm in length, from plantlets produced from shoot-tip explants and growing in vitro) gave the best results. Sections from older and longer leaves died or did not proliferate.

Surface Sterilization. Explants from plantlets in vitro do not require surface sterilization.

Culture Vessels. Erlenmeyer flasks, culture tubes, and other containers are suitable.

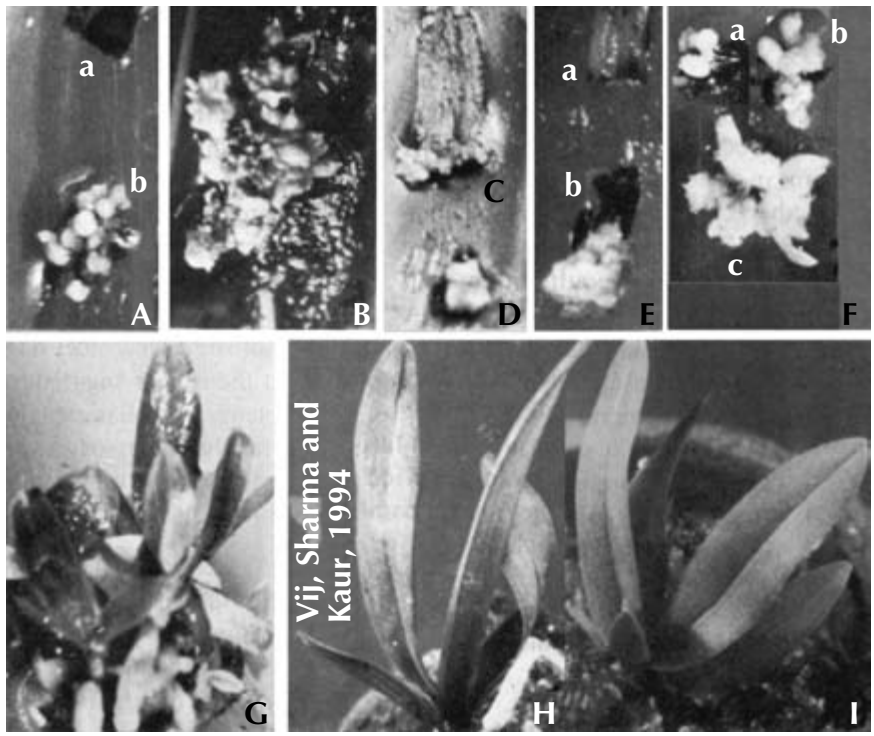


FIG. V-10. **Micropropagation of *Vanda* Kasem's Delight 'Tom Boykin'. A. Explant (a) and PLB (b) formation on a basal explant. B. Multiplication of PLBs. C, D. PLB formation on cut ends. E. Explant (a) and PLBs (b) on the basal ends of older leaves. F. PLB formation (on an explant from a younger leaf in b) and subsequent differentiation (a, c). G. Plantlets forming in 15-week-old cultures. H, I. Plantlets in pots. (Vij et al., 1994*b*.)**

Culture Conditions. The research cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx. Standard culture room conditions will probably also prove to be suitable.

Culture Media. Explants should be cultured on Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) containing per liter 2 g peptone, 5 mg BA, and 1 mg NAA (Table V-39). PLBs produced on this medium proliferate when subcultured on it (Table V-39). PLBs are cultured on a medium containing 1 mg kinetin l^{-1} (Table V-40) and produce plantlets. Twenty-week-old plantlets should be potted in a mixture of moss, pine bark, brick shards, and charcoal pieces (in the ratio of 1 : 1 : 1 : 1) and maintained in partial shade and a relative humidity of 70%.

Procedure. Surface-sterilized leaves are sectioned and the sections (Fig. V-10Aa, C, Ea) are cultured on the first medium (Table V-39). PLBs (Fig. V-10Ab) produced on this medium (Table V-39) should be moved to the kinetin-containing solution (Table V-40) for plantlet production (Fig. V-10G). When the plantlets are 20 weeks old they should be potted and grown in a greenhouse (Fig. V-10H, I).

Developmental Sequence. PLBs are produced at the cut ends of explants (Fig. V-10C, D, Eb, F) cultured on the first medium (Table V-39). They proliferate (Fig. V-10B) when subcultured on the same solution (Table V-39). Plantlets (Fig. V-10G) are formed when the PLBs are cultured on the second medium (Table V-40). New leaves and roots are formed 3 weeks after the plantlets are potted and moved to a greenhouse (Fig. V-10H, I).

General Comments. A major drawback of this procedure is that it does not utilize explants from mature plants. However an effort should be made to culture very young leaves of mature plants. If such leaves are used the procedure may prove to be suitable. Also, even if this method was developed with *Vanda* Kasem's Delight 'Tom Boykin' the chances are that it may prove to be suitable for similar hybrids.

> The name *Vanda* is derived from a Sanskrit word which refers to a sacred mistletoe found on *Vandaca* oak trees. It was coined by Sir William Jones to describe epiphytic plants in general . . . and published posthumously in *Asiatic Researches* volume 4, page 302. (Hew et al., 2002 citing Grove, 1995)

Sir William Jones (London, 1746 to Calcutta, 1794) was a British orientalist who spoke 28 languages including Arabic, Chinese, Latin, Greek, Hebrew, Persian, and Sanskrit and also wrote extensively on Muslim and Indian law. His use of a word which refers to mistletoe in the description of an epiphytic genus is ingenious and reflects his British (i.e., northern rather than tropical) origins (Hew et al., 2002 citing Grove, 1995; Dr. David Grove, pers. comm.). For more information about Jones see Cannon (1993).

TABLE V-39. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the initial culture of foliar explants of *Vanda* Kasems's Delight 'Tom Boykin' (Vij et al., 1994*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	2.2	220.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Cytokinin				
15	Benzylaminopurine (BAP)	5.0	500 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Complex additive				
16	Peptone[g]	2.0 g	No stock	No stock	Weigh
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[h]	To 1000 ml			
	Solidifier				
19	Agar[h]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin fails to dissolve add a few drops of 0.1 KOH or HCl, respectively.

[g]Peptones can differ. Therefore it is advisable to either use the exact same peptone used in the original research or purchase a preparation which is suitable for plant tissue culture. Possible sources are www.sigmaaldrich.com, www.caissonlabs.com, and www.phytotechlab.com.

[h]Add items 1–16 to 900 ml of distilled water (item 18), adjust pH to 5.5, add sugar (item 17), and bring volume to 1000 ml with distilled water (item 18). Bring the solution to a gentle boil and add the agar (item 19) slowly while stirring. The agar (item 19) can also be added to the cold water which is then brought to a boil and stirred. When the agar (item 19) is completely dissolved pour the solution into culture vessels and autoclave. Agar (item 19) is not used in liquid media. As a rule media which contain vitamins (items 8–13), hormones (items 14 and 15), and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

TABLE V-40. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for plantlet production from foliar-explant-derived protocorm-like bodies of *Vanda* Kasems's Delight 'Tom Boykin' (Vij et al., 1994*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	2.2	220.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Cytokinin				
14	Kinetin	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1.0	
	Sugar				
15	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.5, add sugar (item 15), and bring volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar (item 17) can also be added to the cold water which is then brought to a boil and stirred. When the agar (item 17) is completely dissolved, pour the solution into culture vessels and autoclave. Agar (item 17) is not used in liquid media. As a rule media which contain vitamins (items 8–13), hormones (item 14), and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

Micropropagation of *Papilionanthe* (*Vanda*) *teres* through the Culture of Foliar Explants

The genus *Vanda* was established in 1795 by Sir William Jones (1746, London to 1794, Calcutta). *Vanda teres* was discovered in northern India by Nathaniel Wallich (1786–1854) who introduced it into cultivation in 1829 and was named by John Lindley (1799–1865) in 1833. In 1915 Rudolph Schlechter (1872–1925) made it the type species of a *Papilionanthe*, a genus he segregated from *Vanda*. The new genus was not accepted widely until Leslie Garay treated it in 1972 (Bechtel et al., 1992). This species is one of the parents of *Vanda* Miss Joaquim, a natural hybrid and a foundling which became the national flower of Singapore (Hew et al., 2002). It is found in dry areas from the Himalayas to Burma, Thailand, and Laos. Insufficient reproduction in its native areas, habitat destruction, and overexploitation are reducing its natural population and this led to the development of a micropropagation procedure from young leaves of mature plants (Pathak and Vij, 2001).

Plant Material. "Young leaves up to 1 cm in length excised from greenhouse grown plants were segmented into 0.3–0.5 cm long basal (with leaf tip) parts and used as explants." It is not clear from this quote (taken verbatim from Pathak and Vij, 2001) which segments were used as explants because a "basal" part cannot have a "leaf tip." Therefore it is possible that what were used were either basal segments (Fig. V-11Nb), or basal and tip sections (Fig. V-11Nb, d), or explants consisting of very young short leaves (Fig. V-11M). In any case "the basal explants responded

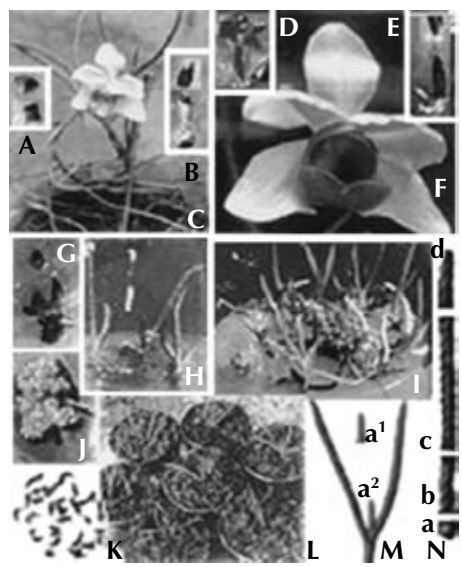


FIG. V-11. **Micropropagation of *Vanda teres*. A, B. Callus formation on basal ends. C. Plant. D, E, G. Differentiation of PLBs and subsequent plantlet formation. F. Flower. H, I. Plantlets. J. Multiplication and differentiation of PLBs. K. Somatic chromosomes, $2n = 38$. L. Potted plantlets. M. Shoot top showing different size leaves, with the youngest leaf (a^2) possibly being used as an explant (a^1). N. Sectioned leaf showing remnants of stem (a), basal (b), median (c), and tip (d) sections. (Sources: A–L, Pathak and Vij, 2001; M, N, Hew et al., 2002.)**

better than the apical ones." This suggests that the most suitable explants are basal ones (Fig. V-11Nb) or very young short leaves (Fig. V-11Ma[1]).

Surface Sterilization. Explants should first be washed with a mild detergent and running water [this step is not part of the procedure recommended by Pathak and Vij (2001)]. After the wash the explants should be dipped in 70% ethanol (74 ml 95% ethanol diluted to 100 ml with distilled water) for 5 s, immersed in 0.01% (w/v) streptomycin (available from www.sigmaaldrich.com and other sources) for 5 min, submerged in 7% calcium hypochlorite (7 g calcium hypochlorite suspended in 100 ml distilled water and agitated and allowed to settle three times before decanting and using the supernatant), and washed three times with sterile distilled water.

Culture Vessels. Erlenmeyer flasks, culture tubes, and other containers can be used.

Culture Conditions. The research cultures were maintained at 25 ± 2°C under 12-h photoperiods of 3500 lx. Standard culture room conditions will probably also prove to be suitable.

Culture Media. The explants should be cultured on MS medium (Murashige and Skoog, 1962) supplemented with 1 mg BA l^{-1} (Table V-41). Callus and PLBs that form in the initial cultures should be subcultured on the same medium (Table V-41). Plantlets formed on this medium can be cultured on it or on one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate (BH) or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal (AC) should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. After being surface-sterilized the leaves are sectioned and the sections are cultured on the BA-containing MS (Table V-41). Callus and PLBs can be subcultured on the same medium (Table V-41) and plantlets can be grown on it. Or, they can be moved to one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, and V-4) with or without BH or AC. If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). AC should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*). When plantlets are large enough they should be removed from the culture vessels and potted in community pots.

Developmental Sequence. Meristematic areas are formed 3 weeks after the explants are placed in culture. Up to ten plantlets can be obtained per explant.

General Comments. This seems to be an efficient and safe procedure.

TABLE V-41. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of foliar explants of *Vanda teres* (Pathak and Vij, 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
10	Benzyladenine (BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of HCl.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.5, add sugar (item 14), and adjust volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Agar is not added to liquid media. Amino acids, vitamins, and hormones may be heat-labile and are generally not autoclaved. However in this case they are added before autoclaving.

Plantlet Production from Leaf Explants of *Vanda teres*

The flowers of *Vanda teres* are "large rose-colored . . . 10 cm across . . . long lasting and fragrant." A micropropagation method using leaf explants taken from aseptic seedlings was developed at the Botanical Survey and Herbarium, Department of Medicinal Plants, Kathmandu, Nepal (Niraula and Rajbhandary, 1988).

Plant Material. In the original research, 10-cm-long leaves were excised from 6–8-month-old seedlings maintained on Vacin and Went medium (Vacin and Went, 1949). Sections, 5–8 mm, of these leaves were cultured.

Surface Sterilization. Explants taken from seedlings growing in vitro do not require surface sterilization.

Culture Vessels. Erlenmeyer flasks, 100-ml capacity, were used in the original research. Other containers (culture tubes, jar, plastic vessels) can also be used.

Culture Conditions. The research cultures were maintained at 25°C under 16-h photoperiods of 3000 lx (the light sources were not described). Standard culture room conditions will probably also be suitable.

Culture Media. Explants should be cultured initially on MS medium (Murashige and Skoog, 1962) supplemented with 1 g casein hydrolysate (CH) l^{-1} and 5 mg l^{-1} each of IAA and kinetin (Table V-42). PLBs that form on this medium should be moved to Vacin and Went medium (Table V-43) for plantlet production.

Procedure. Leaves are taken from seedlings and sectioned. The sections are cultured on the modified MS (Table V-42). When PLBs form on this medium they are transferred to the Vacin and Went substrate (Table V-43).

Developmental Sequence. Explants cultured on the MS-based medium (Table V-42) thicken, especially at the cut ends, after 8 weeks of culture and form small, greenish structures. After 3 months of culture the explants turn brown, but the small green structures continue to develop and produce a mass of PLBs at the end of 4 months (Fig. V-12). On being moved to Vacin and Went medium (Table V-43), the PLBs produce leaves after 4–6 weeks. Roots appear later and plants are developed 6 months from the start of culture. Plants that can be potted in a greenhouse are produced after 6–8 months.

General Comments. This is an interesting procedure but it cannot be used to select specific clones since the quality of seedlings is not known. The authors seem to use the words "plantlet" and "seedling" interchangeably. This is incorrect.

TABLE V-42. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of foliar explants of *Vanda teres* (Niraula and Rajbhandary, 1988)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indoleacetic acid (IAA)	5.0	500–300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Kinetin	5.0	500 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
15	Casein hydrolysate (CH)	1.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–7, 9, and 15 to 900 ml of distilled water (item 17), adjust pH as required, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into a 2-l flask and autoclave. Add the amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media. In this case items 1–15 can be added to the medium before autoclaving.

TABLE V-43. **Vacin and Went (VW) medium (Vacin and Went, 1949) for plantlet production from protocorm-like bodies derived from foliar explants of *Vanda teres* (Niraula and Rajbhandary, 1988)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[e]	To 1000 ml			
	Solidifier				
10	Agar[e]	12–15 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 9) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Add items 1 and 3–7 to the 500–700 ml of distilled water (item 9) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 9), adjust pH to 5–5.4, add sugar (item 8), and adjust volume to 1000 ml with distilled water (item 9). Bring the solution to a gentle boil and add the agar (item 10) slowly while stirring. The agar can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

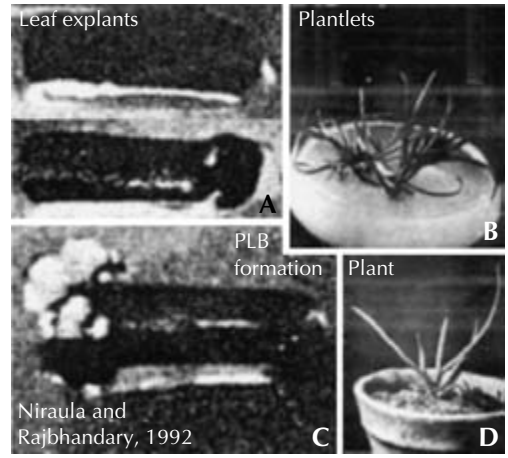


FIG. V-12. **Plantlet production from leaf explants of *Vanda teres*.**

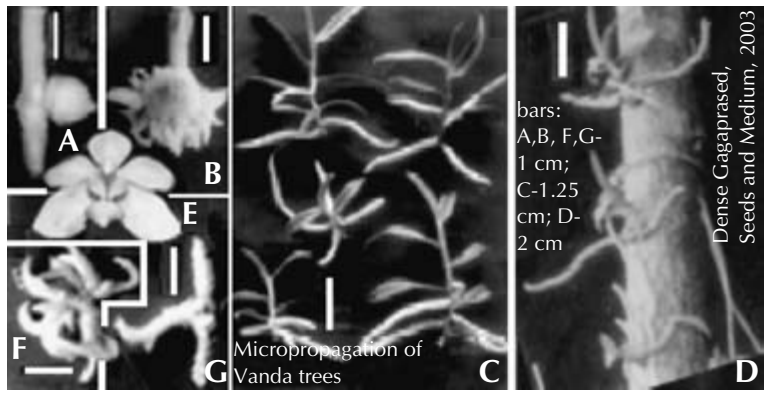


FIG. V-13. **Micropropagation of *Vanda spathulata*. A. Swollen axillary bud. B. Multiple shoot formation from an axillary bud. C. Rooted shoots. D. Plantlets established on a tree trunk 120 days after reintroduction. E. Flower. F. Axillary shoots after 120 days of culture. G. Shoots after 60 days of culture.**

Micropropagation of *Vanda spathulata*

A species with large golden flowers (Fig. V-13E), *Vanda spathulata* is used extensively in breeding and as part of an ayurvedic preparation called Guggul or Guggulu, which is presumed to lead to weight loss, promote flexible joints, support healthy heart functions, and reduce cholesterol and triglycerate levels. Its natural populations have been reduced severely as a result of overcollection and habitat destruction. A micropropagation method was developed at the Plant Biotechnology Division, Tropical Botanic Garden and Research Institute, Palode, Trivandrum, India (Decruse et al., 2003*a*, 2003*b*).

Plant Material. Terminal shoot cuttings are taken from flowering plants, trimmed to 2–2.5 cm, and inserted 3–5 mm deep in culture medium. When these explants produce 2–5-cm-long shoots (Fig. V-13F, G), nodes of 0.5–1 cm in length should be excised and cultured. This can be repeated three times.

Surface Sterilization. The cuttings should be washed with running tap water and then soaked in 2% Labolene (Qualigens Fine Chemicals, Mumbai, India, a Division of Glaxo, India) or Teepol (www.sigmaaldrich.com) for 15 min and rinsed thoroughly with running tap water. After the last tap water wash the cuttings should be submerged in 5% Steriliq (Combichem, Delhi, India) or 20% Clorox or a similar household bleach (20 ml bleach made up to 100 ml with distilled water) for 20 min, immersed in 0.1% mercuric chloride for 12 min, and rinsed three times with sterile distilled water.

Culture Vessels. Erlenmeyer flasks, culture tubes, and other containers can be used.

Culture Conditions. Cultures can be maintained at 25 ± 2°C under 12-h photoperiods of 30–50 μmol m^{-2} s^{-1} or standard culture room conditions.

Culture Media. Explants should be cultured first in Mitra, Prasad, and Roychowdhury (MPR) medium (Mitra et al., 1976) supplemented with 44.4 μmol BA l^{-1} and 17.1 μmol IAA l^{-1} (Table V-44). MPR containing 22.2 μmol BA l^{-1} and 5.7 μmol IAA l^{-1} (Table V-45) should be used to subculture the nodal explants. One problem with this medium is that the abstract of the original paper states that 5.7 μmol l^{-1} should be added to the medium whereas the text lists the concentration as 1.7 μmol l^{-1}. The concentration recommended here is based on a graph (Decruse et al., 2003*a*, fig. 1 on p. 200) which seems to indicate that 5.7 μmol l^{-1} is suitable. For rooting, shoots should be cultured on MPR containing 75 g banana homogenate and 5.7 μmol IAA l^{-1} (Table V-46).

Procedure. Explants are placed on the first medium (Fig. V-44) and maintained on it for 3 months or until they produce buds (Fig. V-13A, B). Bud-bearing explants should be subcultured on the second medium (Table V-45) for "further multiplication." This step was repeated three times in the original research and the same can be done in practical laboratories. Shoots are moved to the third medium (Table V-46) for rooting.

Developmental Sequence. The axillary buds on the explants bulge and produce a spherical structure (Fig. V-13A) 60 days after the start of culture on the first medium (Table V-44). Within 2–3 months this structure produces numerous axillary shoots (Vig. V-13B). Nodes taken from the shoots produced in vitro produce vegetative buds within 30 days. When moved to the second medium (Table V-45), additional shoots (up to an average of 6.1 ± 4.4 per node), 3–5 cm long, are produced in 3–4 months. On being cultured on the banana-containing medium (Table V-46), more than 95% of the shoots produce 2–5 roots which can vary in length from 2 to 7 cm in 40–60 days (Fig. V-13C). When moved to community pots, 90–95% of these plants survive. After these plants become established on the pots they can be moved to ecorestoration sites (Fig. V-13D) where 43–79% can be expected to survive.

General Comments. Given the continued habitat destruction and overcollection, the use of mass, rapid, clonal propagation for ecorestoration can be justified on some levels especially since most orchids are heterozygous. However, it has also been suggested that restoration through the use of clones can lead to genetic pauperization. Since *Vanda* seeds are easy to germinate, the use of seedlings in ecorestoration may be preferable.

TABLE V-44. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Vanda spathulata* nodes (Decruse et al., 2003*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Cytokinin				
14	Benzyladenine (BA)	10.0	500 mg 50 mg^{-1} 95% ethanol[e,f]	1	
	Auxin				
15	Indoleacetic acid (IAA)	3.0	300 mg 100 mg^{-1} 95% ethanol[e,f]	1	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Another name for benzyladenine (BA) is benzylaminopurine (BAP). If the auxin or cytokinin fails to dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.6, add sugar (item 16), and bring volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar (item 18) can also be added to the cold water (item 17) which is then brought to a boil and stirred. When the agar (item 18) is completely dissolved pour the solution into culture vessels and autoclave. Agar (item 18) is not used in liquid media. As a rule media which contain vitamins (items 8–13), hormones (items 14 and 15), and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

TABLE V-45. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the culture of *Vanda spathulata* shoots produced from nodes in vitro (Decruse et al., 2003*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}		
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Cytokinin				
14	Benzyladenine (BA)	5.0	250 mg 50 mg^{-1} 95% ethanol[e,f]	1	
	Auxin				
15	Indoleacetic acid (IAA)	1.0	100 mg 100 mg^{-1} 95% ethanol[e,f]	1	
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Another name for benzyladenine (BA) is benzylaminopurine (BAP). If the auxin or cytokinin fails to dissolve add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust pH to 5.6, add sugar (item 16), and bring volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. The agar (item 18) can also be added to the cold water (item 17) which is then brought to a boil and stirred. When the agar (item 18) is completely dissolved pour the solution into culture vessels and autoclave. Agar (item 18) is not used in liquid media. As a rule media which contain vitamins (items 8–13), hormones (items 14 and 15), and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

TABLE V-46. **Mitra, Prasad, and Roychowdhury medium (Mitra et al., 1976) modified for the rooting of *Vanda spathulata* shoots produced from nodes in vitro (Decruse et al., 2003*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	100.0	10.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	200.0	20.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	180.0	18.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	0.6	60.0 mg l^{-1}	10	One solution
(b)	Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.05	5.0 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.05	5.0 mg l^{-1}		
(d)	Manganese chloride, $MnCl_2 \cdot 4H_2O$	0.4	40.0 mg l^{-1}		
(e)	Potassium iodide, KI	0.03	3.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.05	5.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.05	5.0 mg l^{-1}		
	Vitamins				
8	Biotin	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
9	Folic acid	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
10	Niacin (nicotinic acid)	1.25	500 mg 100 ml^{-1} 95% ethanol[e]	0.25	
11	Pyridoxine (vitamin B_6)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
12	Riboflavine (vitamin B_2)	0.05	20 mg 100 ml^{-1} 95% ethanol[e]	0.25	
13	Thiamine (vitamin B_1)	0.3	120 mg 100 ml^{-1} 95% ethanol[e]	0.25	
	Auxin				
14	Indoleacetic acid (IAA)	1.0	100 mg 100 mg^{-1} 95% ethanol[e,f]	1	
	Complex additive				
15	Banana homogenate[g]	75.0 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar[h]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Originally the recipe called for 3 ml of commercial $Na_2FeEDTA$, which may not be easily available or if used may not be the same as that in the original formulation. The substitute suggested here is used very commonly in tissue culture media for orchids and other plants. A product called Edathamil or Komplexon (NaFeEDTA) is available from www.sigmaaldrich.com.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin fails to dissolve add a few drops of 0.1 N KOH.

[g]Opinions differ regarding the use of banana in culture media. The predominant opinion is that only homogenized pulp of ripe banana should be used. Other views are that: (1) unripe (green) bananas are preferable, (2) the peel rather than the pulp should be used, (3) unpeeled banana homogenate should be incorporated in media, and (4) slices, ripe or unripe, peeled or unpeeled, rather than homogenate are most effective. Opinions also differ regarding which is the most effective banana variety (Gros Michel, Raja, Pisang Susu, and others). Since there is no experimental evidence to support any view the best approach is to follow the recommendations in the original reports and/or use what is suggested there.

[h]Add items 1–15 to 800 ml distilled water (item 17) in a homogenizer and homogenize until the banana is completely dispersed, adjust pH to 5.6, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 18) slowly while stirring. Agar (item 18) can also be added to the cold water (item 17) which is then brought to a boil and stirred. When the agar (item 18) is completely dissolved pour the solution into culture vessels and autoclave. As a rule media which contain vitamins (items 8–13), hormones (items 14 and 15), and/or other heat-labile components should not be autoclaved without prior determination that this will not destroy or damage any of these components. This medium may be autoclaved (Mitra, 1971; Mitra et al., 1976).

Culture of *Vanda pumila* Shoot Apices

A method for the culture of shoot apices of protocorms of *Vanda pumila* was developed as part of a cryopreservation research project (Na and Kondo, 1995, 1996; Kondo and Na, 1997).

Plant Material. Seeds from a capsule of *V. pumila* were germinated on MS medium (Murashige and Skoog, 1962). Shoot tips, 1–2 mm in diameter, were excised from 60-day-old protocorms and were cultured.

Surface Sterilization. Explants from seedlings in vitro do not require sterilization.

Culture Vessels. Culture tubes, 30 × 200 mm, containing 25 ml medium were used in the original research. Other containers are also suitable.

Culture Conditions. In the original research the cultures were maintained on a rotary shaker (2 rpm; Fig. V-14A) at 22°C under continuous illumination (a halogen light was used in the original research) of 4000–6000 lx (Na and Kondo, 1997) or 2000–10000 lx (Kondo and Na, 1997).

Culture Media. Shoot tips should be cultured on liquid B5 medium (Gamborg et al., 1968) containing 0.02 mg BA l^{-1} (Table V-47). Tissues, PLBs, and callus (Fig. V-14B, C) produced on this medium develop plantlets when cultured on half-strength hormone-free B5 (Table V-48). No medium is suggested for the culture of plantlets to more advanced stages. Plantlets can be cultured on one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate (BH) or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal (AC) should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. Seeds are germinated on MS and allowed to develop protocorms. Shoot tips are excised from these protocorms and cultured on the first medium (Table V-47). Tissue which develops on this medium (Table V-47) should be moved to the second solution (Table V-48) for plantlet production. For further growth the plantlets can be moved to one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, Cym-1 to Cym-3, Cym-5, Cym-24, C-19, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, and V-4) with or without BH or AC. If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). AC should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

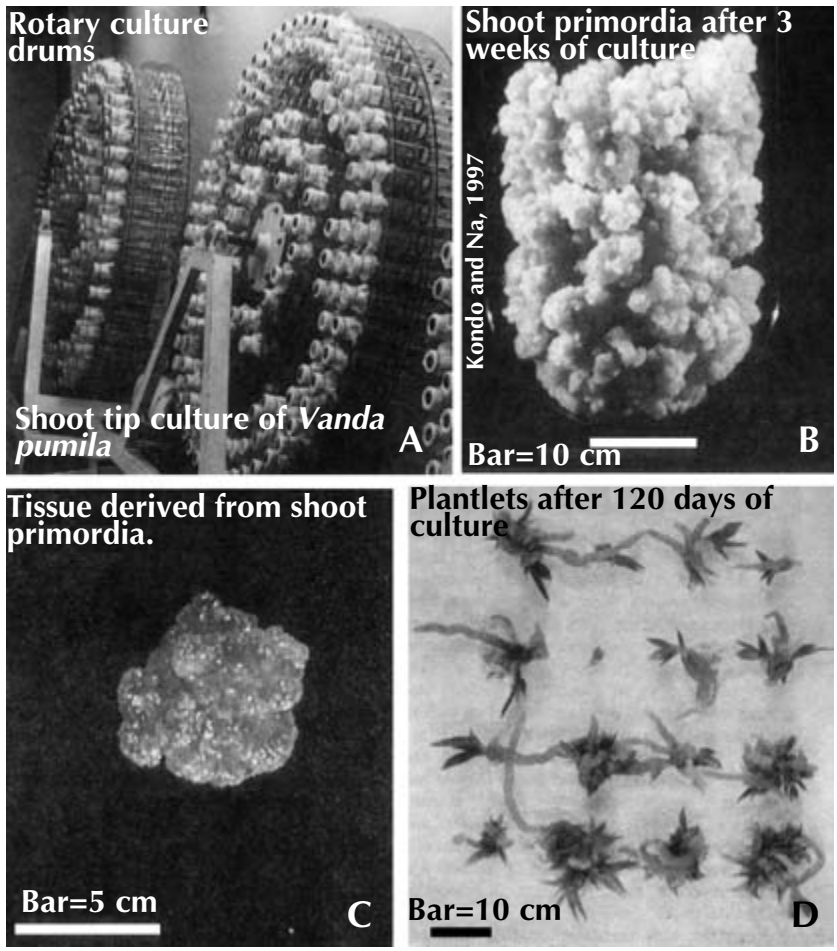


FIG. V-14. **Culture of *Vanda pumila* shoot tips.**

Developmental Sequence. The explants produce shoot primordia (Fig. V-14B, C), PLBs, and callus after 3 months of culture on B5. Plantlets develop after 120 days of culture on half-strength B5. Several additional months are required for the production of community-pot-size plants.

General Comments. The protocol was developed as a means of producing material for cryopreservation. It is not suitable for mass, rapid, clonal propagation of selected cultivars because the explants are taken from seedlings whose characteristics are not known.

TABLE V-47. **B5 medium (Gamborg et al., 1968) modified for the culture of shoot tips of *Vanda pumila* seedlings (Na and Kondo, 1995, 1996; Kondo and Na, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	134.0	13.4 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	150.0	15.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	2500.0	250.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	150.0	15.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.0	300.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	10.0	1.0 g l^{-1}		
(e)	Potassium iodide, KI	0.75	75.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.0	200.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Cytokinin				
9	Benzylaminopurine (BAP)	0.02	2 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
10	Niacin (nicotinic acid)	1.0	10 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[g]	To 1000 ml			
	Solidifier				
15	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH to 5.8, add sugar (item 13), and adjust volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar (item 15) can also be added to the cold solution which is then brought to a boil and stirred. When the agar (item 15) is completely dissolved pour the medium into culture vessels and autoclave. Hormones (item 9) and vitamins (items 10–12) can be or are heat-labile. Therefore it is preferable to autoclave 1 l of medium in a 2-l flask and add heat-labile substances after autoclaving with sterilized pipettes under sterile conditions. All components are added to this medium before autoclaving.

TABLE V-48. **Half-concentration B5 medium (Gamborg et al., 1968) for plantlet production from callus, protocorm-like bodies and primordia derived from seedling shoot tips of *Vanda pumila* (Na and Kondo, 1995, 1996; Kondo and Na, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	67.0	6.7 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	75.0	7.5 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	125.0	12.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1250.0	125.0 g l^{-1}	10	
5	Monosodium phosphate, $NaH_2PO_4 \cdot H_2O$	75.0	7.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.0	300.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot H_2O$	10.0	1.0 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.75	75.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	2.0	200.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
9	Niacin (nicotinic acid)	1.0	10 mg 100 ml^{-1} 95% ethanol[e]	1	
10	Pyridoxine (vitamin B_6)	1.0	100 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Thiamine (vitamin B_1)	10.0	1000 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
12	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
13	Water, distilled[f]	To 1000 ml			
	Solidifier				
14	Agar[f]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. In this table only the macroelements are reduced to half their concentrations in the B5 medium.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add the salts to the same 1 l of distilled water and stir and/heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Add items 1–11 to 900 ml of distilled water (item 13), adjust pH to 5.8, add sugar (item 12), and adjust volume to 1000 ml with distilled water (item 13). Bring the solution to a gentle boil and add the agar (item 14) slowly while stirring. The agar (item 14) can also be added to the cold solution which is then brought to a boil and stirred. When the agar (item 14) is completely dissolved, pour the medium into culture vessels and autoclave.

Plantlet Production from Thin Sections of *Vanda coerulea* Shoot Tips

Vanda coerulea, the Blue Vanda of Asia, is threatened with extinction in India due to overexploitation and habitat destruction. A rapid multiplication method on a medium containing the cytokinin, TDZ, was developed at the Department of Botany, Karnatak University (Malabadi et al., 2003).

Plant Material. In the original research, plants collected in the Jaintia and Khasi Hills in India and grown in a greenhouse served as explant sources. Shoot tips (4–8 mm) were harvested and surface-sterilized before longitudinal sections, 1–5 mm thick, were cut from them and cultured.

Surface Sterilization. The research shoot tips were "carefully washed with distilled water." This is not necessary. Washing with running tap water and a mild detergent is a common and effective step which can be used with these explants. After the wash the explants should be dipped in 0.1% (w/v) streptomycin (www.sigmaaldrich.com, www.vwr.com, www.fishersci.com, and other sources) for 20 s and 70% ethyl alcohol (74 ml of 95% ethanol diluted to 100 ml with distilled water) for 30 s, immersed in 0.1% (w/v) mercuric chloride ($HgCl_2$) for 2 min, and washed three times with sterile distilled water.

Culture Vessels. Glass tubes, 25 × 145 mm, containing 15 ml medium were used in the original research. Other containers can also be employed.

Culture Conditions. The research cultures were maintained at 25 ± 2°C, and a relative humidity of 55–60% under illumination of 100 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (the length of the photoperiod is not listed, but it seems reasonable to assume that 8–24-h light periods would be suitable).

Culture Media. The abstract of the original paper states that "Vacin and Went (VW) (1949) basal medium supplemented with 11.35" μmol TDZ was used. However, in the body of the paper the medium is described as "Vacin and Went's (VW) (1949) basal medium with 3.0% sucrose, 0.7% agar (Hi-media), 1.0 g l^{-1} meso-inositol, 2 g l^{-1} casein hydrolysate, 0.5 g l^{-1} L-glutamine, 250 mg l^{-1} Peptone and supplemented with . . ." TDZ. This is the medium suggested here (Table V-49). Shoots produced on this medium should be moved to half-strength Vacin and Went supplemented with 11.42 μmol IAA l^{-1} (Table V-50). "Well rooted shoots" should be potted in charcoal chips, coconut husk, and broken tiles (in a ratio of 2 : 2 : 1) or another suitable potting medium.

Procedure. Shoot tips are taken from mature plants, washed, and surface-sterilized. Explants are taken after that and placed on the first medium (Table V-49). Small bud-like structures which are formed by the explants after 2 weeks and PLBs are subcultured on fresh medium of the same kind (Table V-49) after a month. Shoots formed by the PLBs are moved to the second medium (Table V-50) for rooting.

TABLE V-49. **Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the culture of shoot-tip sections of *Vanda coerulea* (Malabadi et al., 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	500.0	50.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	200.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250.0	25.0 g l^{-1}	10	
4	Monosobsic potassium phosphate, KH_2PO_4	250.0	25.0 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	525.0	52.5 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750.0 mg l^{-1}	10	
	Polyol				
8	*myo*-inositol	1.0 g	No stock	No stock	Weigh
	Amino acid				
9	Glutamine	500.0	No stock	No stock	Weigh
	Cytokinin				
10	Thidiazuron (TDZ)	2.5	250 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additives				
11	Casein hydrolysate	2.0 g	No stock	No stock[f]	Weigh
12	Peptone	250.0	No stock	No stock[g]	
	Sugar				
13	Sucrose	30.0 g[h]	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[i]	To 1000 ml			
	Solidifier				
15	Agar[i]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 14) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]Keep frozen between uses.

[f]Not all casein hydrolysates are the same. Their compositions and analyses vary depending on the methods by which they were produced. A product sold by Merck (www.merck.de) as casein hydrolysate (CH) is obtained by degrading casein with hydrochloric acid; vitamins and other growth-promoting compounds are largely destroyed in this process. The CH sold by Sigma-Aldrich (www.sigmaaldrich.com) is pancreatic hydrolysate of casein. There is also a CH which is hydrolysed by enzymes. Becton, Dickinson and Company (www.bd.com) sells Difco products including a number of different casamino acid products all of which are casein hydrolysates. Therefore if a method specifies a source for the CH in its culture medium it is best to obtain the additive from the same vendor. If a source is not listed it is best to purchase CH which is described as being suitable as a supplement for culture media. Failing that, CH should be purchased from a source which specializes in tissue culture and micropropagation supplies. One such source is www.caissonlabs.com. They list casein rather than CH as Stock #: SPCA205. Another source for CH is www.duchefa.com (item 1301). Additional sources include www.fishersci.com, www.thomassci.com, and others.

[g]Peptones vary. Use one which is recommended or certified for plant tissue cutlure.

[h]The original formulation of the Vacin and Went medium contains 20 g sucrose l^{-1}.

[i]Add items 1 and 4–12 to the 500–700 ml of distilled water (item 14) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 14), adjust pH to 5.8, add sugar (item 13), and adjust volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar (item 15) can also be added to the cold water which is then brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

TABLE V-50. **Half-strength Vacin and Went (VW) medium (Vacin and Went, 1949) modified for the rooting of shoots produced by tissues derived from shoot-tip sections of *Vanda coerulea* (Malabadi et al., 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)_2SO_4$[b]	250.0	25.0 g l^{-1}	10	
2	Calcium phosphate, $Ca_3(PO_4)_2$[c]	100.0	No stock	No stock	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	125.0	12.5 g l^{-1}	10	
4	Monobasic potassium phosphate, KH_2PO_4	125.0	12.5 g l^{-1}	10	
5	Potassium nitrate, KNO_3[b]	262.5	26.25 g l^{-1}	10	
6	**Chelated iron**[d]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
	Microelement				
7	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	3.75	375.0 mg l^{-1}	10	
	Auxin				
8	Indoleacetic acid (IAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[f]	To 1000 ml			
	Solidifier				
11	Agar[f]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. Only the concentrations of items 1–5 and 7 are reduced in half.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]This salt is hard to dissolve (heating does not help). Therefore it is not possible to prepare a stock solution. To facilitate preparation of the medium 200 mg of the salt (item 2) should be dissolved in 500–700 ml of distilled water (item 10) before adding the other components.

[d]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. Ferric citrate, $Fe_2(C_4H_4O_6) \cdot 2H_2O$, 28 mg l^{-1} is used in the original recipe, but chelated iron as suggested here is preferable. The difference (2.8 mg l^{-1}) between the amount suggested here and that in the original formulation is negligible and can be ignored.

[e]If the auxin does not dissolve add a few drops of 0.1 N KOH. Keep frozen between uses.

[f]Add items 1 and 3–8 to the 500–700 ml of distilled water (item 10) which contain the calcium phosphate (item 2), bring volume to 900 ml with distilled water (item 10), adjust pH to 5.8, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar (item 11) can also be added to the cold water which is then brought to a boil and stirred. When the agar (item 11) is completely dissolved, pour the solution into culture vessels and autoclave. There are no microelements other than manganese (item 7) in the original recipe of the Vacin and Went medium (Vacin and Went, 1949) and it is not clear whether the addition of such nutrients may/would be of benefit. If incorporation of additional microelements becomes necessary, those used as part of the Murashige–Skoog (MS) medium will probably be suitable.

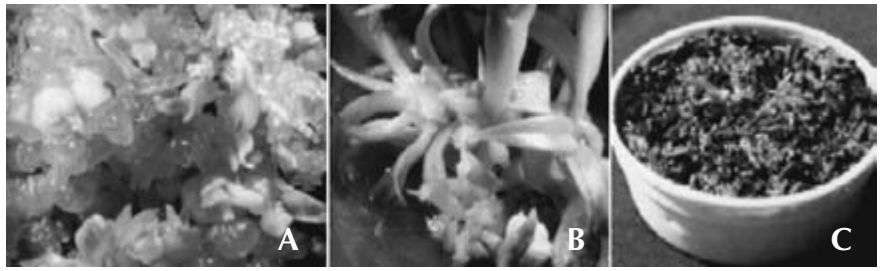


FIG. V-15. **Micropropagation of *Vanda coerulea* through the culture of thin sections of shoot tips. A. Bud-like structures that form after 2 weeks of culture. B. Shoots that form after 4–8 weeks of culture. C. Plantlets, 12 weeks old, in a pot. (Malabadi et al., 2003.)**

Developmental Sequence. The explants produce bud-like structures 2 weeks after being placed in culture (Fig. V-15A). PLBs are also formed. Shoots with 2–3 leaves are produced after 4–8 weeks (Fig. V-15B). These shoots form roots on the second medium; they are then moved to pots (Fig. V-16C), at first 2–4 per pot. After 5 months, the plants are potted individually in 22-cm pots.

General Comments. TDZ, first assumed to be a defoliant and used commercially as such, is very effective as a cytokinin in culture media. It is usually added to orchid culture media in much lower concentrations than the one employed in this protocol. What, if any, mutagenic effects the high concentration may have is not clear. The use of thin sections of shoot tips is clever and the protocol seems effective, but the paper which describes the procedure is not written as well as it should have been.

Vandofinetia

Vandofinetia is a hybrid genus. The first hybrid, *Vandofinetia* Premier, was produced in Hawaii, but many of the recent hybrids have originated in Japan.

Undescribed Method for the Micropropagation of *Vandofinetia*

A culture method is mentioned but is not described in an article on *Phalaenopsis* (Intuwong and Sagawa, 1974).

Micropropagation of *Vandofinetia* through the Culture of Flower-bud Explants

A method for mass, rapid, clonal propagation of *Vandofinetia* was developed at the Kirin Brewery Laboratory in conjunction with the Tagaki Orchid Nursery (Kishi et al., 1997*a*, 1997*b*).

Plant Material. Shoots, 10 cm long, bearing immature flower buds should be harvested and taken to the laboratory. Floral buds, 3–6 mm long, should be cut with razor blades. After surface sterilization, the buds should be sectioned into 0.5-mm cubes, which are cultured.

Surface Sterilization. The shoots should first be washed with a mild liquid detergent and running water. Gentle scrubbing with a very soft (and/or used) toothbrush is advisable but is not necessary (this initial washing is not part of the original procedure). After that, the floral buds should be washed with 10% benzalkonium chloride (www.sigmaaldrich.com, www.vwr.com, www.fishersci.com, and other sources) for 10 min and dipped for 30 s in 70% ethanol (73–74 ml 95% ethanol diluted to 100 ml with distilled water). This should be followed by a 5-min immersion in 0.5% sodium hypochlorite [10 or 8.3 ml, respectively, of household bleach which contains 5–5.25 or 6% sodium hypochlorite diluted to 100 ml with distilled water to which is added 50 mg Tween 20 (www.sigmaaldrich.com, www.vwr.com, www.fishersci.com, and other sources)], or an equal amount of a mild household detergent, and three washes with sterile distilled water.

Culture Vessels. Culture tubes, 30×200 mm containing 20 ml of medium, were used in the original research. Other containers are also suitable.

Culture Conditions. Cultures should be maintained on a drum-type rotary shaker (the one used in the original research was manufactured by Nihon Ika Kikai Co., Ltd., Tokyo, Japan) at 1 rpm, a temperature of 25 ± 2°C, and a 16-h photoperiod of 3000 ± 500 lx provided by fluorescent lamps. Standard culture room temperature and illumination will probably also be suitable.

Culture Media. Explants should be cultured on MS medium (Murashige and Skoog, 1962) containing 30 g sucrose l^{-1} (Table Vf-1). PLBs produced on this medium (Table Vf-1) can be proliferated on quarter-strength MS containing 10 g sucrose l^{-1} and 6.95 mg $FeSO_4 \cdot 7H_2O$ instead of FeEDTA (Table Vf-2). After that PLBs should be cultured on a Hyponex medium (Table Vf-3) for 2 months. Plantlets and PLBs produced on this medium (Table Vf-3) should be moved to MS (Table Vf-1) at 2-month intervals.

Procedure. Shoots are washed and after that the floral buds are removed, surface-sterilized, and rinsed with distilled water. The buds are cut into 0.5-mm cubes and these are cultured on the first medium (Table Vf-1). PLBs produced on the first medium (Table Vf-1) are proliferated on the second solution (Table Vf-2) and moved to the third substrate (Table Vf-3) for 2 months. Plantlets and PLBs produced on the third medium (Table Vf-3) are moved to the MS solution (see Tables Anct-1, Ble-1, Cym-16, Cym-19, Den-27, Phal-9, Phal-11, Phal-15, Rhy-4, and Spa-1 are all suitable). They can be subcultured on this medium for 4 months at 2-month intervals. This protocol can produce more that 10,000 plantlets ready for community pots from one bud during a single year.

Developmental Sequence. PLBs are produced on the first medium (Table Vf-1). These PLBs proliferate on the second medium (Table Vf-2). Removal of EDTA from the medium accelerates the process. Plantlet production and growth take place on the third and MS media (Table Vf-3 and other tables as listed above).

General Comments. There are two major advantages to this procedure. One is the source of explants. Flower buds are usually plentiful and their removal does not endanger the donor plant. The second is its yield of 10,000 plants from a single bud per year. However, the high yield is also a possible disadvantage because excessive proliferation can cause undesirable mutations. Fortunately in this case the frequency of somaclonal variations seems to be small.

TABLE VF-1. **One-quarter concentration of Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) as used for the culture of *Vandofinetia* flower-bud explants (Kishi et al., 1997*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	412.5	41.25 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	110.0	11.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	92.5	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	475.0	47.5 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	42.5	4.25 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin[f]				
	Cytokinin[f]				
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise. Only the concentrations of macroelements are one-quarter those of the original formulation. The concentrations of iron and microelements are so small that reducing their levels to one-quarter of the original formulation would not affect the osmolarity of the solution. It is also possible that if reduced to a quarter of the original formulation the levels of these elements would become suboptimal.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. A commercial FeEDTA preparation (obtained from Wako Pure Chemical Inc., Osaka, Japan) was used in the original research at 10.525 mg l^{-1}.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]There is no mention in of hormones in the original paper. That is why there are no entries under the auxin and cytokinin headings.

[g]Add items 1–9 and 10–12 to 900 ml of distilled water (item 14), adjust to 5.8, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14), pour the solution into culture vessels, and autoclave. Heat-labile substances like amino acid (item 8), hormones, and vitamins (items 10–12) are sometimes added to the warm and still liquid solution after autoclaving under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. In this case all components are added to the medium before sterilization. Agar is not added to liquid media.

TABLE VF-2. **One-quarter concentration of Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of protocorm-like bodies derived from flower-bud explants of *Vandofinetia* (Kishi et al., 1997*b*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	412.5	41.25 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	110.0	11.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	92.5	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	475.0	47.5 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	42.5	4.25 g l^{-1}	10	
	Iron[c]				
6	Iron sulfate, $FeSO_4 \cdot 7H_2O$	6.95	0.695	10	
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin[f]				
	Cytokinin[f]				
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise. Only the concentrations of macroelements are one-quarter those of the original formulation. The concentrations of iron and microelements are so small that reducing their levels to one-quarter of the original formulation would not affect the osmolarity of the solution. It is also possible that if reduced to a quarter of the original formulation the levels of these elements would become suboptimal.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]The stock solution will form a brown precipitate on standing and must be shaken vigorously before dispensing an aliquot into the culture medium.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]There is no mention of hormones in the original paper. That is there are no entries under the auxin and cytokinin headings.

[g]Add items 1–9 and 10–12 to 900 ml of distilled water (item 14), adjust to 5.8, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14), pour the solution into culture vessels and autoclave. Heat-labile substances like amino acid (item 8), hormones, and vitamins (items 10–12) are sometimes added to the warm and still liquid solution after autoclaving under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. In this case all components are added to the medium before sterilization. Agar is not added to liquid media.

TABLE VF-3. **Hyponex medium for plantlet production from and culture of protocorm-like bodies derived from flower-bud explants of *Vandofinetia* (Kishi et al., 1997*b*)**

Component	Amount l^{-1}	Comments
Hyponex	2 g	Add components 1 and 2 to 900 ml of water, adjust pH to 5.8, add component 3, raise volume to 1000 ml, bring to gentle boil, add agar (component 4) slowly with stirring. When agar is dissolved pour the medium into culture vessels and autoclave. Use proteose peptone which is intended for tissue culture or microbiological use. Possible sources are www.sigmaaldrich.co, www.caissonlabs.com, www.phytotechlab.com, and others.
Proteose peptone	2 g	
Sucrose	20 g	
Agar	8 g	

Vanilla

The only orchid not grown for its ornamental value, *Vanilla* is the source of the spice vanilla (Lawler, 1984). At one time it was grown in Tahiti, Madagascar, Puerto Rico, Fiji, the Seychelles, and a number of other areas. Currently the major producer of vanilla is Madagascar. *Vanilla* is attacked by a systemic fungus that is transmitted in the cuttings used to propagate the plant. As a result new plantings are diseased from the outset. Clonal propagation through the culture of uninfected explants could be a means of producing healthy plants.

There are approximately 107 *Vanilla* species. They are found in tropical America, South East Asia, New Guinea, Africa, and Madagascar. There are no *Vanilla* species in Australia. The most important species for vanilla production is *Vanilla planifolia*. Two other species that serve as sources of vanilla are *V. pompona* and *V. tahitensis*. Additional *Vanilla* species also produce aromatic fruits, but few if any of them are cultivated to any extent.

In Vitro Propagation of *Vanilla planifolia*

To reduce dependence on cuttings, in vitro propagation of *Vanilla planifolia* was studied at Purdue University (Kononowicz and Janick, 1984).

Plant Material. Nodal stem sections are cultured.

Surface Sterilization. Explants are surface-sterilized by immersing them in 10% Clorox (10 ml Clorox diluted to 100 ml with distilled water) for 20 min. Although not mentioned by Kononowicz and Janick (1984), several rinses with sterile distilled water are advisable following the sterilization.

Culture Vessels. Petri dishes and baby-food jars were used in the original research. Culture tubes, Erlenmeyer flasks, and other containers can also be used.

Culture Conditions. Cultures should be maintained "under low intensity illumination (25–100 μmol s^{-1} m^{-1})" from cool white fluorescent lamps for 16 h daily at 26°C.

Culture Medium. A modified MS medium (Table Vla-1) is used.

Procedure. Place the explants in the culture medium, and allow them to branch and produce plantlets.

Developmental Sequence. Shoot proliferation occurs first, and roots form later. Rooted plants can be transferred to potting medium.

TABLE VLA-1. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for the culture of *Vanilla* explants (Kononowicz and Janick, 1984)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2 EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Cytokinin				
10	Benzylaminopurine (BAP)	0.5	100 mg 100 ml^{-1} 95% ethanol[f,g]	0.5	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
14	Casein hydrolysate	1 g	No stock	No stock	Weigh
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			
	Solidifier				
17	Agar, Difco Bacto[h]	8 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]If cytokinin does not dissolve, add a few drops of dilute HCl.

[g]Keep refrigerated between uses.

[h]Add items 1–7, 9, and 14 to 900 ml distilled water (item 16); adjust pH to 5.7, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Bring solution to a gentle boil, and add agar (item 17) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid, hormone, and vitamins (items 8 and 10–13) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

General Comments. This procedure can be useful for the production of disease-free plantlets. "Callus induction was attempted, . . . but explants did not develop" any calli.

Production of *Vanilla planifolia* Plantlets in Vitro

In an effort to find a long-range solution to the disease problem, Z. Gu, L. P. Nyman, and this author (JA) developed a procedure for callus formation and multiple plantlet production (Gu et al., 1987*a*).

Plant Material. Lateral buds are cultured, and the callus produced by them is used for propagation. Stem sections, 5–8 cm long and each containing one bud, are cut, sterilized, and trimmed to cubes measuring $5 \times 5 \times 1$ mm.

Surface Sterilization. Stem sections are first scrubbed with a very soft toothbrush under running water and then sprayed with 70% aqueous ethanol. After being sectioned the stems are immersed in 50% Clorox (50 ml Clorox diluted to 100 ml with distilled water) for 20 min and rinsed three times with distilled water.

Culture Vessels. Erlenmeyer flasks of 125- and 250-ml capacity were used in the original research. Other containers can also be utilized.

Culture Conditions. The cultures should be maintained under 16-h photoperiods of 0.8 mW cm^{-2} provided by Sylvania Gro Lux fluorescent tubes at $22 \pm 3°C$.

Culture Media. A modified Linsmaier–Skoog medium (Table Vla-2) is used for callus induction and maintenance. The callus is moved to another modification (Table Vla-3) for shoot and root production.

Procedure. Place explants on the first medium (Table Vla-2); when callus forms, subculture it on the same solution. When enough callus is available, move sections to the second medium (Table Vla-3) for plantlet formation. Fully developed plantlets can be moved to a potting mix.

Developmental Sequence. Bud explants can produce single (Fig. Vla-1A) or multiple (Fig. Vla-1B, C) shoots that develop roots (Fig. Vla-1D) or callus (Fig. Vla-1E). Shoots (Fig. Vla-1F) form on the callus and can develop into plantlets (Fig. Vla-1H). Callus can also form on leaf bases (Fig. Vla-1G). The callus forms between weeks 2 and 4, but in the original research only 14.6% of the explants produced calli. On the second medium (Table Vla-3), 53% of the callus section produced shoots and 44% formed roots.

General Comments. This procedure can be useful for the production of fungus-free plantlets.

TABLE VLA-2. **Modified Linsmaier–Skoog medium (Linsmaier and Skoog, 1965) for callus induction from *Vanilla* explants (Gu et al., 1987*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2 EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100	No stock	No stock	Weigh
	Amino acid				
9	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Cytokinins				
10	Kinetin	0.5	100 mg 100 ml^{-1} 95% ethanol[f,g]	0.5	
11	Benzylaminopurine (BAP)	0.5	100 mg 100 ml^{-1} 95% ethanol[f,g]	0.5	
	Vitamins				
12	Niacin	0.5	200 mg 100 ml^{-1} 95% ethanol[g]	0.25	
13	Pyridoxine	0.5	200 mg 100 ml^{-1} 95% ethanol[g]	0.25	
14	Thiamine (vitamin B_1)	0.4	160 mg 100 ml^{-1} 95% ethanol[g]	0.25	
	Complex additive				
15	Casein hydrolysate	1 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, stir and/or heat until dissolved. Dispense volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If cytokinins do not dissolve, add a few drops of dilute HCI. The original recipe for this medium gives the concentration of IAA as being "2.0 (1–30) mg/l," and the level of kinetin as "0.03–0.2 (0.001–10) mg/l" and adds in a footnote that "IAA and kinetin levels vary with the type of growth desired." In this case the intent is to induce callus formation, but the medium contains only cytokinins and no auxin(s).

[g]Keep refrigerated between uses.

[h]Add items 1–8 and 15 to 900 ml distilled water (item 17), adjust pH to 5.7, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l Erlenmeyer flask and autoclave. Add amino acid (item 9) and cytokinins (items 10 and 11) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

TABLE VLA-3. **Modified Linsmaier–Skoog medium (Linsmaier and Skoog, 1965) for plantlet production from *Vanilla* callus (Gu et al., 1987*a*)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Polyol				
8	*myo*-Inositol	100	No stock	No stock	Weigh
	Amino acid				
9	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.2	80 mg 100 ml^{-1} 95% ethanol[f,g]	0.25	
	Cytokinin				
11	Benzylaminopurine (BAP)	0.1	40 mg 100 ml^{-1} 95% ethanol[f,g]	0.25	
	Vitamins				
12	Niacin	0.5	200 mg 100 ml^{-1} 95% ethanol[g]	0.25	
13	Pyridoxine	0.5	200 mg 100 ml^{-1} 95% ethanol[g]	0.25	
14	Thiamine (vitamin B_1)	0.4	160 mg 100 ml^{-1} 95% ethanol[g]	0.25	
	Complex additive				
15	Casein hydrolysate	1 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[h]	To 1000 ml			
	Solidifier				
18	Agar, Difco Bacto[h]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, stir and/or heat until dissolved. Dispense volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin do not dissolve, add a few drops of dilute KOH or HCl, respectively. The original recipe for this medium gives the concentration of IAA as being "2.0 (1–30) mg/l," and the level of kinetin as "0.03–0.2 (0.001–10) mg/l" and adds in a footnote that "IAA and kinetin levels vary with the type of growth desired." The medium described in this table includes the auxin NAA in place of IAA, and the cytokinin BAP instead of kinetin.

[g]Keep refrigerated between uses.

[h]Add items 1–8 and 15 to 900 ml distilled water (item 17), adjust pH to 5.7, add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid, auxin, and cytokinin (items 9–11) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels.

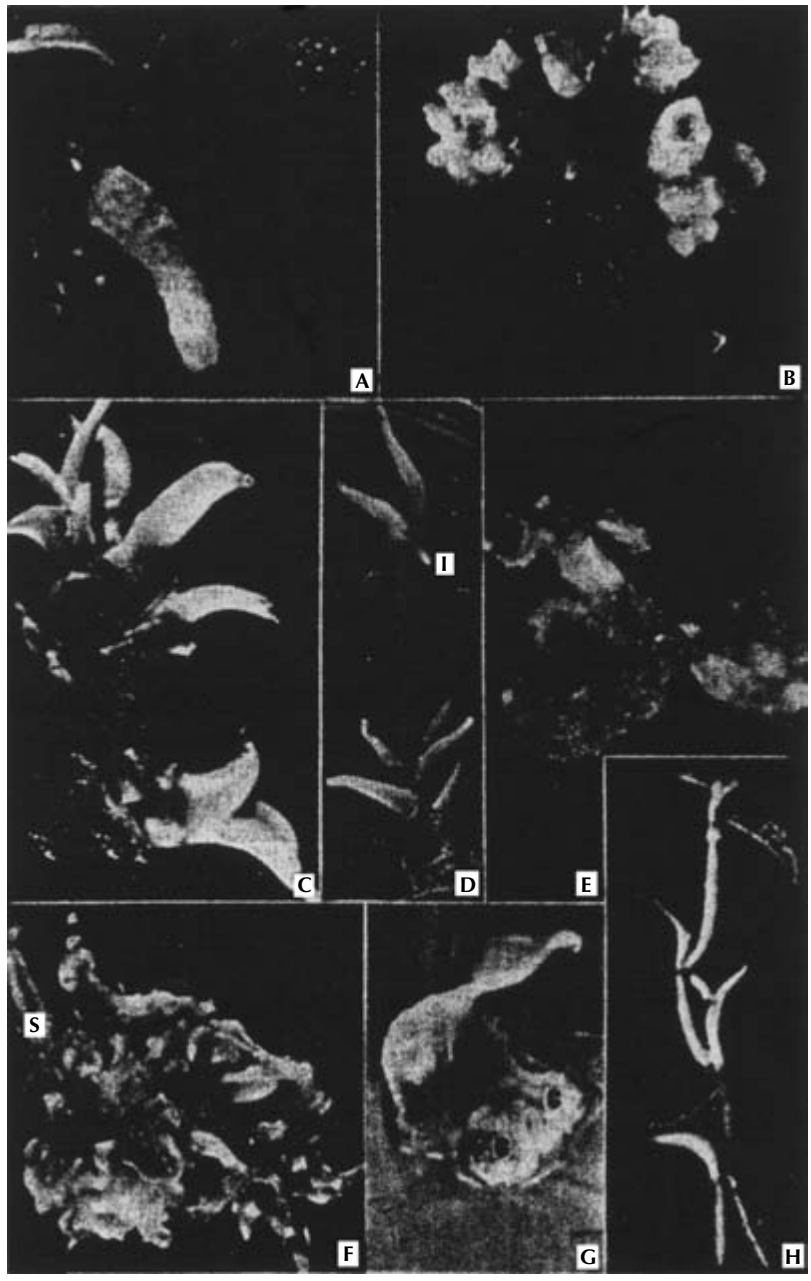


FIG. VLA-1. **Tissue culture and plantlet differentiation of *Vanilla*. A. Shoot produced from a bud explant within 2 weeks of culture on modified Linsemaier–Skoog medium containing 1 mg BA l^{-1} (× 2.9). B. Multiple shoots (s) forming on a bud explant cultured on modified Linsemaier–Skoog medium (× 2.9). C. Multiple shoots (s) after 5 weeks of culture on modified Linsemaier–Skoog medium (Table Vla-2; × 2.9). D. Elongated shoot with emerging root (r) after 5 weeks of culture (× 1.8). E. Friable yellow callus with numerous surface hairs on modified Linsemaier–Skoog medium (Table Vla-2) after 3 weeks of culture (× 2.9). F. Shoots (s) forming after 4 weeks (× 2.6). G. Small callus masses (c) at the base of a young leaf on modified Linsemaier–Skoog medium (× 2.9). H. Plantlets growing in the greenhouse after 6 weeks in a potting mix (× 0.6). (Source: Professor Z.–P. Gu, Department of Biology, Lanzhou University, Lanzhou, People's Republic of China.)**

Clonal Propagation of *Vanilla planifolia* from Aerial Root Rips

As already mentioned,

> a major threat to *Vanilla* cultivation is a root rot disease (*Fusarium batatatis* var *vanillae* Tucker) which often has a devastating effect and completely destroys the plantation. . . . Techniques of rapid and large scale multiplication of disease-free plants would be of considerable value. (Philip and Nainar, 1986)

Plant Material. (1) Stem discs that are 2–3 cm long, have a node in the middle, and are excised from near apices, and (2) aerial root tips that are 2 cm long, are cultured.

Surface Sterilization. Explants are surface-sterilized by immersing them in 0.5% mercuric chloride, $HgCl_2$ (500 mg per 100 ml distilled water) for 10 min and then rinsed three times with sterile distilled water. $HgCl_2$ is a very toxic, dangerous substance and must be used with great caution.

Culture Vessels. Culture tubes and Erlenmeyer flasks can be used.

Culture Conditions. Cultures should be maintained under continuous illumination of 7000–8000 lx at 24 ± 2°C. Stem sections and root tips are first cultured on filter-paper bridges placed in liquid medium (Fig. Vla-2).

Culture Media. Stem sections produce PLBs when cultured in liquid modified Knudson C medium (Table Vla-4). Root tips are cultured on modified MS medium (Table Vla-5).

Procedure. Place stem explants on paper bridges in the first medium (Table Vla-4), and allow them to form PLBs, which eventually produce plantlets. These can be cultured on unmodified Knudson C medium (see Table Aranda-7) with and without banana homogenate. Allow root tips to remain on the paper bridges in the second medium (Table Vla-5) for 3 months, and then move them to a solid version of the same solution.

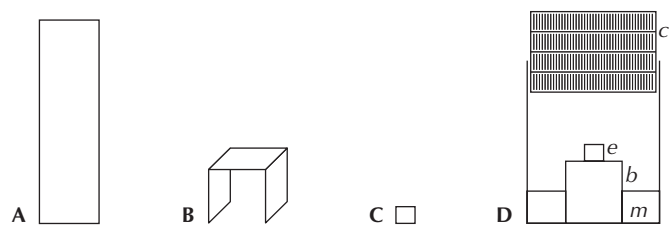


FIG. VLA-2. **Preparation of a filter-paper bridge for the culture of *Vanilla* and/or other explants. A. A rectangular strip cut from a filter-paper disk; dimensions would depend on the size of the culture tube. B. The filter-paper strip folded to form a bridge. C. Explant. D. Culture tube with cover (c), filter-paper bridge (b) in culture medium (m) with explant (e).**

TABLE VLA-4. **Modified Knudson C medium (Knudson, 1946) for the culture of *Vanilla* shoot explants (Philip and Nainar, 1986)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium sulfate, $(NH_4)SO_4$[b]	500	50 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	1 g	100 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250	25 g l^{-1}	10	
4	Monopotassium phosphate, KH_2PO_4	250	25 g l^{-1}	10	
	Iron[c]				
5	Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	25	2.5 g l^{-1}	10	
	Microelement				
6	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	7.5	750 mg l^{-1}	10	
	Auxin				
7	Naphthaleneacetic acid (NAA)	1	100 mg 100 ml^{-1} 95% ethanol[d]	1	
	Cytokinin				
8	Kinetin	0.1	40 mg 100 ml^{-1} 95% ethanol[d]	0.25	
	Sugar				
9	Sucrose	20 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[e]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]It is preferable to chelate the iron. To prepare a stock solution dissolve 3.73 g chelating agent (Na_2EDTA) and 2.78 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 1 l of water. Stir and/or heat until both are completely dissolved. Add 10 ml of this solution to culture medium.

[d]Keep refrigerated between uses. If the auxin or cytokinin do not dissolve, add a few drops of KOH or HCl, respectively.

[e]Add items 1–6 to 900 ml distilled water (item 10), adjust pH to 5.0–5.5, add sugar (item 9), and adjust volume to 1000 ml with distilled water (item 10). Pour solution into a 2-l flask and autoclave. Add auxin (item 7) and cytokinin (item 8) to autoclaved medium under sterile conditions with sterilized pipettes, swirl well several times to insure complete mixing, and dispense into culture vessels. According to Knudson (1946), the pH of this solution made without agar (i.e., liquid) can be close to 4.6–4.7. If necessary adjust it upward with a potassium hydroxide (KOH) solution (see Chapter 2). When agar is added, especially if dissolved in the autoclave, the pH can be 5.4–5.5. Use a dilute solution of hydrochloric acid (HCl) to lower the pH if necessary.

Developmental Sequence. Each stem explant can produce 10–15 PLBs that are round, glistening, and have rhizoids. Every protocorm develops at its tip a bud that produces leaves and eventually a plantlet. The root tips swell within 2 months and after 3 months crack to expose spherical bulges. On the solid medium, multiple meristems form, which give rise to shoots and roots. Well-developed plantlets form within 9 months.

General Comments. This seems to be a useful and effective method.

Medicinal uses of vanilla include: aromatic stimulant in cases of asthenic fevers, rheumatism, and hysteria; digestion and stomach problems; general stimulant; antidote to poison and the bite of venomous animals; emmenagogue and irregular menstruation; acceleration of childbirth and abortion of dead fetuses; dissipation of flaturs; and a cure for fever (Lawler, 1984).

TABLE VLA-5. **Modified Murashige–Skoog medium (Murashige and Skoog, 1962) for the culture of *Vanilla* root tips (Philip and Nainar, 1986)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}		
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	2,4-Dichlorophenoxyacetic acid (2,4-D)	1	100 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
11	Kinetin	0.1	40 mg 100 ml^{-1} 95% ethanol[f,g]	0.25	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Sugar				
15	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[h]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin do not dissolve, add a few drops of KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]Add items 1–7 and 9 to 900 ml distilled water (item 16), adjust pH to 5.0–5.5 as required, add sugar (item 15), and adjust volume to 1000 ml with distilled water (item 16). Pour solution into a 2-l flask and autoclave. Add amino acid, hormones, and vitamins (items 8 and 10–14) to autoclaved solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels.

Propagation in Vitro of *Vanilla planifolia*

Vanilla plants were propagated from axillary buds on modified MS medium (Cervera and Madrigal, 1980) by a procedure that was not described in sufficient detail to justify its inclusion here.

Isolation of *Vanilla* Protoplasts

The method developed for *Acampe praemorsa* was used to isolate 16.5×10^4 and 3.9×10^4 protoplasts from leaves and roots, respectively, of *Vanilla wightiana* (Seeni and Abraham, 1986).

In Vitro Multiplication of *Vanilla planifolia* using Axillary Bud Explants

The constantly increasing demand for vanilla flavoring has created a great demand for plants. This led to the development of a micropropagation method through the culture of axillary buds (George and Ravishankar, 1997).

Plant Material. Stem-node sections should be excised from field-grown plants. In the original research the nodes were taken from 1-year-old plants. If plants of this age are not available, nodes should be taken from young growths on older vines. Every section should be 1.5–2.0 cm long with an axillary bud in the middle (Fig. Vla-3C). Leaves should be removed by cutting the petiole flush with the stem.

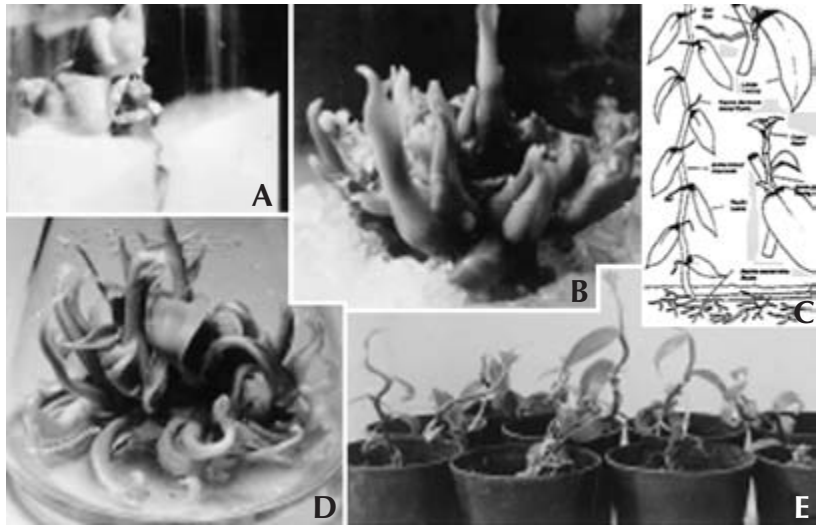


FIG. VLA-3. **Culture of *Vanilla planifolia* axillary buds. A. Explant on medium. B. Multiple shoots. C. Vine (left) and leaf explants prior to removal of leaves (right). D. Multiple shoots following proliferation. E. Potted plantlets being hardened. (George and Ravishankar, 1997.)**

Surface Sterilization. Sections should be first washed with running water and a mild liquid detergent (Tween 20 was used in the original research; it is available from www.sigmaaldrich.com, www.fishersci.com, www.vwr.com, www.duchefa.com, and other sources; a mild household liquid detergent can also be used) for 10 min. After that the explants should be immersed in 0.15% (w/v) mercuric chloride (this is a toxic substance which must be handled with care) for 5 min and then washed three times with sterile distilled water (Table Vla-6).

TABLE VLA-6. **Surface sterilization of *Vanilla* explants**

Step	Agents	Duration
1	Running water and Tween 20 or a mild household detergent	10 min
2	Running water to remove detergent	1 min
3	Mercuric chloride, 0.15% (w/v)[a]	5 min
4	Sterile distilled water rinse	3–5 min
5	Sterile distilled water rinse	1–3 min
6	Sterile distilled water rinse	1–3 min

[a]Caution, this is a very toxic substance.

Culture Vessels. Erlenmeyer flasks, culture tubes, or other containers can be used depending on the available shaker.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under continuous illumination of 37.5 μE m^{-2} s^{-1} (Philips fluorescent lamps were used in the original research). Standard culture room conditions are also suitable. Liquid cultures should be placed on a rotary shaker at 90 rpm.

Culture Media. For multiple shoot initiation, the explants should be cultured on modified MS medium (Murashige and Skoog, 1962) containing 2 mg BA l^{-1}, 1 mg NAA l^{-1}, and 10 g agar l^{-1} (Table Vla-7). Liquid MS containing 1 mg BA l^{-1} and 0.5 mg NAA l^{-1} (Table Vla-8) is used for shoot proliferation. After proliferation, n shoots should be cultured on semisolid MS (Table Vla-9). Well-developed shoots should be moved to a half concentration of MS darkened with charcoal (Table Vla-10).

Procedure. Discolored edges should be removed from explants after the final wash, leaving at least 0.5 mm of tissue on each side of the bud. The bases of explants must be inserted in the first medium (Fig. Vla-3A, Table Vla-7). Explants (Fig. Vla-3C) should be cultured on this medium for 21–28 days until multiple shoots are formed (Fig. Vla-3B). The shoots should be moved to the liquid medium (Fig. Vla-3D, Table Vla-8) and maintained on a shaker for a month or until they have proliferated well. After that, they should be transferred to MS (Table Vla-9) for 30 days or until the shoots are well developed. These shoots are moved to darkened medium (Table Vla-10) for rooting. Rooted plantlets should be potted (Fig. Vla-3E), hardened in a greenhouse for 30 days, and moved to the field.

Developmental Sequence. Explants produce multiple shoots on the first medium (Table Vla-7). These shoots proliferate on the second solution (Table Vla-8). They grow and produce leaves on the third substrate (Table Vla-9). Roots appear on the third medium (Table Vla-10), and plantlets harden in pots.

TABLE VLA-7. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of axillary-bud explants of *Vanilla planifolia* (George and Ravishankar, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	N^6-benzyladenine (BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH 5.7, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) are heat-labile and are often added to the warm and still liquid medium after autoclaving under sterile conditions with sterilized pipettes. In this case all components are added before sterilization.

TABLE VLA-8. **Liquid Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the proliferation of shoots produced by axillary-bud explants of *Vanilla planifolia* (George and Ravishankar, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	N^6-benzyladenine (BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH 5.7, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Pour the solution into culture vessels and autoclave. Amino acid (item 8), hormones (items 10 and 11) and vitamins (items 12–14) are heat-labile and are often added after autoclaving under sterile conditions with sterilized pipettes. In this case all components are added before sterilization.

TABLE VLA-9. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the culture of proliferated shoots derived from axillary-bud explants of *Vanilla planifolia* (George and Ravishankar, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[f,g]	To 1000 ml			
	Solidifier				
15	Agar[f,g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]The original paper does not indicate whether hormones were added. Therefore none are included in this formulation.

[g]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH 5.7, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, pour the solution into culture vessels and autoclave. Amino acid (item 8), hormones, and vitamins (items 10–12) are heat-labile and are often added to the warm and still liquid medium after autoclaving under sterile conditions with sterilized pipettes. In this case all components are added before sterilization. The original states that semisolid medium was used. In a way this is misleading because 10 g of agar is the usual amount of solidifier for MS.

TABLE VLA-10. **Half-strength Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for the rooting of shoots derived from axillary-bud explants of *Vanilla planifolia* (George and Ravishankar, 1997)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	825.0	82.5 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	220.0	22.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	85.0	8.5 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	3.1	31.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.013	1.3 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.013	1.3 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	11.2	1.12 mg l^{-1}		
(e)	Potassium iodide, KI	0.42	42.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.13	13.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	4.3	430.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	10.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[f]	To 1000 ml			
	Solidifier				
15	Agar[g]	10.0 g	No stock	No stock	Weigh
	Darkening agent				
16	Activated charcoal[h]	2.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]The original paper does not indicate whether hormones were added. Therefore none are included in this formulation.

[g]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH to 5.7, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, add the darkening agent (item 16) slowly with vigorous stirring. When the charcoal (item 16) is completely dispersed pour the solution into culture vessels and autoclave. Amino acid (item 8), hormones, and vitamins (items 10–12) and heat-labile and are often added after autoclaving to the still hot and liquid solution under sterile conditions with sterilized pipettes, mixed well, and the medium is distributed to preautoclaved culture vessels. In this case all components are added before sterilization.

[h]Only activated pure vegetable charcoal should be used. One possible source is www.sigmaaldrich.com. There are undoubtedly other sources also. Charcoal functions not only by darkening the medium, but also by absorbing toxic substances and perhaps by adding aeration. Therefore darkening agents such as lamp black and graphite, which are very different in nature, should be used only if required by a specific procedure or under special circumstances. It is always advisable to check the pH of the medium after the addition of charcoal. To keep the electrode free of dark and sticky agar and avoid having to calibrate some pH meters for a hot solution and since small changes in pH (approximately 0.1 of a unit) make no difference, pH paper strips can be used for this measurement. Such paper strips are available from all biological supply houses. One possible source is www.vwr.com, but there are many others.

General Comments. This protocol was described as "an efficient micropropagation system." The description may be apt, but this method may have a major weakness: If explants are taken from diseased plants, plantlets produced through this method may also carry pathogens. Also, the original paper should have been organized better.

Cell Suspension Cultures of *Vanilla*

Cell suspension cultures of *Vanilla* are used for studies of biosynthesis and metabolism of vanillin, phenolics, and other substances (for examples see Romagnoli and Knorr, 1988; Havkin-Frenkel et al., 1996; Yuanna and Verponte, 2002; for a review see Dörnenburg and Knorr, 1996). The culture method used by several investigators was developed in Switzerland (Funk and Brodelius, 1990).

Plant Material. Cell suspension cultures of *Vanilla planifolia* were provided by Danisco Biotechnology in Denmark (www.danisco.com; an inquiry to the company elicited the following reply: "We have only some confidential internal reports which we cannot reveal."). The cell suspension cultures were initiated from a callus produced in 1986 by an explant taken from a fresh fruit.

Surface Sterilization. Cell suspension cultures do not require surface sterilization.

Culture Vessels. Erlenmeyer flasks, 500-ml capacity, containing 100 ml of medium, should be used.

Culture Conditions. Cultures should be maintained on a gyratory shaker at 120 rpm in the dark at 26°C. An attempt to culture the cells on a shaker under standard laboratory conditions in the light could be worthwhile but results may not be useful.

Culture Media. Liquid MS medium (Murashige and Skoog, 1962) containing 1 mg 2,4-D l^{-1} (Table Vla-11) was used in the original research. It may be interesting to determine whether the cells will form callus masses, PLBs, or plantlets if cultured on the same medium in solid form or one of the other media used for micropropagation of *Vanilla* in the dark or under illumination.

Procedure. Cultures should be centrifuged to collect cells. Every 10 days, 7 g fresh weight (FW) of cells should be transferred to fresh medium. Introducing 10–20-ml suspension culture aliquots into fresh medium as a means of subculturing the cells may be worth trying.

Developmental Sequence. Cultures reach a growth curve plateau and a dry weight of 15 g l^{-1} 8–9 days after being started (Fig. Vla-4). During the very active growth phase (days 4–8) the doubling time is approximately 36 h.

General Comments. This may well be the only long-term orchid cell suspension culture. It is a useful achievement. The cells can be used for metabolic and biochemical research. Unfortunately no plants were produced from these cultures.

TABLE VLA-11. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) for *Vanilla* cell suspension cultures (Funk and Brodelius, 1990)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	2,4-Dichlorophenoxyacetic acid (2,4-D)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.5 (this is a guess, the original paper does not list a pH), add sugar (item 14), raise volume to 1000 ml with distilled water (item 15). Pour the solution into culture vessels and autoclave. Agar is not added to liquid media. Amino acid (item 8), hormones (item 10), and vitamins (items 11–13) are heat-labile and are often added after autoclaving to the still hot and liquid solution under sterile conditions with sterilized pipettes, mixed well, and the medium is distributed to preautoclaved culture vessels. In this case all components are added before sterilization.

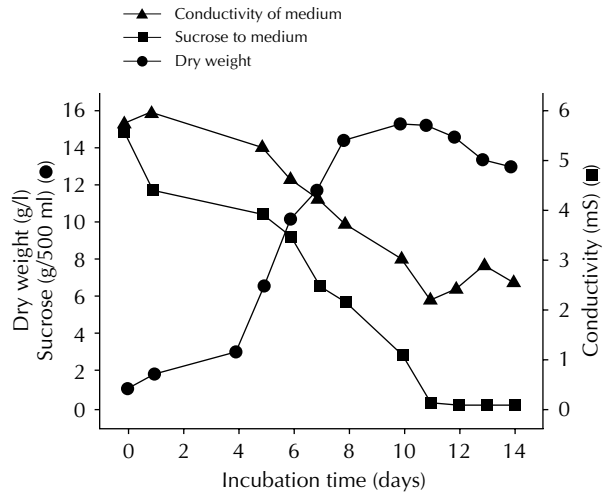


FIG. VLA-4. **Growth of *Vanilla planifolia* cells in in suspension cultures (Funk and Brodelius, 1990).**

Plantlet Production from *Vanilla* Root Tips

"In an attempt to evolve an effective method of clonal propagation of the commercial orchid, *Vanilla planifolia*, without sacrificing the monopodial mother plant" a method was developed involving "the lysis of the root cap of the explant [which] sets in train a series of histological events which leads to plantlet formation" (Philip and Nainar, 1988).

Plant Material. Aerial root tips, 2 cm long, should be cultured.

Surface Sterilization. Root tips should be washed with running water, immersed in 0.15% mercuric chloride ($HgCl_2$; this is a toxic substance which must be handled with care) for 10 min, and after that rinsed three times with sterile distilled water.

Culture Vessels. In the original research the explants were cultured on filter-paper bridges in tubes (see Fig. Vla-2). Culture tubes were probably used because it is somewhat easier to build filter-paper bridges in narrow containers. Erlenmeyer flasks, 100-ml capacity, were used for cultures maintained on solid medium.

Culture Conditions. The research cultures were maintained at 24 ± 2°C under continuous illumination of 7000–8000 lx. Similar conditions should also prove to be suitable.

Culture Media. The original paper is not very clear on this subject. It starts by stating that the explants should be cultured "on filter paper bridges [see Fig. Vla-2] in tubes containing liquid MS (Murashige and Skoog, 1962) medium supplemented with

IAA (0–10 mg l^{-1}) and 0.2 mg l^{-1} KN" (Table Vla-12). It also suggests that "2 cm long root tips excised from young aerial roots and cultured on liquid MS medium supplemented with 1–5 mg l^{-1} of IAA and 0.2 mg of KN grew in length for about 10 d[ays] . . . [but] . . . Growth in length of the young tips, however, ceases after 1 d[ay] . . ." and eventually meristemoids are produced leading to leaf whorls after 9 weeks. Roots are "initiated only after four to five leaves had formed" presumably on the filter paper bridges and the liquid medium levels (given the range suggested for IAA the concentration in Table Vla-12 is a supposition). Caution is needed with the plantlets because it is possible that the filter-paper bridges will collapse under their weight. Therefore, once leaves are formed the shoots may have to be transferred to solid medium (Table Vla-12). But this is not all. The original paper also states that "after three months . . . when the root tips had swelled considerably, the tips (approx. 7 mm length) were excised under sterile conditions and planted on . . ." solidified medium (Table Vla-12). And, figure 2D of the original paper (Philip and Nainar, 1988) shows "formation of a plantlet . . . three weeks after transfer to solidified MS medium." This suggests that the tips should be cultured on filter-paper bridges and liquid MS (Table Vla-12) until they become large enough to cause sagging and should then be transferred to solid substrate (Table Vla-12). Rooted plants can be cultured on one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate (BH) or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal (AC) should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. After sterilization the shoot tips should be placed on filter-paper bridges (see Fig. Vla-2), which are inserted in liquid MS medium (Table Vla-12). There are several possibilities after that. The tips can be allowed to remain in the original culture tubes for differing amounts of time:

- For 3 months until they swell. Then their tips (7 mm) can be cut and moved to solid medium (Table Vla-12).
- Until the tips and/or plantlets they produce cause the filter-paper bridges to sag. Then the tips and/or plants should be transferred to solid medium (Table Vla-12).
- For as long as 3 months or until they swell. Then their tips (7 mm) can be cut, moved to new filter-paper bridges in fresh medium (Table Vla-12), and cultured on it until the tips and/or plantlets they produce cause the filter-paper bridges to sag. Then the tips and/or plants should be transferred to solid medium (Table Vla-12).

In all cases plantlets can be cultured to potting size on one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, and V-4) with or without BH or AC. If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and

TABLE VLA-12. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Vanilla* root tips (Philip and Nainar, 1988)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indoleacetic acid (IAA)	2.5	250 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Kinetin	0.2	20 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH to 5.8, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved pour the solution into culture vessels. Do not add agar to liquid medium. Amino acid (item 8), hormones (items 10 and 11), and vitamins (items 12–14) are heat-labile and are often added after autoclaving to the still hot and liquid solution under sterile conditions with sterilized pipettes, mixed well, and the medium is distributed to preautoclaved culture vessels. In this case all components are added before sterilization.

Cym-24). AC should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

Developmental Sequence. The root tips swell considerably after 3 months in the initial culture tubes (see Fig. Vla-2, Table Vla-12). After 9 weeks of culture on filter-paper bridges and liquid medium (and perhaps also on solid medium, Table Vla-12) some explants will form shoot apices surrounded by whorls of leaves. Roots appear on these shoots only after 4–5 leaves are formed.

Root tips on liquid medium grow in length for 10 days before growth ceases. Lysis of the root cap follows. Cell division in the quiescent center becomes more frequent and after 4 weeks the number of cells increases to the point of causing the originally pointed apex to assume "a hemispherical outline." Subsequent cell divisions produce a meristemoid. These meristemoids enlarge and form shoot meristems, which give rise to leaves.

General Comments. This protocol could prove to be efficient and productive if it can be made to work. Its main advantage is the use of root tips, which are in plentiful supply. Care should be exercised in taking explants to insure that they do not contain mycorrhizal or pathogenic fungi. The research paper that describes this procedure is focused on development, not on micropropagation. Therefore it was difficult to extract enough details for a practical procedure.

Callus Formation and Shoot Regeneration in *Vanilla planifolia*

> The high demand for natural vanilla flavor led to attempts to establish [a] culture of *Vanilla planifolia* for flavor production and for shoot regeneration from callus. (Davidonis and Knorr, 1991)

Plant Material. Shoots "obtained from J. Janick (Purdue University)" were used in the original research. No other details were given. Shoots, about 2–5 cm in length, taken from young growths are probably appropriate.

Surface Sterilization. All leaves should be removed from the shoot tips after which they should be washed with a soft brush (used tooth brush) in tap water with a mild household detergent, and rinsed with distilled water after the wash (this step is not part of the original procedure). After that they should be treated with 30% hydrogen peroxide (H_2O_2; available in drug stores and pharmacies) and rinsed with sterile distilled water three times before immersing them in 20% "commercial bleach" (this presumably means 20 ml household bleach diluted to 100 ml with distilled water). The bleach should be followed by another rinse with sterile distilled water.

Culture Vessels. Culture tubes, Erlenmeyer flasks, and other containers filled with medium to 20–30% of their volume can be used.

Culture Conditions. Shoot tips should be pretreated for at least 45 days under 4.5 $\mu E\ m^{-2}\ s^{-1}$. The original paper does not list the temperature and day length during

this period, but 25 ± 2–3°C and 8–12-h photoperiods should prove to be suitable. Callus induction from shoot-tip sections and subsequent proliferation should be carried in the dark at 25°C. The culture of callus taken from the proliferation medium and conditions should be at 25 ± 2–3°C and 8–12-h photoperiods of 4.5 μE m^{-2} s^{-1}.

Culture Media. Shoot tips should be placed initially on salts of MS (SMS) medium (Murashige and Skoog, 1962) with 1 g agar l^{-1} (water-acetone agar was used in the original research but such high-purity agar is not necessary for practical applications; Table Vla-13). Sections taken from the shoot tips should be placed on another combination of SMS and supplements (Table Vla-14). Callus should be proliferated on a third medium (Table Vla-15). The callus is maintained on a fourth medium (Table Vla-16). Shoots form on a fifth combination (Table Vla-17). No medium is suggested for plantlet production or the culture of plantlets to maturation. Plantlets can be cultured on one of the versions of the Knudson C medium (Knudson, 1946) with (see Tables C-19, Cym-3, and Cym-24) or without (see Tables Aranda-7, Aranda-8, C-3, C-6, Cym-1, and Cym-2) banana homogenate (BH)or charcoal (see Table Pln-1) or Vacin and Went solution (Vacin and Went, 1949; see Tables Aranda-6, Arnth-5, Cym-5, Den-2, Den-12, Den-21, Onc-5, Rntda-1, and V-4). If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). Activated charcoal (AC) should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (see Table Pln-1, footnote *f*).

Procedure. After surface sterilization, shoot tips should be placed on the first medium (Table Vla-13) in the light for 45 days. After that the shoot tips should be sectioned into 1–2-mm disks. These disks should be cultured for 2 weeks on the second medium (Table Vla-14) in the dark for callus induction. Disks that form callus should be moved to fresh medium (Table Vla-14) for an additional 2 weeks in the dark. Callus that forms on these sections should be proliferated in the dark on the third medium (Table Vla-15). The callus is maintained on the fourth medium (Table Vla-16) in the light and must be transferred to fresh medium (Table Vla-16) every 2 weeks. Callus must be moved to yet another medium (Table Vla-17) for shoot production. For further growth and plantlet development, the shoots can be moved to one of the other media (see Tables Aranda-6 to Aranda-8, Arnth-5, C-3, C-6, C-19, Cym-1 to Cym-3, Cym-5, Cym-24, Den-2, Den-12, Den-21, Onc-5, Pln-1, Rntda-1, and V-4) with or without BH or AC. If BH is not listed in the recipe for a medium it can/should be added according to the instructions for media that contain it (see Tables C-19, Cym-3, and Cym-24). AC should be added to the medium selected for use as suggested for Knudson C that contains this darkening agent (Table Pln-1, footnote *f*).

Developmental Sequence. The pretreatment presumably prepares the shoot tips for sectioning and the sections for callus formation. Callus is initiated when shoot sections are cultured in the dark. The callus proliferates on the proliferation medium and may develop further on the maintenance medium. Shoots form on the medium with 0.1 mg BA l^{-1} and 0.2 mg NAA l^{-1}. The shoots and plantlets grow further on the last medium.

TABLE VLA-13. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for pretreatment of *Vanilla planifolia* shoot tips (Davidonis and Knorr, 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Cytokinin				
8	Benzyladenine (BA)	0.4	40 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[g]	To 1000 ml			
	Solidifier				
11	Agar[g]	1.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The original paper (Davidonis and Knorr, 1991) does not mention a sugar as part of this medium, but 20 g sucrose is suggested here. A sugar-free medium may be tested also.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–8 to 900 ml of distilled water (item 10), adjust pH to 5.8, add sugar (item 9), and raise volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the solution into culture vessels and autoclave. Agar is not added to liquid media.

According to one source, *Vanilla* was first described by Olof Swartz (1760–1818) in 1799 in volume 6, page 66, figure 5 of *Nova Acta Regiae Societatis Scientarum Upsaliensis* (Schultes and Pease, 1963). Another view is that the first description was by Philip Miller in the *Gardener's Dictionary*, 4th edition, 1754 (Bechtel et al., 1992; Pridgeon et al., 2003). The name is derived from the Spanish *vainilla* which means little pod and is in reference to the long and slender fruit, which is pod-like (Schultes and Pease, 1963) but is in fact a capsule and definitely not a bean.

TABLE VLA-14. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Vanilla planifolia* shoot-tip sections (Davidonis and Knorr, 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	1000.0	No stock	No stock	Weigh
	Auxin				
9	Naphthaleneacetic acid	4.0	400 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
10	Benzyladenine (BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamin				
11	Thiamine (vitamin B_1)	5.0	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
12	Casein hydrolysate	500.0	No stock	No stock	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	7.6 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.8, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

General Comments. This is a fairly complicated procedure which is presented in a paper that does not include all the necessary details, is not well organized, and is not written as well as it should be.

TABLE VLA-15. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the proliferation of *Vanilla planifolia* callus-derived shoot-tip sections (Davidonis and Knorr, 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	1000.0	No stock	No stock	Weigh
	Auxin				
9	Naphthaleneacetic acid	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
10	Benzyladenine (BA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamin				
11	Thiamine (vitamin B_1)	5.0	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
12	Casein hydrolysate[g]	500.0	No stock	No stock	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[h]	To 1000 ml			
	Solidifier				
15	Agar[h]	7.6 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Not all casein hydrolysates are the same. Their compositions and analyses vary depending on the methods by which they were produced. A product sold by Merck (www.merck.de) as casein hydrolysate (CH) is obtained by degrading casein with hydrochloric acid; vitamins and other growth-promoting compounds are largely destroyed in this process. The CH sold by Sigma-Aldrich (www.sigmaaldrich.com) is pancreatic hydrolysate of casein. There is also a CH which is hydrolysed by enzymes. Becton, Dickinson and Company (www.bd.com) sells Difco products, including a number of different casamino acid products all of which are casein hydrolysates. Therefore if a method specifies a source for the CH in its culture medium it is best to obtain the additive from the same vendor. If a source is not listed it is best to purchase CH which is described as being suitable as a supplement for culture media. Failing that, CH should be purchased from a source which specializes in tissue culture and micropropagation supplies. One such source is www.caissonlabs.com. They list casein rather than CH as Stock #: SPCA205. Another source for CH is www.duchefa.com (item 1301). Additional sources include www.fishersci.com, www.thomassci.com, and others.

[h]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH to 5.8, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the solution into culture vessels and autoclave. Agar is not added to liquid media.

TABLE VLA-16. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the maintenance of *Vanilla planifolia* shoot-tip-derived callus (Davidonis and Knorr, 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Auxin				
8	Naphthaleneacetic acid	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
9	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
10	Water, distilled[g]	To 1000 ml			
	Solidifier				
11	Agar[g]	7.6 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The original paper (Davidonis and Knorr, 1991) does not mention a sugar as part of this medium, but 20 g sucrose is suggested here. A sugar-free medium may be tested also.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Add items 1–8 to 900 ml of distilled water (item 10), adjust pH to 5.8, add sugar (item 9), and raise volume to 1000 ml with distilled water (item 10). Bring the solution to a gentle boil and add the agar (item 11) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

At one time or another *Vanilla* was or still is cultivated, or attempts were made to cultivate it, on the Comoro Islands, Dutch East Indies (now Indonesia), Fiji, Guadeloupe, Madagascar, Martinique, Mauritius, Mexico, Philippines, Puerto Rico, Reunion, Seychelles, Ceylon (now Sri Lanka), Tahiti, and Uganda.

Professor Charles Morren at the University of Liege in Belgium was the first to hand pollinate *Vanilla* in 1836. Edmond Albius, a slave on Reunion Island, may have learned Morren's method and could have adapted it to practical use. He may not have invented the method. Albius's reward was freedom.

TABLE VLA-17. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the shoot production from *Vanilla planifolia* shoot-tip-derived callus (Davidonis and Knorr, 1991)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Auxin				
8	Naphthaleneacetic acid	0.2	20 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	Benzyladenine (BA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	7.6 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The original paper (Davidonis and Knorr, 1991) does not provide many details about this medium. Because of that the recipe in this table is based on several assumptions.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–9 to 900 ml of distilled water (item 11), adjust pH to 5.8, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

In Vitro Shoot Multiplication of *Vanilla planifolia*

Increasing demand for vanilla and *Vanilla* plants led to the formulation of a number of micropropagation methods. One of them utilizes the auxin-like properties of phenylacetic acid (Giridhar et al., 2003).

Plant Material. In the original research, shoot tips and node explants, 2–3 cm long, were excised from 3-year-old vines. Similar explants or ones taken from young growths of more mature plants can be used in practical micropropagation.

Surface Sterilization. Explants should be washed with tap water and Tween 20 (www.sigmaaldrich.com, www.fishersci.com, www.vwr.com, www.wako-chem.co.jp/english/), baby shampoo, or a mild household detergent and then rinsed with tap water. After that they should be immersed in 0.15% (w/v) mercuric chloride ($HgCl_2$; available from the sources above; this toxic substance must be handled with care) for 10 min, and rinsed five times with sterile distilled water.

Culture Vessels. Erlenmeyer flasks, culture tubes, or other containers can be used.

Culture Conditions. Cultures should be maintained at $25 \pm 2°C$ under continuous illumination of 45 $\mu mol\ m^{-2}\ s^{-1}$. Standard culture room conditions may also be suitable.

Culture Media. Initial explants should be cultured on a modified MS medium (Table Vla-18; Murashige and Skoog, 1962). Shoots produced by them should be moved to a second modification of MS (Table Vla-19). Nodal segments from shoots on the second medium (Table Vla-19) should be moved to a third substrate (Table Vla-20). Shoots produced by the nodal segments should be cultured on an elongation medium (Table Vla-21) and then moved to a rooting substrate (Table Vla-22).

Procedure. Surface-sterilized shoot tips are cultured on the shoot initiation medium (Table Vla-18) for 60 days. The shoots they produce are moved to a second medium (Table Vla-19) for growth. Node segments taken from shoots on the second medium (Table Vla-19) are cultured on a third solution (Table Vla-20) for 4–5 weeks. Shoots produced on this solution (Table Vla-20) are moved to an elongation medium (Table Vla-21) for 30 days and cultured on a rooting substrate on reaching a height of 2.0–2.5 cm (Table Vla-22). Well-developed and rooted plantlets are potted.

Developmental Sequence. Shoots are produced on the first medium (Table Vla-18) and grow on the second solution (Table Vla-19). Nodal sections from shoots on the second solution are cultured on the third substrate (Table Vla-20). Shoots which form on this medium are moved to the fourth solution of elongation (Table Vla-21). Elongated shoots, 2.0–2.5 cm in height, are moved to the fifth medium (Table Vla-22) for rooting.

General Comments. This protocol utilizes five different media but seems to be effective. Parts of the original paper are not very clear. This meant that a number of assumptions had to be made in preparing the recipe tables (Tables Vla-18 to Vla-22).

TABLE VLA-18. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for shoot initiation on *Vanilla planifolia* shoot explants (Giridihar et al., 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Auxin				
8	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	Benzyladenine (BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
10	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	6.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–9 to 900 ml of distilled water (item 11), adjust pH to 5.7 ± 0.2, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to pre culture vessels and autoclave. Agar is not added to liquid media.

TABLE VLA-19. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Vanilla planifolia* shoots (Giridihar et al., 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Auxin				
8	Indole-3-butyric acid (IBA)	0.1	10 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	Benzyladenine (BA)	0.01	1 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Sugar				
10	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[g]	To 1000 ml			
	Solidifier				
12	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The original paper (Giridihar et al., 2003) does not indicate whether sugar is present in this medium. Because of that the recipe in this table is based on the assumption that it does. A test with sugar-free medium is advisable.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–9 to 900 ml of distilled water (item 11), adjust pH to 5.7 ± 0.2, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to pre culture vessels and autoclave. Agar is not added to liquid media.

TABLE VLA-20. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Vanilla planifolia* nodal segments (Giridihar et al., 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Auxin				
8	Phenylacetic acid (PAA)	1.25	125 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	Benzyladenine (BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[g]	To 1000 ml			
	Solidifier				
15	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively. The original report suggests 0.5–2.0 mg phenylacetic acid (PAA) l^{-1} and 2 or 5 mg benzyladenine (BA) l^{-1}; the amounts suggested here were selected as appropriate without being too small or excessive.

[g]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH to 5.7 ± 2, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

TABLE VLA-21. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the elongation of *Vanilla planifolia* nodal shoots (Giridihar et al., 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Auxin				
8	Phenylacetic acid (PAA)	1.13	113 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
9	Benzyladenine (BA)	3.0	300 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
13	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
14	Water, distilled[g]	To 1000 ml			
	Solidifier				
15	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively. The original report suggests 0.5–2.0 mg phenylacetic acid (PAA) l^{-1} and 2 or 5 mg benzyladenine (BA) l^{-1}; the amounts suggested here were selected as appropriate without being too small or excessive.

[g]Add items 1–12 to 900 ml of distilled water (item 14), adjust pH to 5.7 ± 2, add sugar (item 13), and raise volume to 1000 ml with distilled water (item 14). Bring the solution to a gentle boil and add the agar (item 15) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

TABLE VLA-22. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the rooting of *Vanilla planifolia* nodal shoots (Giridihar et al., 2003)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Indolebutyric acid (IBA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamin				
11	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	7.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The original paper states: "Finally . . . 2.0–2.5 cm long shoots . . . were rooted on MS media [sic] containing 2.0 mg/l IBA." This implies the complete MS medium despite the fact that only parts of it are used in Tables Vla-18–Vla-21.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH. There is no cytokinin in this medium.

[g]Add items 1–7 and 9 to 900 ml of distilled water (item 15), adjust pH as required, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

Multiplication of *Vanilla planifolia* through the Use of Silver Nitrate

Silver nitrate, an ethylene inhibitor reported to enhance shoot multiplication, was used to develop "improved methodology for micropropagation of *Vanilla* plants" (Giridhar et al., 2001).

Plant Material. Shoot tips and nodal explants, 2–3 cm long, from 3-year-old vines were used in the original research. Explants from younger vines or new growths of older ones may also be suitable. Some explants were placed on a shoot initiation medium (see Table Vla-18). Others were cultured directly on a rooting medium (Table Vla-23). Shoots and nodal sections produced in vitro were also cultured on a silver nitrate ($AgNO_3$)-containing medium (Table Vla-24).

Surface Sterilization. Explants should be washed with tap water and Tween 20 (www.sigmaaldrich.com, www.fishersci.com, www.vwr.com, www.wako-chem.co.jp/english/), baby shampoo, or a mild household detergent and then rinsed with tap water. After that they should be immersed in 0.15% (w/v) mercuric chloride ($HgCl_2$; available from the sources above; this toxic substance must be handled with care) for 10 min and then rinsed five times with sterile distilled water.

Culture Vessels. Erlenmeyer flasks, culture tubes, or other containers can be used.

Culture Conditions. Cultures should be maintained at $25 \pm 2°C$ under continuous illumination of 37.5 $\mu mol\ m^{-2}\ s^{-1}$ provided by Philips, India fluorescent (type not described) lamps. Standard culture room conditions may also be suitable.

Culture Media. Shoot tips and nodal explants should be placed in a modified MS medium (Murashige and Skoog, 1962) for shoot initiation (see Table Vla-18). Shoots that form on this medium after 60 days of culture should be moved to another modification of MS (see Table Vla-19) to bring about the production of shoots and nodal sections for further multiplication. Nodes taken from this medium can be cultured on a third modification of MS (see Table Vla-20) and placed on a fourth version of MS (see Table Vla-21) for elongation. The elongated shoots should be rooted on a rooting medium (see Table Vla-22). Explants from in vitro shoots and field-grown plants can be rooted on yet another modification of MS (Table Vla-23). In-vitro-grown nodal explants can also be cultured on a medium containing $AgNO_3$ (Table Vla-24).

Procedure. Nodal explants of shoot tips from field-grown plants are surface-sterilized and cultured first on the shoot initiation medium (see Table Vla-18) or rooted on one of the rooting media (Table Vla-23). Shoots from the shoot initiation medium are moved to another medium (see Table Vla-19) for further growth. Nodal sections and shoots from this medium are moved to a medium designed to bring about the production of new shoots (see Table Vla-20) which are moved to an elongation medium for further growth (see Table Vla-21). The elongated shoots are rooted on a rooting medium (see Table Vla-22). Shoot tips and nodal sections

TABLE VLA-23. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the rooting of nodal explants from in-vitro- and field-grown nodal sections (Giridhar et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
9	Indolebutyric acid (IBA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
13	Malt extract	25.0	No stock	No stock	Weigh
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	6.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.6, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium into culture vessels and autoclave. Agar is not added to liquid media.

TABLE VLA-24. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Vanilla planifolia* shoots (Giridhar et al., 2001)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	0.25	25 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	1.0 g	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (IAA)	0.2	20 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamin				
12	Thiamine (vitamin B_1)	5.0	500 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
13	Casein HCl	500.0	No stock	No stock	Weigh
	Silver				
14	Silver nitrate, $AgNO_3$	3.4	340 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	7.5 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. Silver nitrate is toxic and

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–13 to 900 ml of distilled water (item 16), adjust pH to 5.7, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, pour the solution into a 2-l flask and autoclave. Add the silver (item 14) to the hot solution under sterile conditions with sterilized pipettes, mix well, and distribute the medium to preautoclaved culture vessels. Agar is not added to liquid media.

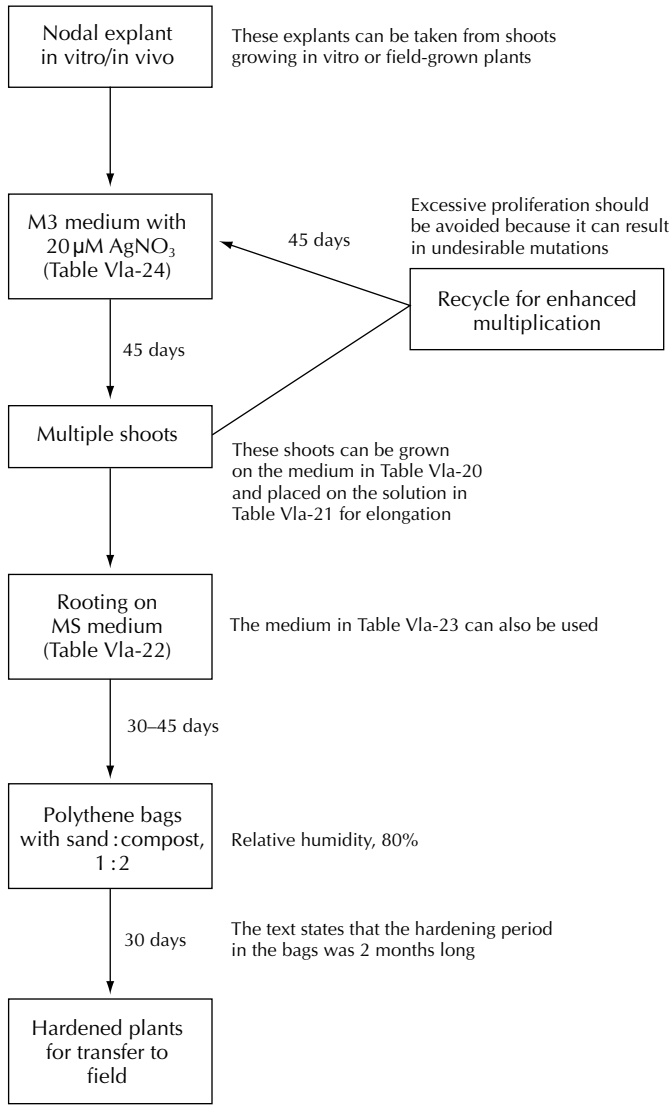


FIG. VLA-5. **Micropropagation of *Vanilla planifolia* (Giridhar and Ravishankar, 2004).**

from field-grown plants are rooted directly on a medium formulated for this purpose (Table Vla-23).

The procedure for explants that are cultured on medium containing $AgNO_3$ is outlined clearly in the original paper. This outline is presented in Fig. Vla-5 with several additions.

Preparation and Addition of Silver Nitrate. Silver nitrate can stain human skin and even cause burns. It is also absorbed by skin and subsequent exposure to light causes

it to turn black. In the event that silver nitrate gets on skin, the affected site must be rinsed immediately with plenty of water. If inhaled, silver nitrate is destructive to mucous membranes and upper respiratory tract tissues. Inhalation symptoms may include laryngitis, wheezing, coughing, shortness of breath, burning sensation, headache, nausea, and vomiting. Silver nitrate is corrosive and swallowing it can cause severe burns of the mouth, throat, and stomach. It can also cause sore throat, vomiting, and diarrhea. In some cases it can even be fatal if ingested. It can cause blurred vision, redness, pain, severe tissue burns, and eye damage on getting into eyes. Altogether, silver nitrate is a great boon to humanity because it is the basis of film photography, but it is a nasty and poisonous chemical which should be avoided. And this should be easy to do since there are no good reasons for using this protocol.

The silver nitrate stock solution should be kept in a black bottle. In the original research, the silver nitrate solution was filter-sterilized and added to the medium after autoclaving. However, since silver nitrate is very soluble in alcohol a stock solution in 95% ethanol is preferable.

Developmental Sequence. Shoots are produced on the first medium (see Table Vla-18) and grow on the second solution (see Table Vla-19). Nodal sections from shoots on the second solution produce shoots on a third medium (see Table Vla-20), and are moved to the fourth solution for elongation (see Table Vla-21). Elongated shoots, 2.0–2.5 cm in height, produce roots on the fifth medium (see Table Vla-22). Shoots from field-grown plants or in vitro cultures produce roots when placed on the appropriate solution (Table Vla-23). Explants on the medium containing $AgNO_3$ (Table Vla-24) produce shoots and roots.

General Comments. This procedure is included here only because it was published. It was formulated and published before the previous procedure (Giridhar et al., 2003) which is not only preferable for practical applications, but also much safer. The theoretical basis for the use of $AgNO_3$ is the inhibition of ethylene action by the silver ion.

Micropropagation of *Vanilla planifolia* on Media Containing Coconut Water and Several Hormones

In the never ending quest for an improved micropropagation method for *Vanilla planifolia* a protocol using coconut water (erroneously called "coconut milk" in the original paper), TDZ, and zeatin (ZN) was developed at the Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore, India (Giridhar and Ravishankar, 2004).

Plant Material. In the original research, shoot tips and node explants, 2–3 cm long, were excised from 1-year-old field-grown vines. Similar explants or ones taken from young growths of more mature plants can probably be used in practical micropropagation.

Surface Sterilization. Explants should be washed with tap water and Tween 20 (www.sigmaaldrich.com, www.fishersci.com, www.vwr.com, www.wako-chem.co.jp/english/), baby shampoo, or a mild household detergent and then rinsed with tap water. After that they should be immersed in 0.15% (w/v) mercuric chloride ($HgCl_2$; available from the sources above; this toxic substance must be handled with care) for 10 min and then rinsed five times with sterile distilled water.

Culture Vessels. Erlenmeyer flasks, culture tubes, or other containers can be used.

Culture Conditions. Cultures should be maintained at 25 ± 2°C under continuous illumination of 37.5 μmol m^{-2} s^{-1}. Standard culture room conditions may also be suitable.

Culture Media. Explants should be cultured on a shoot initiation medium (see Table Vla-18). Shoots which form on this medium should be maintained on a modified MS solution (see Table Vla-19; Murashige and Skoog, 1962). To induce multiple shoots, explants should be moved to a modified MS medium containing 10.56 μmol BA and 4.56 μmol ZN per liter (Table Vla-25). Explants that form axillary buds (called "shoot bud sprouts" in the original paper) should be moved to modification VS of MS (Table Vla-26) to bring about shoot proliferation and elongation. Shoots from this medium can be moved to one of two other media for further growth and root formation: a modification of the Nitsch (1969) medium designated as N69 (Table Vla-27) or a substrate designated as RS (Table Vla-28). Plants from both media N69 and RS should be moved for rooting and maturation to modified MS (see Table Vla-23). The report states that this medium contains 9.8 μmol IBA, which amounts to ($9.8 \times 203.23 = 1.99$) 2 mg, but the schematic representation (Giridihar and Ravishankar, 2004, fig. 3 on p. 118) indicates 1 mg l^{-1}. Since these authors suggest 2 mg l^{-1} in other protocols and one of their figures, this is the amount recommended here. It is also possible to culture explants on a medium containing coconut water (erroneously referred to as coconut milk) and TDZ (Table Vla-29).

Procedure. An excellent diagram (Fig. Vla-6) and an informative illustration (Fig. Vla-7) in the original report show the procedure and the manner in which explants should be inserted in media very clearly. The diagram (Fig. Vla-6) was enhanced by additional descriptions, added steps, annotations regarding figures, and references to tables which contain media recipes.

Developmental Sequence. Explants placed on the shoot induction medium (see Table Vla-18) produce shoots which grow and develop further on the maintenance solution (see Table Vla-19). Nodal explants taken from shoots on the maintenance medium (Table Vla-19) and grown either on BA and ZN (Table Vla-25) or TDZ (Table Vla-29) containing media produce shoot bud sprouts (Fig. Vla-8A, B) after 30 days in culture. These shoot buds produce multiple shoots when cultured 45 days on the VS medium (Table Vla-26). If these shoots are moved to the RS medium (Table Vla-28) they form roots. The same shoots elongate if moved to the N69 substrate (Fig. Vla-8C, D; Table Vla-27) and cultured on it for a month. Rooted plantlets from the RS medium (Table Vla-28) and elongated ones from the N69 solution (Table

TABLE VLA-25. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of nodal explants of *Vanilla planifolia* (Giridhar and Ravishankar, 2004)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	If any was used the original report does not mention it. Should an auxin be required use 1 mg naphthaleneacetic acid l^{-1}				
	Cytokinins				
11	Zeatin (ZN)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
12	Benzyladenine (BA)	2.4	250 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
13	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
16	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[g]	To 1000 ml			
	Solidifier				
18	Agar[g]	6.0	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]Add items 1–15 to 900 ml of distilled water (item 17), adjust to 5.7, add sugar (item 16), and raise volume to 1000 ml with distilled water (item 17). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to culture vessels. Agar is not added to liquid media.

TABLE VLA-26. **VS [modified Murashige–Skoog (MS)] medium (Murashige and Skoog, 1962) for the culture of shoot-bud sprouts of *Vanilla planifolia* (Giridhar and Ravishankar, 2004)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Auxin				
9	Naphthaleneacetic acid (NAA)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
10	Benzyladenine (BA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamin				
11	Biotin	20.0	No stock	No stock	82 µmol; this is a very high concentration
	Sugar				
12	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
13	Water, distilled[g]	To 1000 ml			
	Solidifier				
14	Agar[g]	6.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The original paper states "MS basal" which suggests that the following are not part of this medium. They can be added if necessary.

	Amino acid				
	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
	myo-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–11 to 900 ml of distilled water (item 13), adjust pH to 5.7, add sugar (item 12), and raise volume to 1000 ml with distilled water (item 13). Bring the solution to a gentle boil and add the agar (item 14) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to autoclaved culture vessels. Agar is not added to liquid media.

TABLE VLA-27. **Nitsch (N69) medium (Nitsch, 1969) as used for the elongation of multiple shoots of *Vanilla planifolia* (Giridhar aand Ravishankar, 2004)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	720.0	72.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2$	166.0	16.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	68.0	6.8 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	10.0	1.0 g l^{-1}		
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	25.0	2.5 g l^{-1}	10	One solution
(d)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(e)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	10.0	1.0 g l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Gibberellin				
10	Gibberellic acid	0.01	1 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Cytokinin				
11	Benzyladenine (BA)	0.25	25 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Biotin	0.05	5 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Folic acid	500.0	No stock	No stock	1132 µmol, this is a very high concentration
14	Niacin (nicotinic acid)	5.0	500 mg 100 ml^{-1} 95% ethanol[e]	1	
15	Pyridoxine HCl (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
16	Thiamine HCl (vitamin B_1)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
17	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
18	Water, distilled[g]	To 1000 ml			
	Solidifier				
19	Agar[g]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]If the gibberellin or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively. This is one of very few media which include giberrellic acid.

[g]Add items 1–16 to 900 ml of distilled water (item 18), adjust pH to 5.5, add sugar (item 17), and raise volume to 1000 ml with distilled water (item 18). Bring the solution to a gentle boil and add the agar (item 19) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

TABLE VLA-28. **Murashige–Skoog (MS) medium (RS; Murashige and Skoog, 1962) modified for the rooting of *Vanilla planifolia* shoots (Giridhar and Ravishankar, 2004)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Polyol				
8	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
9	Indolebutyric acid (IAA)	2.0	200 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
10	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
11	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
12	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Complex additive				
13	Malt extract	25.0	No stock	No stock	Weigh
	Sugar				
14	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[g]	To 1000 ml			
	Solidifier				
16	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin does not dissolve, add a few drops of 0.1 N KOH.

[g]Add items 1–13 to 900 ml of distilled water (item 15), adjust to 5.7, add sugar (item 14), and raise volume to 1000 ml with distilled water (item 15). Bring the solution to a gentle boil and add the agar (item 16) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

TABLE VLA-29. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of nodal explants of *Vanilla planifolia* (Giridhar and Ravishankar, 2004)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}	10	One solution
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}		
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Cytokinin				
8	Thidiazuron (TDZ)	0.5	50 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Complex additive				
9	Coconut water (CW)[g]	100.0 ml	No stock	No stock	Measure
	Sugar				
10	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[h]	To 1000 ml			
	Solidifier				
12	Agar[h]	6.0 g	No stock	No stock	Weigh
	Amino acid				
	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
	myo-inositol	100.0	No stock	No stock	Weigh
	Auxin				
	Indoleacetic acid (IAA)	1.0–30	100–300 mg 100 ml^{-1} 95% ethanol[e,f]	1	Or another auxin
	Vitamins				
	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	

[a]Amounts are given in mg unless indicated otherwise. The original report states that basal MS medium was used and supplemented with TDZ and CW. This implies that the substances listed below were excluded because the standard use of "basal" implies only the mineral component of a medium. If necessary they can be added. There is no indication if sugar was included in this medium, but it is reasonable to assume that it should be.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the cytokinin does not dissolve, add a few drops of 0.1 N HCl.

[g]The correct term for the liquid endosperm of coconuts is coconut water (CW), not coconut milk which should be applied to the white milky liquid obtained by grating and/or squeezing copra (the solid part of the endosperm or "meat" inside the nut). It is preferable to use water from unripe (green) nuts. In countries where coconut palms do not grow, unripe nuts may be available in stores which specialize in Asian foods. The outer (green) covering of these nuts is often peeled by the store and they are white in appearance. If unripe (green or white) nuts are not available, water from ripe (brown) ones can be used. Water (i.e., the liquid endosperm) of both ripe and unripe nuts should be filtered through a Whatman No. 1 filter before it is added to culture media. Filtered CW must be stored in a freezer until it is used. Canned or commercially frozen coconut water must not be used because it may contain sugar (which will increase the concentration in the culture medium) and/or preservatives (that may have undesirable effects on cultures).

[h]Add items 1–9 to 800 ml of distilled water (item 11), adjust pH to 5.7, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

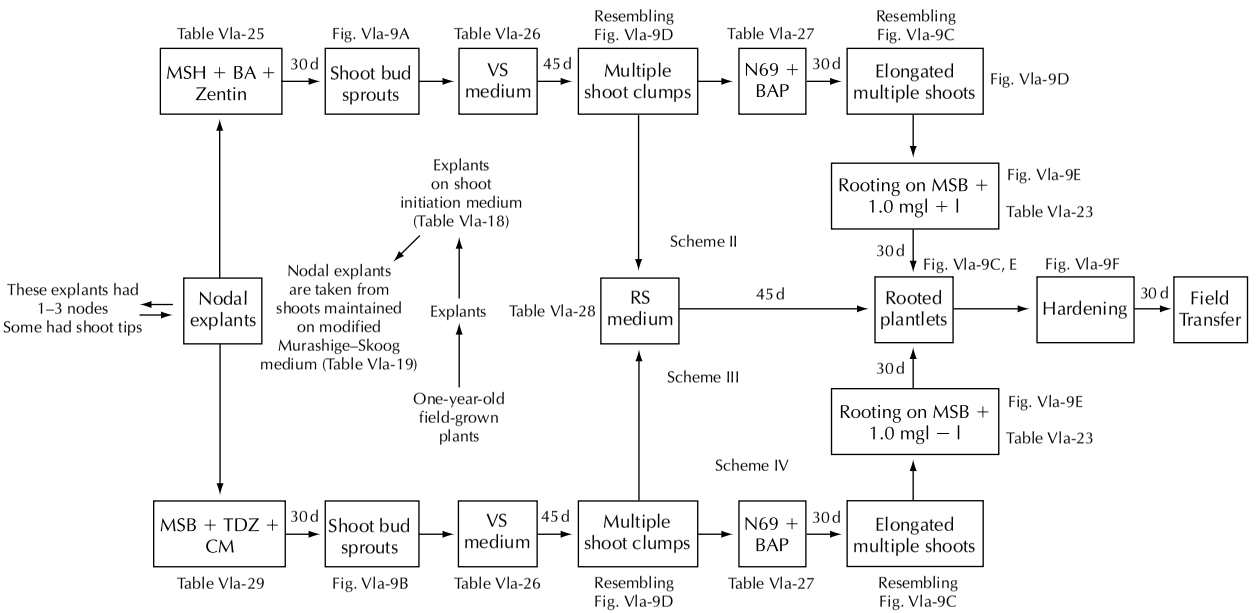


FIG. VLA-6. **Procedure for culturing *Vanilla planifolia* nodal explants (Giridhar and Shenkar, 2004 with additional annotations). BA, benzyladenine; BAP, benzylaminopurine; CM, coconut water; I, indolebutyric acid; MSB, basal Murashige–Skoog medium; N69, Nitsch (1969) medium; RS and VS, modified MS media.**

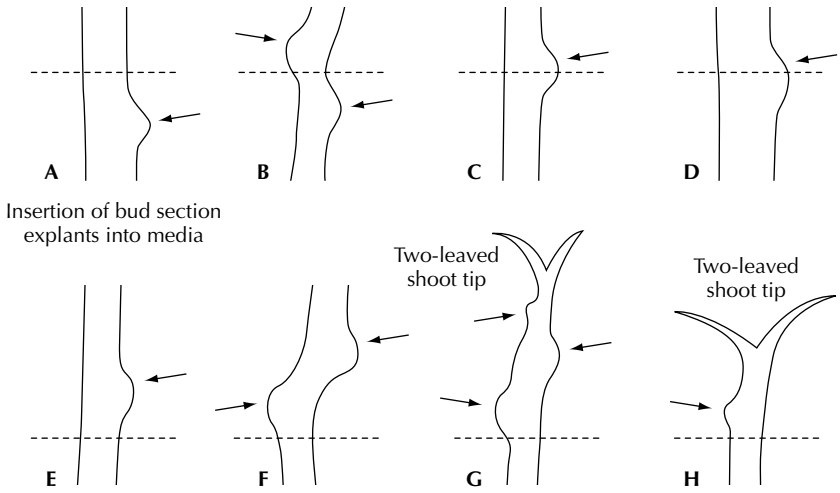


FIG VLA-7. **Insertion of *Vanilla planifolia* bud section explants in culture media. In most cases the manner of insertion makes little or no difference (Giridhar and Shankar, 2004).**

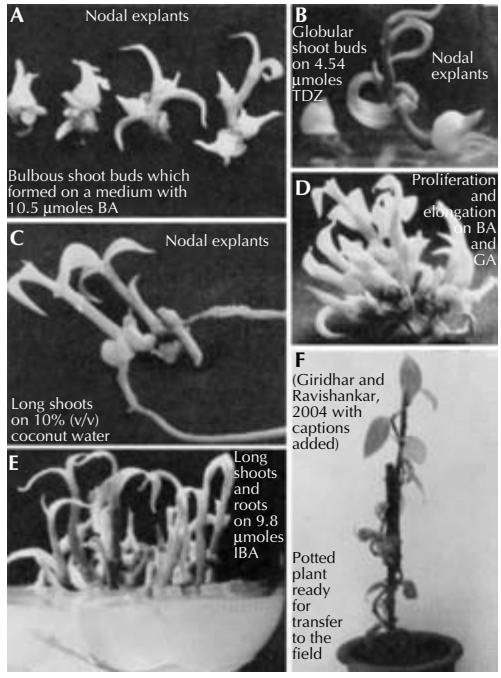


FIG. VLA-8. **Growth and development of *Vanilla planifolia* (Giridhar and Shankar, 2004).**

Vla-27) form roots (Fig. Vla-8C, E) after 30 days on the IBA-containing substrate (see Table Vla-23). The rooted plants can be planted in the field after 30 days of hardening (Fig. Vla-8F).

General Comments. This is a well thought out and carefully researched procedure which is complex and involves eight media, five vitamins, three cytokinins (BA, TDZ, and ZN), two auxins (IBA and NAA), two basal media (MS and Nitsch), one complex additive (malt extract), one gibberellin (GA_3), one polyol (*myo*-inositol), and many steps. Those who may wish to use this protocol should prepare for it in advance, plan carefully, and be sure that a simpler method will not be equally effective.

In Vitro Propagation of *Vanilla planifolia* using Two Media

"Mass propagation [of *Vanilla planifolia*] requires a simple, economical, rapidly multiplying and highly reproducible protocol without an intervening callus or protocorm phase as to give rise to true-to-type clones" (Geetha and Shetty, 2000). Such a protocol was developed by the plant tissue culture laboratory of Kahland Biotechs Private Limited in India (Geetha and Shetty, 2000).

Plant Material. Shoot tips and nodal explants can be used.

Surface Sterilization. In the original research explants were pretreated for 4 h with a solution of 0.2% Bavistin (www.BASF.com), 0.2% streptocycline, and 5–6 drops of liquid Dettol (www.dettol.com.uk), and then washed with Tween 20 (www.sigmaaldrich.com) and sterile distilled water. This step may not be necessary. A simple wash with tap water, a mild household detergent, and a soft toothbrush may be sufficient. After the wash the explants should be sterilized with 0.1% mercuric chloride ($HgCl_2$) for 10 min (this sterilant is toxic and should be handled with care) and rinsed 3–5 times with sterile distilled water.

Culture Vessels. Jars were used in the original research. Other containers are also suitable.

Culture Conditions. The research cultures were maintained at $25 \pm 1°C$ under 12-h photoperiods of 2500 lx provided by cool white fluorescent tubes. Standard culture room conditions should also prove to be suitable.

Culture Media. Explants and elongated shoots should be cultured in MS medium (Murashige and Skoog, 1962) containing 1 mg BA l^{-1} (Table Vla-30). Explants with breaking buds and initial and young proliferating shoots should be cultured on a modification of the Nitsch medium (Nitsch, 1969) for *Nicotiana* androgenesis (Table Vla-31).

Procedure. The original report includes an excellent diagram that outlines the protocol and is presented here with some additions and incorporates photographs of cultures (Fig. Vla-9).

TABLE VLA-30. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of *Vanilla planifolia* explants (Geetha and Sheety, 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650.0	165.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440.0	44.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370.0	37.0 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900.0	190.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170.0	17.0 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620.0 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 mg l^{-1}	10	One solution
(e)	Potassium iodide, KI	0.83	83.0 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860.0 mg l^{-1}		
	Amino acid				
8	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100.0	No stock	No stock	Weigh
	Auxin				
10	Table 1 in the original report makes no mention of an auxin. Should one prove to be necessary, 1 mg naphthaleneacetic acid l^{-1} may be appropriate				
	Cytokinin				
11	Benzylaminopurine (BAP)	1.0	100 mg 100 ml^{-1} 95% ethanol[e,f]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
15	Sucrose	30.0 g	No stock	No stock	Weigh
	Solvent				
16	Water, distilled[g]	To 1000 ml			
	Solidifier				
17	Agar[g]	10.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. The difference will probably have little or no effects.

[e]Keep frozen between uses.

[f]If the auxin (should one be used) or cytokinin does not dissolve, add a few drops of 0.1 N KOH or HCl, respectively.

[g]Add items 1–14 to 900 ml of distilled water (item 16), adjust pH as required, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

TABLE VLA-31. **Nitsch medium (Nitsch, 1969) modified for the culture of *Vanilla planifolia* shoots (Geetha and Shetty, 2000)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	720.0	72.0 g l^{-1}	10	
2	Calcium chloride, $CaCl_2$	166.0	16.0 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	185.0	18.5 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	950.0	95.0 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	68.0	6.8 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	10.0	1.0 g l^{-1}	10	One solution
(b)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(c)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	25.0	2.5 g l^{-1}		
(d)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25.0 mg l^{-1}		
(e)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	10.0	1.0 g l^{-1}		
	Vitamins				
8	Biotin	0.05	5 mg 100 ml^{-1} 95% ethanol[e]	1	
9	Folic acid	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Sugar				
10	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
11	Water, distilled[f]	To 1000 ml			
	Solidifier				
12	Agar[f]	8.0 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise. The original paper states that the basal medium was used and mentions only BAP, biotin, and folic acid as additives. This implies that the following substances were not included. They can be added as necessary.

	Amino acid				
	Glycine	2.0	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
	myo-inositol	100.0	No stock	No stock	Weigh
	Vitamins				
	Niacin (nicotinic acid)	5.0	500 mg 100 ml^{-1} 95% ethanol[e]	1	
	Pyridoxine HCl (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	
	Thiamine HCl (vitamin B_1)	0.5	50 mg 100 ml^{-1} 95% ethanol[e]	1	

[b]Solutions containing ammonium and/or nitrate can become contaminated on standing. Therefore stock solutions should not be prepared. If made they must be kept frozen between uses.

[c]Add the chelating agent (item 6a) and the iron salt (item 6b) to the same 1 l of distilled water and stir and/or heat until both are dissolved. This is the iron solution used for the Murashige–Skoog medium (Murashige and Skoog, 1962) and many other media, not the one listed by in the original paper describing this substrate (Nitsch, 1969).

[d]Add the salts to the same 1 l of distilled water and stir and heat until all are dissolved. Dispense the volume indicated per liter of culture medium.

[e]Keep frozen between uses.

[f]Add items 1–9 to 900 ml of distilled water (item 11), adjust pH to 5.5, add sugar (item 10), and raise volume to 1000 ml with distilled water (item 11). Bring the solution to a gentle boil and add the agar (item 12) slowly while stirring. The agar can also be added to the cold water which is brought to a boil and stirred. When the agar is completely dissolved, distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

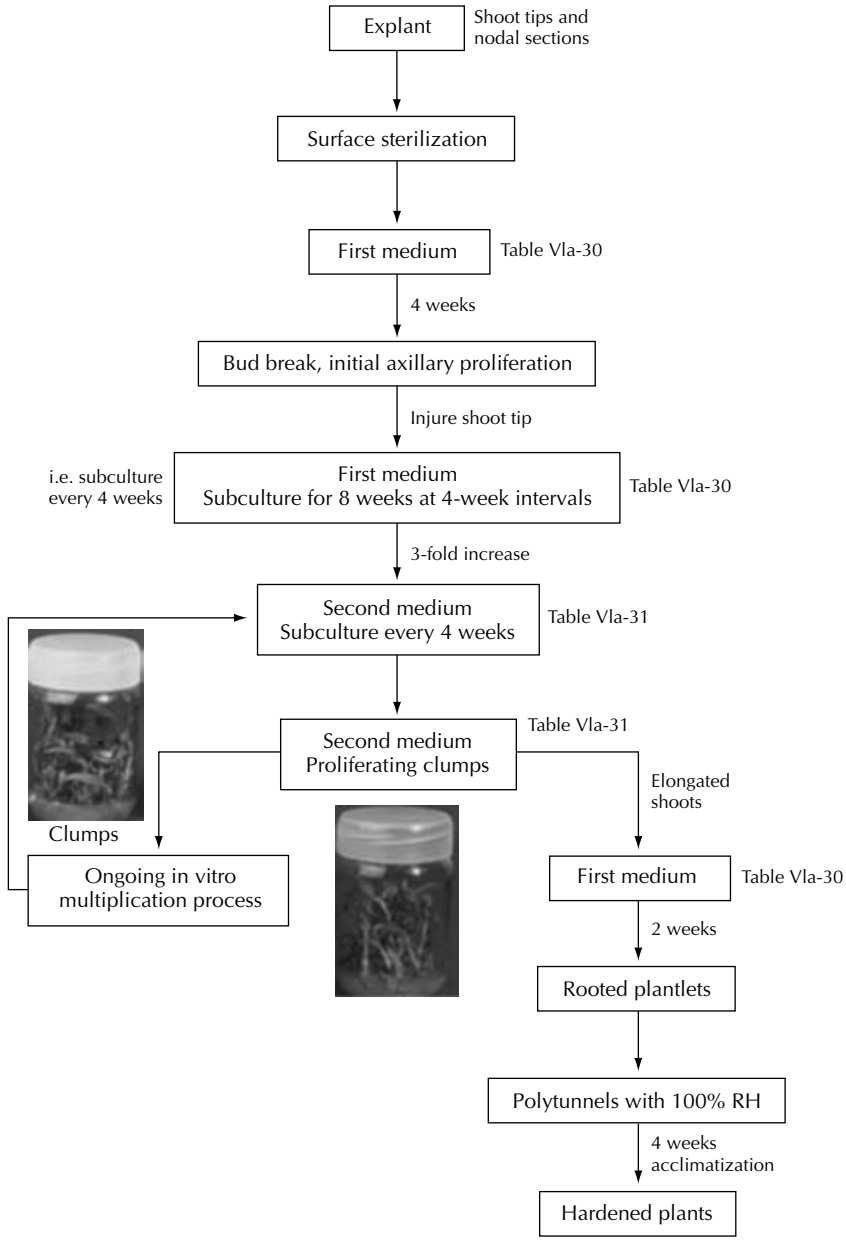


FIG. VLA-9. **Protocol for micropropagation of *Vanilla planifolia* (photographs and diagram from Geetha and Shetty, 2000).**

Developmental Sequence. The explants produce shoots that proliferate, elongate, and form roots (Fig. Vla-9), all over a relatively short period.

General Comments. This seems to be a simple procedure, which is as effective as much more complex and expensive protocols. It should be given serious consideration by those intending to propagate *Vanilla planifolia*.

Movement of *Vanilla* Plants and Germplasm

Vanilla is an important and high value crop in some parts of the world. Because of that plants are exchanged internationally. This carries the danger of accidental transport of pests and diseases. To prevent or at least reduce such incidents, the Food and Agriculture Organization (FAO) of the United Nations and the International Board of Plant Genetic Resources (IBPGR) have produced guidelines for the safe movement of *Vanilla* germplasm (Pearson et al., 1991). The intent is laudable, but the manner in which the FAO/IBPGR proceeded in respect to *Vanilla* leaves much to be desired because "the present guidelines for vanilla [sic] were not prepared by a panel meeting [presumably of experts]; instead a contract was awarded to a principal coordinating author, Dr. M. Pearson. . . ." As a result those involved in the preparation of the guidelines included only one orchid expert (Dr. Pearson, a noted orchid virologist who has published many excellent research papers in major journals) and no one with proven expertise in orchid propagation, tissue culture, or micropropagation. This is evident in the some of the technical recommendations, only a few of which will be addressed here.

Seeds

The section on the movement of seeds seems to imply, or at least creates the impression, that *Vanilla* can or maybe even should be propagated by seed germination. This is not the case for practical *Vanilla* cultivation because growers want, or at least prefer, established clones. Seed propagation is useful only for breeders and can be of interest to hobby growers. Also, the statement "preferably, seed should be germinated *in vitro*" is misleading because even if it is theoretically possible to germinate *Vanilla* symbiotically ex vitro, this is not being done and there are no published procedures for such germination. Since this book does not deal with seed germination, relevant procedures will not be discussed here.

A recommendation regarding the transfer of seeds is incomplete and incorrect. If seeds are to be surface-sterilized by soaking them in a saturated solution of calcium hypochlorite, it is necessary to dry them completely and rapidly under sterile conditions and place them in a sterilized container for shipping. Also, instructions should have been included regarding the preparation and use of saturated calcium hypochlorite.

Plantlets in Vitro

As has been pointed out elsewhere in this book and in the first edition (Arditti and Ernst, 1993), the promise that micropropagation (shoot-tip cultures, tissue culture) would free orchids of viruses was never fulfilled. In fact there are more virus-infected orchids now than before the introduction of shoot-tip culture. And, even more importantly, nodal cultures [Gu et al., 1987*a*; this method was developed in my (JA's) laboratory] in particular cannot free orchids of viruses. This is not pointed out in the guidelines, even if there is a suggestion to index each plant. However, there is a major problem with the latter also. Indexing is not simple or easy and is probably beyond the capability of most practical tissue culture laboratories. Therefore the suggestion to move plantlets in sterile cultures may do more harm than good, encouraging unqualified laboratory operators to culture infected plants and ship them to unsuspecting recipients.

Methods for freeing orchids of virus through the use of antiviral agents have been developed by several laboratories, but none are for *Vanilla*. Therefore the danger of transporting virus-infected orchid plants remains as high as ever.

Recommendations

Until proven and simple methods are developed for indexing *Vanilla* plants or freeing them of viruses through the use of antiviral agents in vitro, transport should be regulated very strictly and must be limited to laboratories with a proven ability to detect viruses. Laboratories that do not have such expertise should never assume that *Vanilla* plants propagated in vitro are virus-free. Practical growers should become thoroughly familiar with the symptoms of virus diseases of *Vanilla* [this section of the guidelines (Pearson et al., 1991) is excellent] and cull infected plants.

In the year 2000, 38,525 ha were planted with *Vanilla* worldwide and the yield was 4728 metric tonnes. The USA, Germany, and France use 60–80% of the world vanilla production. The USA alone uses 30–60%.

The Aztec Emperor Montezuma drank *choclatl*, a beverage prepared from ground cocoa seeds and flavored with vanilla, which the Aztecs called *tlilxochitl*. The word was derived from *tlili* which means black and *xochitl* meaning pod. According to legend, Cortez was also served *choclatl*. He repaid the favor with murder and pillage. The priests who accompanied him forced their religion on the Aztecs. One purveyor of religion, Bernardino de Sehagun, a Franciscan friar who came to Mexico in 1529, wrote that the Aztecs mixed vanilla with cocoa and honey, but his work was only published in 1829–1830, 300 years after he wrote it.

Francisco Hernandez, who was sent to Mexico by Spanish King Philip II, translated *tlilxochitl* as "black flower" in an illustrated description of vanilla in his *Rerum Medicarum Novae Hispaniae Thesaurus* (published in Rome in 1651). This erroneous translation remained in the literature for many years.

www.guweb2.gonzaga.edn www.learnersonline.com www.redescolarilce.edn.mx www.ihnca.edi.ni

Bernal Diaz (A), an officer serving under Herman Cortez (B) was the first European to notice vanilla. (C) Bernardino de Sahagun. (D) Francisee Hermandez.

Vascostylis

The procedure used for *Ascofinetia* (Intuwong and Sagawa, 1973) is also appropriate for *Vascostylis*.

Vuylstekeara

The procedures used for *Cymbidium* (Morel, 1960, 1963, 1964*a*, 1965*b*, 1970, 1974) were described as being also suitable for *Vuylstekeara*. A more specific procedure was developed in Poland (Kukułczanka et al., 1989).

Plant Material. In the original research, shoots that were 10–12 cm long and bearing two to three leaves were taken from plantlets of *Vuylstekeara* Cambria maintained in vitro.

Surface Sterilization. The explants are taken from axenic plantlets and need not be surface-sterilized.

Culture Vessels. Test tubes were used in the original research, but other vessels are also suitable.

Culture Conditions. The cultures were maintained at 22–26°C "in daylight supplemented by an artificial lighting with the photoperiod prolonged to 14–15 hrs" (Kukułczanka et al., 1989). Conditions used for *Cymbidium, Cattleya, Odontoglossum, Oncidium*, and *Laelia* are also suitable.

Culture Medium. A modification of the MS medium is used (Table Vuyl-1).

Procedure. Place the explants in culture, and allow them to develop offshoots. Subculture plantlets formed in this fashion, and grow them until large enough to be moved to pots.

Developmental Sequence. Lateral shoots develop after 5 weeks in culture. PLBs, adventitious shoots, and roots form after 10 weeks.

General Comments. This method could be used for the micropropagation of *Vuylstekeara*, but its suitability for explants from mature greenhouse- or field-grown plants is not clear at present.

Zygopetalum

A method used for *Cymbidium* (Morel, 1960, 1963, 1964*a*, 1965*b*, 1970, 1974) could also be employed with *Zygopetalum*.

TABLE VUYL-1. **Murashige–Skoog medium (Murashige and Skoog, 1962) modified for the culture of *Vuylstekeara* Cambria (Kukułczanka et al., 1989)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium, ml	Remarks
	Macroelements				
1	Ammonium nitrate, NH_4NO_3[b]	1650	165 g l^{-1}	10	
2	Calcium chloride, $CaCl_2 \cdot 2H_2O$	440	44 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370	37 g l^{-1}	10	
4	Potassium nitrate, KNO_3[b]	1900	190 g l^{-1}	10	
5	Monopotassium phosphate, KH_2PO_4	170	17 g l^{-1}	10	
6	**Chelated iron**[c]				
(a)	Chelating agent, Na_2EDTA	37.3	3.73 g l^{-1}	10	One solution
(b)	Iron sulfate, $FeSO_4 \cdot 7H_2O$	27.8	2.78 g l^{-1}		
7	**Microelements**[d]				
(a)	Boric acid, H_3BO_3	6.2	620 mg l^{-1}		
(b)	Cobalt chloride, $CoCl_2 \cdot 6H_2O$	0.025	2.5 mg l^{-1}		
(c)	Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025	2.5 mg l^{-1}		
(d)	Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3	2.23 g l^{-1}	10	One solution
(e)	Potassium iodide, Kl	0.83	83 mg l^{-1}		
(f)	Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25	25 mg l^{-1}		
(g)	Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6	860 mg l^{-1}		
	Amino acid				
8	Glycine	2	200 mg 100 ml^{-1} 95% ethanol[e]	1	
	Polyol				
9	*myo*-inositol	100	No stock	No stock	Weigh
	Auxin				
10	Naphthaleneacetic acid (NAA)	0.2	20 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Cytokinin				
11	Benzyladenine (BA)	0.2	20 mg 100 ml^{-1} 95% ethanol[f,g]	1	
	Vitamins				
12	Niacin (nicotinic acid)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
13	Pyridoxine (vitamin B_6)	0.5	50 mg 100 ml^{-1} 95% ethanol[g]	1	
14	Thiamine (vitamin B_1)	0.1	10 mg 100 ml^{-1} 95% ethanol[g]	1	
	Complex additive				
15	Peptone[h]	1 g	No stock	No stock	Weigh
	Sugar				
16	Sucrose	30 g	No stock	No stock	Weigh
	Solvent				
17	Water, distilled[i]	To 1000 ml			
	Solidifier				
18	Agar, Difco Bacto[i]	10 g	No stock	No stock	Weigh

[a]Amounts are given in mg unless indicated otherwise.

[b]Solutions containing ammonium and/or nitrate may become contaminated. Therefore stock solutions should not be prepared. If made, keep frozen between uses.

[c]Add chelating agent (item 6a) and iron salt (item 6b) to the same 1 l of distilled water, and stir and/or heat until both are dissolved.

[d]Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ at the same concentration. This difference probably has little effect.

[e]Keep frozen between uses.

[f]If auxin or cytokinin do not dissolve, add a few drops of KOH or HCl, respectively.

[g]Keep refrigerated between uses.

[h]The brand of peptone used is Peptobak-Bacutil. Its composition and effects may not be the same as those of peptones available in the United States and other countries. Should the brand of peptone be critical, it may be necessary to contact the authors at the Botanical Garden, University of Wrocllaw, 50–335 Wroclaw, Poland.

[i]Add items 1–7, 9, and 15 to 900 ml distilled water (item 17). Adjust pH as required; no details are given in the original research, but pH of media used for *Cymbidium* would be suitable. Add sugar (item 16), and adjust volume to 1000 ml with distilled water (item 17). Bring solution to a gentle boil, and add agar (item 18) slowly while stirring. Agar can also be added to cold solution, which is then brought to a boil and stirred. When agar is completely dissolved, pour solution into a 2-l flask and autoclave. Add amino acid, hormone, and vitamins (items 8 and 10–14) to hot solution under sterile conditions with sterilized pipettes, mix well, and distribute medium to preautoclaved culture vessels. Omit agar if preparing liquid medium.

CHAPTER FOUR

Summary

Tissue culture (which includes the culturing of organs, cells, and protoplasts) is still an empirical science to some extent. Much has been learned during the last decade about procedures, media, effects of and requirements for a variety substances, methods which can be used with individual plants or groups of plants, and approaches which can bring about desired results; but success often hinges on intuition, good guesses, and fortune's smile. It is usually not easy and in some cases impossible to predict the type of explant, media, and conditions that would be suitable for a specific genus, species, hybrid, or clone. And, explaining why one combination of medium components and culture conditions can lead to success whereas another brings failure remains difficult and frequently unfathomable or beyond explanation. Nevertheless, some broad generalizations can be made.

Culture Conditions

A survey of the conditions and media used for the tissue culture of orchids shows a wide diversity (Arditti, 1977*b*; Arditti and Ernst, 1993; Dodds and Roberts, 1995).

State of the Medium

Both liquid and solid media can be used, but suitability must be determined empirically. Proliferation is generally faster and more extensive in liquid media on a shaker. However, liquid media have not proved suitable for all genera. Differentiation is always better on solidified substrates or on solid supports placed in culture vessels which contain liquid media. For example, bridges made of filter paper are recommended for some procedures (Gandawijaja, 1980) which utilize a liquid medium. Glass wool was tried (Ernst, 1974) and found to be unsuitable since it is not a pleasant or safe material to work with and because roots become entwined in it so extensively that they cannot be removed without breakage and damage. Cotton gets soggy, becomes poorly aerated, and roots can also become enmeshed in it.

The best solid support for cultures on a liquid medium may well be the Life Raft™, which is produced by Osmotek Ltd (www.osmotek.com). It consists of a membrane film (0.3 or 25 μm pore size for explants which benefit from less or more contact,

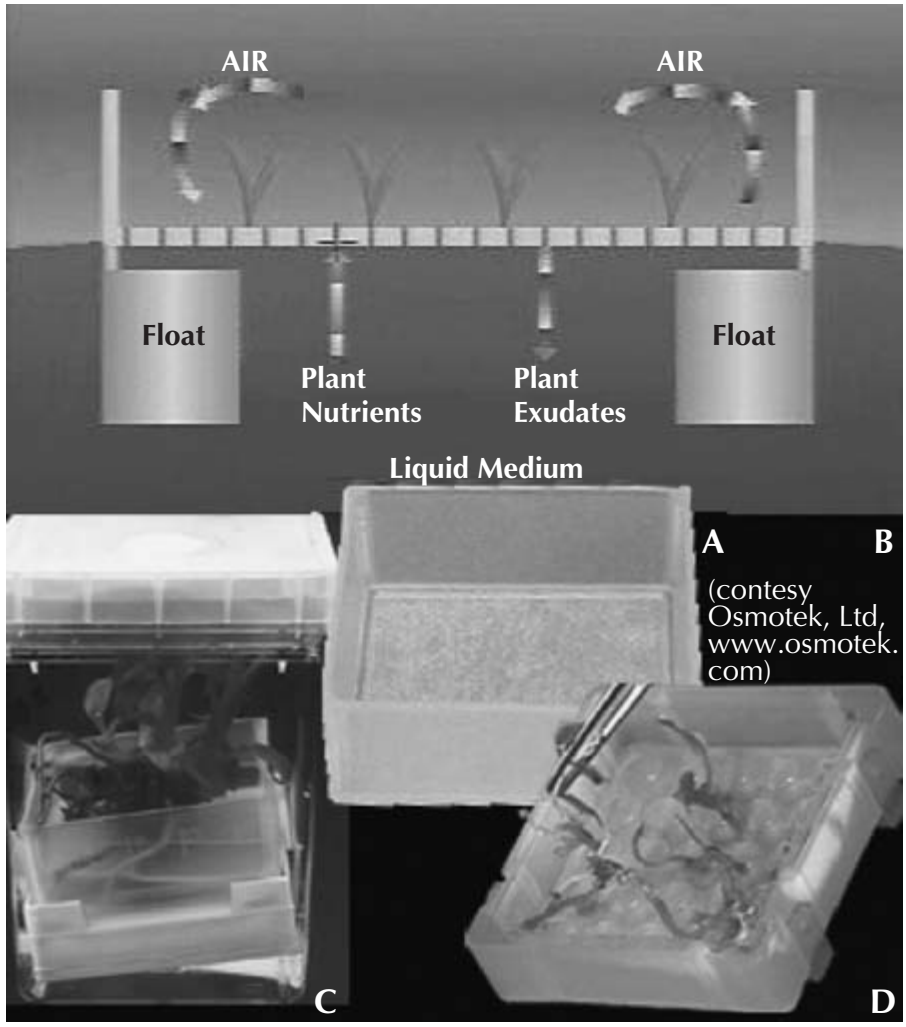


FIG. 4-1. **Life Raft™. A. An empty raft. B. Diagrammatic representation of a Life Raft™, its construction and its functions. C. Life Raft™ with plants inside a culture vessel. D. Plants inside a Life Raft™.**

respectively, with the medium) welded to a polypropylene frame which floats on the medium being supported by a buoyant float (Fig. 4-1). A solid support over liquid medium allows for better aeration as well as a quicker and faster dispersion of exudates (see also the section below, Solidifying Agents and Alternative Supporting Media).

Agitation

Liquid media should be or are usually agitated. However, there are also reports of success with stationary liquid media that are shaken only occasionally by hand (Kako,

1973). Numerous commercial shakers are available, including wheels that rotate in a horizontal or vertical plane and oscillate in a gyratory or horizontal manner. Rates of agitation vary considerably, with recommended speeds ranging from a low of 0.2 rpm (Jasper, 1966) to a high of 200 rpm (Scully, 1966). The choice of culture vessels may also influence the selection of the shakers and speed. If appropriate conditions are not specified, they must be determined experimentally. If specified, it is best to use the type and speed of rotation indicated for each procedure. There are a number of reasons why agitation may enhance the proliferation of some cultures. Among them are:

1 Elimination of polarity (sometimes referred to as disorientation).
2 Retardation and/or prevention of root and shoot development (Wimber, 1963, 1965; Scully, 1967).
3 Improved aeration.
4 Increased surface area.
5 Accelerated dilution of toxic metabolites (such as the polyphenol oxidates) and/or other exudates that can be harmful.
6 Stimulation of protocorm-like-body (PLB) formation.

As a rule liquid media and agitation are usually not beneficial when differentiation is desired.

Illumination

A wide range of light intensities seems to be suitable for orchid tissues. These range from darkness to 2000 ft-c (ft-candles; see the section on light in Chapter 2 and Appendix 4). Most cultures are illuminated by fluorescent lights, with or without additional incandescent bulbs. Numerous light sources have been listed in the literature, but they are reasonably interchangeable. However, the many units used to describe light intensity in individual papers are not always easily interconvertible due to the different characteristics of the sources of illumination. For example, the photosynthetically active radiation (PAR) in a light intensity of 2000 ft-c produced by a warm white fluorescent tube is not the same as in the same number of foot candles emitted by a halide lamp because the emission spectra of the two sources are different (see the section on light in Chapter 2 and Appendix 4).

Photoperiods

Photoperiods used for orchid tissue culture vary from continuous darkness or constant illumination to light periods of a few to many hours. The available evidence suggests that photoperiods of 12–18 h are suitable for most tissues. In general, photoperiods and light intensities useful for orchid seed germination and seedling growth are also suitable for tissue culture.

pH

As with seed culture, the pH range of most orchid tissue culture media is 4.8 to 5.5. In cases where the acidity of a medium increases during sterilization, the pH range

of the media should be adjusted slightly upward (to 5.6–5.9). There are some indications that an initial pH in the range of 4 to 8 may have no effect on PLB growth or plantlet development of several orchids (Piriyakanjanakul and Vajrabhaya, 1980). The pH of media adjusted by these researchers to 4, 5, 6, 7, and 8 changed to 4 after 2 weeks and remained near this level until the plantlets were harvested. A drop in initial pH to a level below 4 has also been observed with *Phalaenopsis* seed cultures (Ernst, 1967*a*). When the initial pH is very acid prior to autoclaving, hydrolysis or destruction of some media components may result. Hydrolysis of agar, for example, can be detrimental because it may result in semisolid solutions and toxic hydrolysis products. It should also be noted that explants, cells, and protoplasts have generally more exacting requirements than seed cultures.

Temperature

Orchid tissue cultures are usually maintained at temperatures ranging from 22 to 26°C. However, successful cultures have been maintained at much higher temperatures in the Asian tropics. Despite this relatively wide range, some tissues may have a narrower tolerance or specific optima. Therefore if specific temperatures are indicated it is best to provide them.

Media Components

Media, whether for seed culture or tissue culture, must provide the following inorganic macro- (or major) and micro- (minor) elements:

Macroelements

The terms macroelements or major elements are based on the fact that plants require large amounts of these minerals. They include nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and iron (Fe) (Table 4-1).

Nitrogen

This element is usually supplied as nitrate (NO_3; Fig. 4-2A) and/or ammonium (NH_4; Fig. 4-2B) ions. Roots of *Dendrobium* Multico White plantlets took up ammonium faster than nitrate (Hew and Lim, 1989). The uptake of nitrate was linear in the presence or absence of sugar (Hew and Lim, 1989). However, the uptake of ammonium in the absence of sugar reached a plateau after 30 days; in the presence of sugar, the uptake of ammonium remained linear for 2 months (Hew and Lim, 1989). The plantlets took up 64.6% of the ammonium when sugar was absent and 80.3% in its presence. The parallel figures for nitrate were 15.7 and 22.1%, respectively (Hew and Yong, 1997). Callus tissue of *Aranda* Noorah Alsagoff took up nitrate at pH 4.5, 5.0, 5.5, 6.0, and 7.0 (Lee et al., 1987). The uptake of ammonium occurred only at pH 4.5 and 5.0 (Lee et al., 1987). The tissues took up ammonium preferentially at pH 5.0 and 5.5 (Lee et al., 1987). Depletion of nitrogen (nitrate and

TABLE 4-1. **Macroelements, their formula weights, and pH ranges as employed in the tissue culture of orchids**

Macroelement	Formula	Formula weight[a]	pH range, 5% solution at 25°C[b]
Ammonium chloride	NH_4Cl	53.49	4.5–5.5
Ammonium nitrate	NH_4NO_3	80.05	4.5–6.0
Monoammonium phosphate	$NH_4H_2PO_4$	115.03	3.8–4.4
Diammonium phosphate	$(NH_4)_2HPO_4$	132.07	7.7–8.1
Ammonium sulfate	$(NH_4)_2SO_4$	132.14	5.0–6.2
Calcium chloride	$CaCl_2$	110.99	4.5–8.5
Calcium chloride dihydrate	$CaCl_2{\cdot}2H_2O$	147.02	4.5–8.5
Calcium nitrate	$Ca(NO_3)_2$	164.10	5.0–7.0
Calcium nitrate tetrahydrate	$Ca(NO_3)_2{\cdot}4H_2O$	236.15	5.0–7.0
Monocalcium phosphate hydrate	$CaH_4(PO_4)_2{\cdot}H_2O$	252.07	2.80–2.90
Tricalcium phosphate	$Ca_3(PO_4)_2$	310.20	sp. sol.[c]
Calcium sulfate	$CaSO_4$	136.14	sp. sol.[c]
Calcium sulfate dihydrate	$CaSO_4{\cdot}2H_2O$	172.17	sp. sol.[c]
Ferrous chloride	$FeCl_2$	126.76	2.95–3.05
Ferrous chloride decahydrate	$FeCl_2{\cdot}10H_2O$	306.87	3.45–3.55
Ferrous citrate	$FeC_6H_6O_7{\cdot}H_2O$	263.97	
Ferric citrate	$FeC_6H_5O_7$	244.9	sp. sol.[c,d]
Ferric citrate pentahydrate	$FeC_6H_5O_7{\cdot}5H_2O$	335.03	
Ferrous oxalate dihydrate	$FeC_2O_4{\cdot}2H_2O$	179.9	sp. sol.[c]
Ferric sulfate	$Fe_2(SO_4)_3$	399.88	Slowly hydrolyzes in water
Ferrous sulfate heptahydrate	$FeSO_4{\cdot}7H_2O$	278.02	4.05–4.15
Ferric tartrate	$Fe_2(C_4H_4O_6)_3$	555.9	sp. sol.[c,d]
Magnesium chloride	$MgCl_2$	95.03	4.5–7.0
Magnesium chloride hexahydrate	$MgCl_2{\cdot}6H_2O$	203.30	4.5–7.0
Magnesium nitrate hexahydrate	$Mg(NO_3)_2{\cdot}6H_2O$	256.41	5.0–8.2
Magnesium sulfate	$MgSO_4$	120.37	5.0–8.0
Magnesium sulfate heptahydrate	$MgSO_4{\cdot}7H_2O$	246.47	5.0–8.0
Potassium chloride	KCl	74.55	5.4–8.6
Potassium nitrate	KNO_3	101.10	4.5–8.5
Monopotassium phosphate	KH_2PO_4	136.09	4.1–4.5
Dipotassium phosphate	K_2HPO_4	174.18	9.0–9.4
Dipotassium phosphate trihydrate	$K_2HPO_4{\cdot}3H_2O$	228.23	9.0–9.4
Potassium sulfate	K_2SO_4	174.26	5.5–8.5
Sodium nitrate	$NaNO_3$	84.99	5.5–8.3
Monosodium phosphate monohydrate	$NaH_2PO_4{\cdot}H_2O$	137.99	4.1–4.5
Sodium sulfate	$NaSO_4$	142.04	5.2–9.2
Sodium sulfate decahydrate	$NaSO_4{\cdot}10H_2O$	322.19	5.2–9.2

[a]Formula weight = molecular weight + weight of water of crystallization where applicable.

[b]pH ranges are given only for salts readily soluble at the temperature and concentration of test solutions.

[c]Sparingly soluble.

[d]Use not recommended; chelated iron is preferable.

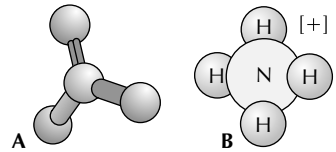


FIG. 4-2. **A. Nitrate. B. Ammonium. (Sources: A, www.chemsoc.org/examplarchem/entries/2001/anderson/acidityregulators.htm; B, www.fishdoc.co.uk/water/ammonia.htm.)**

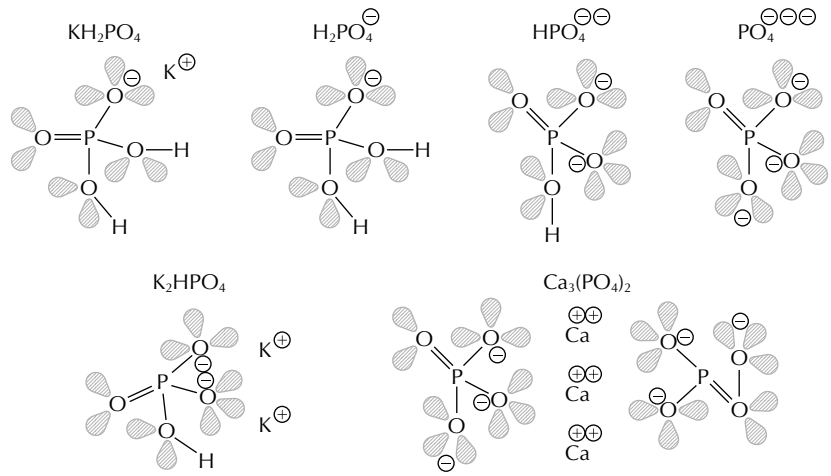


FIG. 4-3. **Phosphates. Dibasic potassium phosphate contains twice as much potassium as the monobasic salt, but the same amount of phosphorus. On a percentage basis K_2HPO_4 contains 44.89% potassium and 17.89% phosphate, whereas KH_2PO_4 has 28.73% potassium and 22.76% phosphate, but these weight-based calculations are not only irrelevant, but also bad science because what is important are the number of molecules or atoms of an element, not how much it/they weigh. (Source: www.jergym.hiedu.cz.)**

ammonium) from the culture medium was maximal at pH 5.0 (Lee et al., 1987). Plantlets of *Darwinara* Pretty Girl took up ammonium preferentially whereas those of *Dendrobium moniliforme* preferred nitrate (Kishi and Takagi, 1997*b*).

Phosphorus

This element is generally added to culture media as a monobasic (KH_2PO_4) and/or dibasic (K_2HPO_4) phosphate salt (Fig. 4-3). Phosphate is also added as calcium and ammonium salts (Fig. 4-3). The $H_2PO_4^-$ ion was absorbed completely by PLBs of *Dendrobium moniliforme* but not by those of *Darwinara* Pretty Girl (Kishi and Takagi, 1997*b*). The uptake of phosphate ions by *Dendrobium* Multico White is higher in the presence of sugar (0.32 mg day^{-1} and 33.3% of total) than in its absence (0.015 mg day^{-1} and 21.6% of total; Hew and Yong, 1997). A combination of the two salts can be used as a buffer with the pH being determined by the concentration of each salt. The German mycorrhiza expert Hans Burgeff seems to have been the first to suggest such a buffer for orchid seed germination media (Burgefff, 1936). However, it is still not clear if it is necessary to buffer media which support the growth of seedlings or plantlets. The pH of media which support seedlings or plantlets does drop, but in most cases this does not seem to have a deleterious effect.

Potassium

This element is supplied most often as salts containing the potassium ion (K^+), as for example potassium phosphate (K_2HPO_4 and KH_2PO_4; Fig. 4-3), potassium

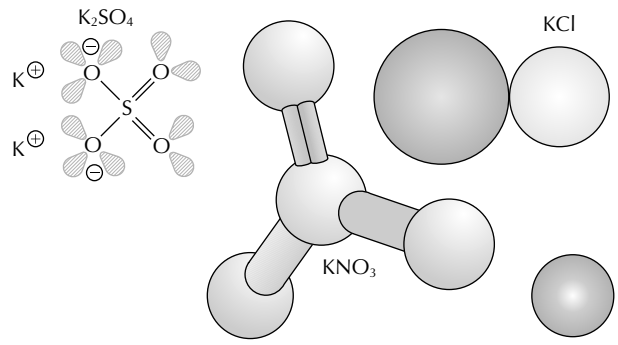


FIG. 4-4. **Potassium salts (sources: A, www.jergym.hiedu.cz; B. www.chemsoc.org).**

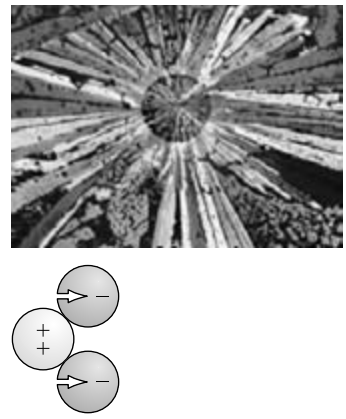


FIG. 4-5. **Magnesium salts (sources: www.mic-d.com; www.southwest.com).**

nitrate (KNO_3; Fig. 4-4), potassium chloride (KCl; Fig. 4-4), and potassium sulfate (K_2SO_4; Fig. 4-4). After 1 month of culture, PLBs of *Darwinara* Pretty Girl took up 40% of the potassium from the medium. Those of *Dendrobium moniliforme* absorbed only 54% (Kishi and Takagi, 1997*b*).

Magnesium

The bivalent cation Mg^{2+} is usually supplied as a chloride, nitrate, or sulfate salt (Fig. 4-5). PLBs of *Dendrobium monilforme* took up 24.2% of the magnesium from the medium during 1 month of culture. Those of *Darwinara* Pretty Girl used 83.5% during the same period (Kishi and Takagi, 1997*b*).

Calcium

A bivalent cation (Ca^{2+}), calcium is usually added as water-soluble nitrate or chloride salts (Fig. 4-6) but is also included in some media as the difficult to dissolve

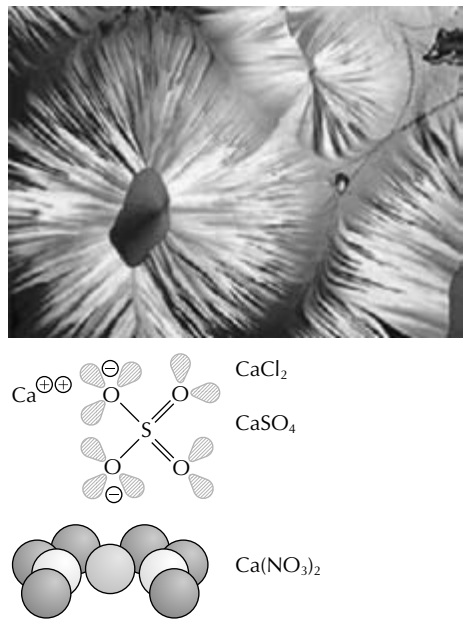


FIG. 4-6. **Calcium salts (sources: www.mic-d.com; www.jergym.hiedue.cz; www.saburchill.com).**

calcium phosphate [$Ca_3(PO_4)_2$] salt (Fig. 4-3). During 1 month of culture, PLBs of *Dawinara* Pretty Girl took up 70.5% of the calcium from the medium (Kishi and Takagi, 1997*b*). The levels of Ca^{2+} in a medium which supported *Dendrobium moniliforme* for the same period contained 119% of the initial level (Kishi and Takagi, 1997*b*).

Iron

This element is usually supplied as the ferrous (Fe^{2+}) or ferric (Fe^{3+}) sulfate salt (Fig. 4-7), and as chelated iron with ethylene diamine tetraacetic acid (EDTA) or other

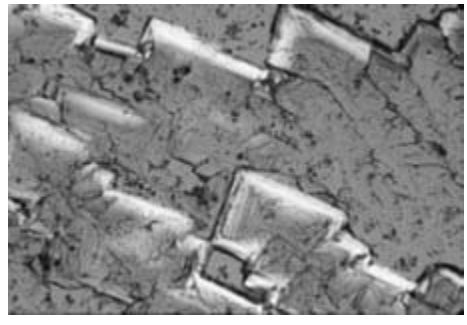


FIG. 4-7. **Ferrous sulfate (www.arsmachina.com/gallery%20micro.htm).**

chelating agents. In earlier media (those generally designed for seedling culture), iron was added as ferrous or ferric tartrate and citrate. These salts of hydroxycarboxylic acids were utilized prior to the advent of chelated iron. The sodium salt of ferric EDTA (NaFeEDTA) is a commonly used form of chelated iron. Chelation prevents the formation and precipitation of insoluble ferrous hydroxide [$Fe(OH_2)$], which can lead to a possible iron deficiency.

Microelements

The terms microelements or minor elements are based on the fact that plants require very small amounts of these minerals. Their importance in plant nutrition is not less than that of the macroelements. A number of culture media, especially older formulae such as Knudson C (Knudson, 1946) and Vacin and Went (Vacin and Went, 1949) solutions, include manganese as the sole microelement. Still other media (Knop, 1884; Morel and Muller, 1964) do not contain microelements. The reason for this is that the low required levels of these microelements are satisfied by the impurities present in nutrient salts and other media components. The same is true for Thomale GD medium (Thomale, 1954) which does not contain added microelements.

Calcium salts are also present as impurities of the agar employed in this and most other solid media. Presently supplied macroelements are much purer than those used decades ago, making the addition of microelements to seed germination media a proper and safe practice.

Micropropagation media in general are different from those used for orchid seed germination because they have included microelements since the earliest days of tissue culture. And, when orchid seed germination media are used for micropropagation, they are often supplemented with microelements. The most often used microelements are boron, chlorine, copper, manganese, molybdenum, sulfur, and zinc, but aluminum, cobalt, iodine, and nickel are also added to some media (Figs 4-8 and 4-9; Table 4-2).

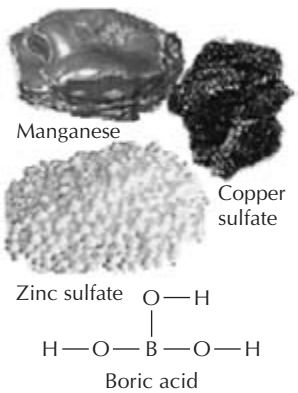


FIG. 4-8. **Some microelements (www.chemsoc.org; www.chss.montclair. edu; www.tetramicro.com; http://chemical.chem.Purdue.edu).**

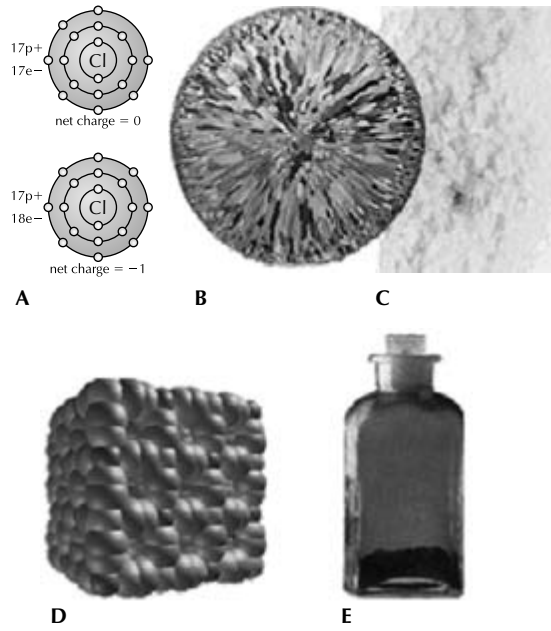


FIG. 4-9. **Five microelements. A. Chlorine atom (top) and ion (bottom). B. Molybdenum ingot. C. Sulfur powder. D. Cobalt molecule. E. Iodine, solid and sublimated. (Sources: A, academic.brooklyn.cuny.edu; B, www.molybdenum.com; C, www.asarco.com; D, www.itg.uiuc.edu; E, www.lighthouse.chtr.k12.ma.us/periodic.)**

TABLE 4-2. **Microelements and their formula weights as employed in the tissue culture of orchids**

Microelement	Formula	Formula weight[a]
Aluminum chloride	$AlCl_3$	133.34
Aluminum chloride hexahydrate	$AlCl_3 \cdot H_2O$	241.43
Ammonium molybdate tetrahydrate	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1235.86
Boric acid	H_3BO_3	61.83
Cobalt chloride hexahydrate	$CoCl_2 \cdot 6H_2O$	237.93
Cobalt nitrate hexahydrate	$Co(NO_3)_2 \cdot 6H_2O$	291.03
Cobalt sulfate heptahydrate	$CoSO_4 \cdot 7H_2O$	281.1
Cupric chloride dihydrate	$CuCl_2 \cdot 2H_2O$	170.48
Cupric nitrate di/trihydrate	$Cu(NO_3)_2 \cdot 2.5H_2O$	232.59
Cupric sulfate	$CuSO_4$	159.60
Cupric sulfate pentahydrate	$CuSO_4 \cdot 5H_2O$	249.68
Manganese chloride	$MnCl_2$	125.84
Manganese chloride tetrahydrate	$MnCl_2 \cdot 4H_2O$	197.90
Manganese nitrate	$Mn(NO_3)_2$	178.95
Manganese sulfate	$MnSO_4$	151.00
Manganese sulfate hydrate	$MnSO_4 \cdot H_2O$	169.01
Nickel chloride hexahydrate	$NiCl_2 \cdot 6H_2O$	237.71
Nickel nitrate hexahydrate	$Ni(NO_3)_2 \cdot 6H_2O$	290.81
Nickel sulfate hexahydrate	$NiSO_4 \cdot 6H_2O$	262.84
Potassium iodide	KI	166.00
Sodium molybdate dihydrate	$NaMoO_4 \cdot 2H_2O$	241.95
Zinc chloride	$ZnCl_2$	136.29
Zinc sulfate heptahydrate	$ZnSO_4 \cdot 7H_2O$	287.54
Zinc sulfate hydrate	$ZnSO_4 \cdot H_2O$	161.44

[a]Formula weight = molecular weight + weight of water of crystallization where applicable.

Aluminum

This element is added very rarely, usually as aluminum chloride.

Boron

This element is commonly supplied as boric acid (H_3BO_3; Fig. 4-8). It is required in very minute amounts and is toxic to most plants at higher levels.

Chlorine

Any chloride salt present in media will satisfy the need for this element (Fig. 4-9). Plantlets of *Dendrobium moniliforme* took up 74.5% of the chloride from the medium during 1 month of culture (Kishi and Takagi, 1997*b*). During the same period, plantlets of *Darwinara* Pretty Girl took up 29.7% percent of the Cl^- (Kishi and Takagi, 1997*b*).

Cobalt

This element(Fig. 4-9) is added as water-soluble cobaltous (Co^{2+}) chloride, sulfate, or nitrate.

Copper

Salts in which this element is present as Cu^{2+} are usually employed, the most common being copper sulfate (Fig. 4-8).

Iodine

Potassium iodide (KI) is a component of the frequently used and well-known Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) and other media.

Manganese

This element (Fig. 4-8) is added to media most often as a sulfate, but chloride and nitrate salts can also be used.

Molybdenum

Usually added as sodium molybdate (Na_2MoO_4), this element (Fig. 4-9) has also been incorporated as molybdenum trioxide (MoO_3).

Nickel

When included in media (which is rarely), nickel is added as a chloride, nitrate, or sulfate salt.

Sulfur

Media contain generally one or more sulfate salts that fully satisfy the needs for this element (Fig. 4-9).

Zinc

The sulfate salt (Fig. 4-8) is generally used, but the chloride salt can also be added.

Substitutions

Should substitutions of either micro- or macroelement salts be needed, they should be made on a molar, not weight, basis. Equal weights of salts of different molecular weights will not supply the same levels of the required minerals (see tables in Chapter 2). Any suggestions that equal weights are appropriate for substitutions are not only erroneous, they are also bad science. And, even more importantly, equal weight substitutions may result in improper or even damaging levels (sub- or supraoptimal concentrations) of a mineral. Another factor to consider in making substitutions is the other substance in a molecule because it may be toxic, undesirable, or dangerous.

Carbohydrates

Most orchid seed and tissue culture media require the addition of sugar as a source of carbon. The disaccharide sucrose (Fig. 4-10) is an entirely satisfactory and inexpensive carbon source (kitchen grade sucrose can be used for practical purposes). Its monomeric components and products of hydrolysis, glucose and fructose (Fig. 4-10), are used frequently singly or in combination. After supporting a culture of *Dendrobium moniliforme* plantlets, a medium contained more glucose and fructose than initially (131.1 and 217.6%, respectively). A medium that supported *Darwinara* Pretty Girl contained 324.9 and 253.7% (Kishi and Takagi, 1997*b*). The relative usefulness of a large number of carbohydrates for orchid culture media has been investigated (Ernst, 1967*a*). Some of them, particularly galactose, have been found to be toxic to orchids (Quednow, 1930; Ernst et al., 1971*b*). Sugar levels in orchid tissue culture media differ but are generally 2–5%. In several procedures, some steps are even carried out without added sugars (Intuwong and Sagawa, 1974).

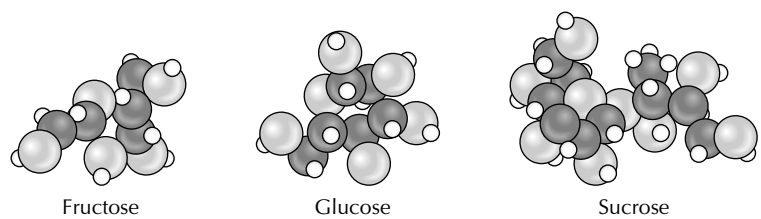


FIG. 4-10. **Sugars (source: www.iconbazaar.com).**

Since the osmolarity of solutions can affect the well being of explants it is very important to keep in mind that equal weights of sucrose and glucose or sucrose and fructose represent different molarities and therefore affect the concentrations of solutions differently. To put it differently, equal weights of sucrose and glucose or glucose and fructose are not the same in terms of concentration because they represent different molarities. Equal weights of glucose and fructose are the same in terms of molarity and concentration.

On the whole, experimental evidence shows that orchid plantlets are heterotrophic under culture conditions (for excellent reviews see Hew and Yong, 1997, 2004). Sugar uptake by *Aranda* and *Dendrobium* tissues shows linear kinetics and "is a function of initial sugar concentration" (Hew and Yong, 1997). The only word which should be added to this statement is "available" before the word "initial" because if larger sugar oligosaccharide molecules are present the tissue probably cannot take them up until after hydrolysis. One of the very best statements regarding the need, utilization, and uptake of sugars by orchid tissue can be found in a book on orchid physiology (Hew and Yong, 1997). Parts of it are quoted here with Professor Hew's permission.

> The overall rate of sugar uptake by orchid callus tissues is determined to a large extent by their surface area-to-volume ratio. The importance of sugar uptake and its subsequent utilization for growth in orchid callus tissues is demonstrated in sugar uptake kinetics studies. *Aranda* callus shows a high specific rate of glucose uptake when grown in glucose-containing media. Depending on its concentration, the rate of glucose uptake is 10–100 times higher than the specific biomass growth rate. This suggests that glucose accumulates in the cells more rapidly than it can be used for growth.

This view is substantiated by the accumulation of starch in protocorms.

> Orchid tissues of different genera show different affinities for the various sugars and this makes formulation of a standard solution difficult.

Proof of this is the preference shown by *Paphiopedilum* for fructose.

Substitution of sugars should be based on the ability of orchids to take up or break down sugar molecules (for example orchids can utilize maltose easily, but not longer glucose chains like maltopentaose), the suitability of all sugars in a molecule (e.g., orchids can utilize the glucose molecule in lactose, but the galactose moiety is toxic), molarity (equal weights of monosaccharides like glucose or fructose and disaccharides such as sucrose or maltose are not the same in terms of their effects on the properties of solutions), and preferences by specific orchids (*Paphiopedilum* seeds and seedlings prefer fructose).

Amino Acids

The amino acid glycine (Fig. 4-11) is a component of the well-known MS medium (Murashige and Skoog, 1962) and many of its modifications. Other amino acids which are added to media include (but are not limited to) isoleucine, glutamic acid, and asparagine. The fact that many orchid tissue culture media do not contain added

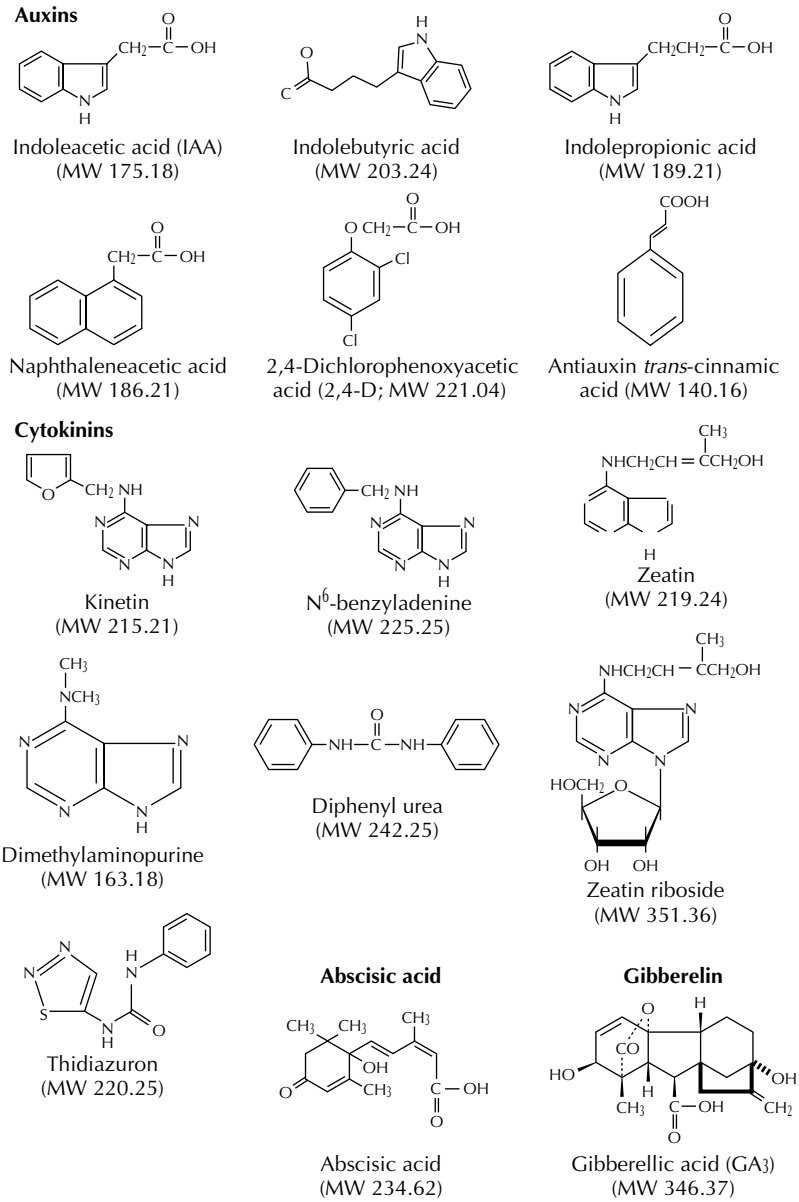


FIG. 4-11. **Vitamins, an amino acid, plant hormones and other substances which are added to media.**

amino acids raises a question as to whether they are really needed. However, it is recommended that they be added where called for in specific media. Their concentrations are generally less than 10 ppm.

To protect these compounds as well as vitamins and hormones from heat destruction, it is best to add them to autoclaved media as ethanol solutions or as

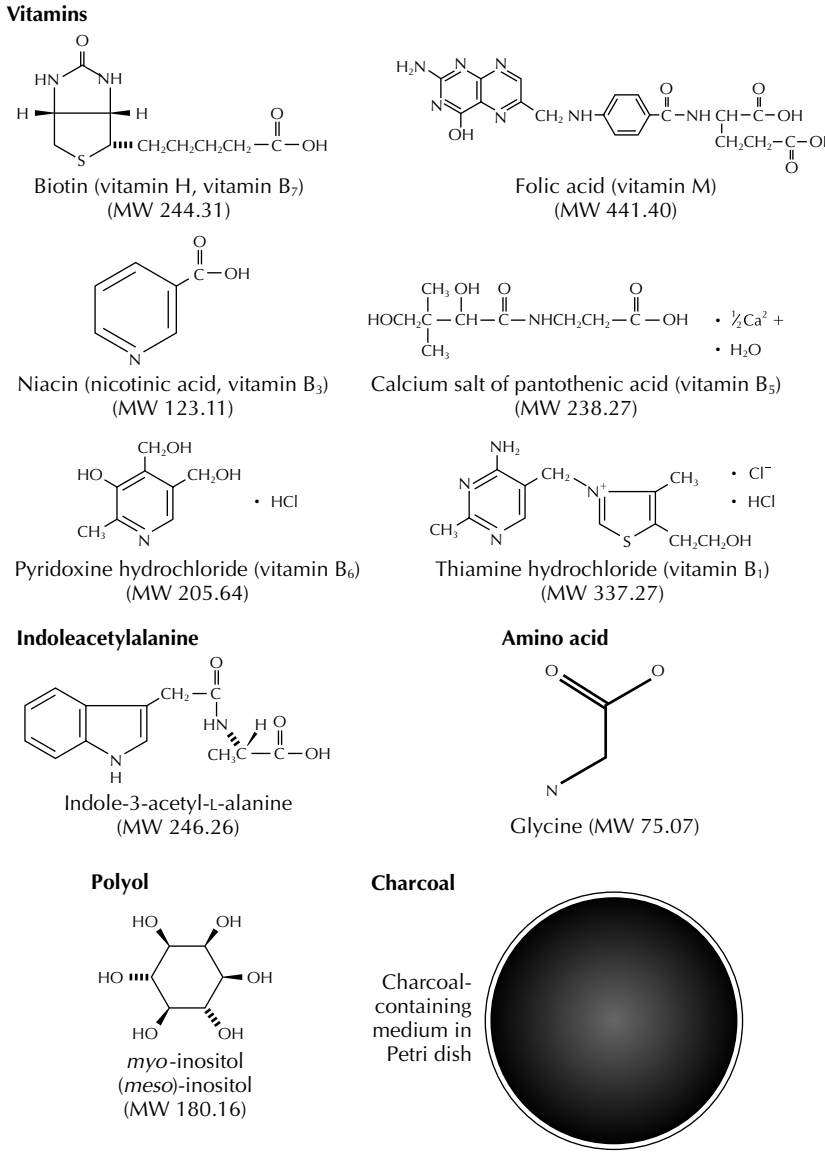


FIG. 4-11. (***Continued***)

cold-sterilized aqueous solutions. Additions should be made under sterile conditions and while the autoclaved media are still liquid to insure good dispersion on agitation.

Amino acids, vitamins, and/or hormones are often added to media before autoclaving. This suggests that: (1) they are not destroyed by the heat and the pressure; (2) a proportion of the added amino acid(s) is (are) destroyed, but the remainder is sufficient for the explants; (3) the destruction product(s) is (are) as effective as the

amino acid(s); and/or (4) all of the added amino acid(s) is (are) destroyed, but this makes no difference because it (they) is (are) not really needed.

Substitutions of amino acids should not be made (or must be made only after prior testing) because some of them may have a specific effect which another amino acid cannot replace.

Vitamins

Absolute requirements for vitamin additions to all or even some orchid media have not been established; as with amino acids, many media do not contain them. However, a large number of media include niacin (nicotinic acid), pyridoxine (vitamin B_6), or thiamine (vitamin B_1; Fig. 4-11). The latter two are added as hydrochlorides. Niacin has been reported to give a slight growth improvement in the culture of *Cymbidium* (Fonnesbech, 1972*b*). A small number of media contain biotin, pantothenic aid, folic acid, and other vitamins. According to Koch (1974*a*), PLBs of *Phalaenopsis* could be cultured for an extended time only if the medium (modified Knudson C) contained pantothenic acid (0.5 ppm); niacin, pyridoxin, and thiamine (0.2 ppm each); and glycine (5 ppm). Regardless of whether requirements for vitamins have been established with certainty or not, they should always be added if a specific recipe includes them. Vitamins are usually destroyed by excessive heat (see the discussion of heat-labile compounds in the section above, Amino Acids).

Vitamins have specific effects and cannot replace each other.

Polyols

The polyalcohol *myo*-inositol (Fig. 4-11), a constituent of coconut liquid endosperm (Pollard et al., 1961), is added to a number of tissue culture media (Murashige and Skoog, 1962; Linsmaier and Skoog, 1965; Nitsch and Nitsch, 1966; Schenk and Hildebrandt, 1972; Anderson, 1978; Gamborg, 1986). Yet, numerous successful tissue culture media do not contain *myo*-inositol. It is considered to be part of the vitamin B_1 complex and is involved in cell wall biosynthesis (Loewus, 1965, 1969). It has been reported that the addition of 100 ppm *myo*-inositol to a culture medium improved growth of *Cymbidium*, but the increase was not significant (Fonnesbech, 1972*a*). Still, it is advisable to include *myo*-inositol in media formulae that suggest it as a component, unless experiments show it not to be useful. If it is required, inositol has specific effects and cannot be replaced by another polyol.

Mannitol, a sugar alcohol, is widely used to prepare isotonic media for the culture of protoplasts. Usually 0.4–0.7-*M* solutions of mannitol are employed, depending on the osmotic potential of the cell line.

Hormones

Most orchid tissues require auxins, cytokinins, and other plant hormones (Fig. 4-11) for growth, the formation of callus or PLBs, proliferation, and plantlet development.

Where both of these hormones are present the ratio between them is usually of critical importance. For example, auxins induce root initiation at high levels, the elongation of roots at low concentrations, and stimulate shoot elongation; whereas cytokinins promote cell division.

Early work involving the tissue culture of *Cymbidium* was carried out using media without added hormones (Morel, 1960, 1964*a*, 1964*b*; Wimber, 1963), showing that not all orchid tissues require them. Indeed, many of the media listed in this book contain no hormones or include only one of them. The original paper describing the widely used MS medium lists a range of 1 to 30 ppm indoleacetic acid (IAA) and 0.4 to 10 ppm kinetin. The upper concentration proposed for the auxin may be too high for orchid tissue culture.

Auxins

Auxins commonly utilized in tissue culture are the naturally occurring indoleacetic acid (IAA) and the synthetics indolebutyric acid (IBA), α-naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D, a herbicide). The synthetics are less subject to enzymatic degradation than is endogenous IAA and therefore persist in culture media for longer periods. For this reason they can be employed at lower levels. Auxins are generally added at levels of about 1–10 ppm, but other concentrations are also used. It is most convenient to add ethanolic solutions to previously autoclaved media, but in many cases auxins are added before autoclaving without deleterious effects.

IAA at 10 pmol l^{-1} (1.8 ppm) produced optimal fresh weight and vigorous PLBs in *Cymbidium* tissue culture (Fonnesbech, 1972*a*). When 2,4-D was used at 1 pmol 1^{-1}, weight increase was high but the PLBs were abnormal (Fonnesbech, 1972*a*). Optimal concentrations of NAA or 2,4-D were 0.1 ppm (Ichihashi and Kako, 1973).

Vanda tissues form roots on 1.25 ppm NAA (Payawal and de Guzman, 1972). On the other hand, *Phalaenopsis* roots produced PLBs on modified MS medium without the addition of auxin or cytokinin (Tanaka et al., 1976).

Shoot formation on *Phalaenopsis* flower-stalk nodes was reduced by the addition of 2 ppm NAA to Knudson C medium (Tse et al., 1971). IAA, NAA (as a potassium salt), or 2,4-D increased initial shoot length from *Phalaenopsis* flower-stalk nodes but inhibited subsequent development (Koch, 1974*a*). However, the combined addition of NAA (0.5–3.0 ppm potassium salt) and 2 ppm benzyladenine (BA) induced the formation of PLBs on thickened basal leaf sections of *Phalaenopsis* flower-stalk buds (Koch, 1974*a*). Care should be exercised with 2,4-D since it may cause mutations.

Acetylamino Acids

Conjugates of the amino acid alanine with IAA (IAA-ala) or of glycine with IAA (IAA-gly) were used as substitutes for the combined addition of auxins and cytokinins (?) in clonal propagation of *Phalaenopsis* from flower-stalk nodes. These conjugates were employed in a PLB proliferation step and could replace conventional auxin–cytokinin combinations (Griesbach, 1983). The question, of course, is why use exotic and costly chemicals when common and inexpensive ones can be equally

effective or more so. There are no good, compelling, scientific, practical, and/or logical reasons for the use of IAA-ala and IAA-gly.

Anti-auxin

The antiauxin effects of *trans*-cinnamic acid (*t*CA) were first observed during the middle of the last century (van Overbeek et al., 1941). *Dendrobium* buds could be released from apical dominance in culture media containing *t*CA and the cytokinin BA (Mosich et al., 1974*b*). The highest shoot development was obtained with 14.8 ppm *t*CA and 2 ppm BA. No shoot development occurred in the absence of anti-auxin. Root development was inhibited but could be initiated by transfer of the shoot-bearing explant to media that were free of these hormones but contained 15% (w/v) banana homogenate (Mosich et al., 1974*b*). A similar release from apical dominance was observed in *Phalaenopsis* flower-stalk buds on Knop's medium containing 1.48–14.8 ppm *t*CA and 2 ppm BA (Ball et al., 1974/75). Later, a "keiki paste" was developed containing 50 mg *t*CA and 5 mg BA per milliliter of lanolin (Griesbach, 1984). However, the high cytokinin content of this paste would be expected to induce plantlet formation on flower-stem buds on its own. In fact most "keiki pastes" do not contain *t*CA and work well. The reason for the formulation of a *t*CA-BA "keki paste" is not immediately apparent and does not seem to make much, if any, sense.

Cytokinins

Representative members of these hormones most often used in tissue culture media are kinetin (N^6-furfuryl adenine), benzylaminopurine (N^6-benzyladenine, BAP, or BA), zeatin [6-(4-hydroxy-3-methyl-but-2-enylamino) purine], zeatin riboside, 6-(dimethylallylamino) purine, and to a lesser extent 1,3-diphenylurea. Some cytokinin activity is exhibited by adenine (aminopurine) itself. Thidiazuron (TDZ), a compound first introduced as a defoliant and found to be a potent cytokinin (Mok et al., 1982; Malik and Saxena, 1992), is being used increasingly in orchid micropropagation media.

Kinetin, which is not a naturally occurring cytokinin, was isolated from herring sperm DNA (Miller et al., 1955*a*, 1995*b*). The first endogenous adenine-derived cytokinins identified were zeatin riboside and its 5′ monophosphate (Letham, 1968). Many substituted adenines were tested for cytokinin activity and the synthetic N^6-benzyladenine (BA) is the most widely used at present. Zeatin and zeatin riboside, both hormones contained in coconut liquid endosperm, are 10–100 times more active than kinetin in a number of growth assays (Letham, 1967, 1968). Cytokinins such as BA are added to orchid tissue culture media at levels below about 10 ppm, although higher concentrations have been used in specific procedures. Extremely high concentrations (up to 5000 ppm) are contained in the so-called "keiki pastes" (Zimmer and Pieper, 1979; Zimmer, 1980).

Root and shoot growth in *Cymbidium* were inhibited by all concentrations of kinetin that were tested (Fonnesbech, 1972*a*). On the other hand, 1–4 ppm kinetin enhanced PLB formation (Payawal and de Guzman, 1972). However, when kinetin is used in combination with NAA in *Vanda* culture, each hormone seems to counteract the other's effects (Payawal and de Guzman, 1972). Shoot development of *Phalaenopsis* flower-stalk nodes was best when a modified Knudson C medium was

supplemented with 1–3 ppm BA (Koch, 1974*a*). Multiple plantlets on *Phalaenopsis* flower-stalk nodes can be obtained with BA levels of 25–125 ppm in vitro. Shoots obtained in this manner must be transplanted onto hormone-free media, preferably containing banana homogenate, to develop roots (Ernst, 1984).

Leaf tissue of *Phalaenopsis* plantlets obtained from flower-stalk nodes can be induced to produce PLBs on a medium containing 1–5 ppm BA (Tanaka and Sakanishi, 1978).

TDZ has been shown to be very effective for a number of orchids including *Cymbidium sinense* (J. T. Chen et al., 2000*b*), *Dendrobium nobile* (Ferreira and Kerbauy, 2002), *Doritaenopsis* (Ernst, 1994), *Phalaenopsis* (Ernst, 1994; Chen and Piluek, 1995), *Vanda coerulea* (Malabadi et al., 2003), and a number of other orchids.

Some hormones are heat-sensitive (see the discussion of heat-labile compounds above in Amino Acids). However, they are often added prior to autoclaving.

Ethylene

This gaseous hormone is not added to orchid tissue cultures and there is no evidence that an exogenous source is required. Ethylene accumulates in the head space of cultures (Hew and Yong, 1997), but if it has any effects they are not obvious.

The effects of hormones (even those which belong to the same group) on particular orchids or explants seem to be specific. Therefore, substitutions or changes in concentrations should not be made without prior testing.

Coconut Water (Coconut Liquid Endosperm, "Coconut Milk")

Several dictionaries of the English language and continued misuse notwithstanding, the correct term for the clear liquid endosperm of coconuts is not coconut milk. It is coconut water (CW). Coconut milk is the milky liquid obtained by grating or squeezing the solid white endosperm, which is also known as copra. Coconut milk is not used as a tissue culture additive.

The first use of CW as an effective tissue culture medium component goes back many years (van Overbeek et al., 1941; for an historical account see Arditti and Krikorian, 1996). It was used extensively in clonal multiplication of carrots from root tissue (Caplin and Steward, 1948; Steward and Caplin, 1952*b*). The first use with orchids was in *Cattleya* seedling cultures (Mariat, 1951). In orchid seed culture of *Paphiopedilum* and *Vanilla*, the combined addition of 10% CW and 3 ppm IBA to nutrients had a stimulating effect (Hegarty, 1955). *Phalaenopsis* seedlings developing on Knudson C medium modified with 15% CW showed substantial proliferation, which did not occur with the addition of numerous other fruit endosperms tested (Ernst, 1967*b*).

The first successful use of CW in orchid tissue culture was also at 15% (v/v) in a medium for shoot tips of *Phalaenopsis* (Intuwong and Sagawa, 1974). Later a liquid Vacin and Went medium modified with 20% CW was used to proliferate PLBs from leaf tissue of *Phalaenopsis* flower-stalk plantlets (Tanaka and Sakanishi, 1977, 1980). Much larger additions of CW (53%) were used in a private micropropagation laboratory in Germany by Dr. Norbert Haas-von Schmude and Mrs. Gudrun Haas-von Schmude (Haas-von Schmude, 1983) in otherwise hormone-free

MS medium for the proliferation of *Phalaenopsis* PLBs. The latter were also differentiated on a similar solid nutrient but with reduced levels (20%) of CW (Haas-von Schmude, 1983).

CW contains a large spectrum of biochemicals that can act as growth factors individually or synergistically (Shantz and Steward, 1952). One such substance isolated from 700 gallons of coconut liquid endosperm was 1,3-diphenylurea (*N*, *N′*-diphenylurea, carbanilide), which shows cytokinin-like activity (Shantz and Steward, 1955). Later, two adenine-type cytokinins, zeatin riboside (Letham, 1968; van Staden and Drewes, 1975) and zeatin (van Staden and Drewes, 1975), were shown to be present in this endosperm. As is the case with several natural products, the activity of CW may depend on such factors as age (whether the coconut is green or ripe), season, and source. Autoclaving does not affect the active principle(s) in CW.

Some workers may not be able to obtain CW from green nuts but liquid endosperm from brown mature nuts (Fig. 4-12A–C) can also be used. In some parts of the USA there are food stores which sell unripe (green) coconuts that are trimmed and partially peeled. These nuts are white and have a conical top and a flat base (Fig. 4-12D). If not available locally from stores, markets, or directly from coconut

FIG. 4-12. **Coconuts. A. Brown mature nut with the typical three "eyes." A hole for draining the liquid endosperm can easily be made by forcing a screwdriver through a soft "eye." B. Mature brown nut which has been cut open to show the white copra. C. A mature nut. D. Trimmed and peeled immature nuts. E. Immature nuts cut open. The copra in such nuts is soft and gelatinous, the backgroundshows immature unpeeled nuts. (Sources: A, www.otan.us; B, www.rpi.edu; C, www.foodsubs.com; D, E, www.thaitradesource.com.)**

plantations, CW can be obtained from suppliers of tissue culture media and their components.

The easiest and simplest method for draining a coconut is to poke a hole with a screw driver through one of the "eyes" (Fig. 4-12A), which is very soft, and invert the nut over a large funnel lined with filter paper made of several layers of cheese cloth. If the "eye" is hard, a drill or hammer and nail can be used to make a hole. To facilitate pouring, a second hole (vent) should also be made. Coconuts should be opened and poured one at a time or into separate containers because the liquid endosperm in some nuts may have fermented or become contaminated. Liquid which is brownish or smelly should be discarded.

Opinions and practices regarding the preparation of CW vary. Some workers use the water after it has been drained and filtered. Others prefer to boil the water to precipitate proteins, allow the liquid to cool, filter it again, and use the filtered CW (Dodds and Roberts, 1995). Both methods seem equally effective.

No other additive can be used in place of CW (but see Bleeding Sap of Birch Trees below). Coconut milk (i.e., the liquid obtained from grated copra, by squeezing it or as an extract) cannot be used as a CW substitute. Banana homogenate, taro extract, potato extract, tomato or apple juice, and other plant homogenates, extracts, or juices may have beneficial effects for some orchids or explants, but they must be established experimentally and tested carefully before extensive use.

Bleeding Sap of Birch Trees

Due to difficulties in obtaining CW in Europe, it was replaced with the bleeding sap of birch trees (BSBT) in the proliferation of *Phalaenopsis* tissues from plantlets derived from flower-stalk nodes (Zimmer and Pieper, 1978). Even if BSBT is as effective as CW (which may or may not be the case), it is much more difficult to find.

Other Complex Organic Additives

Many complex additives to seed and tissue culture media have been used, but comparative data are insufficient to permit a discussion of their effects (Arditti, 1977*b*). A limited number of these additives has been compared in the seed culture of *Phaleanopsis* (Ernst, 1967*b*), *Cymbidium*, *Paphiopedilum*, *Dendrobium*, and a few species of other genera (Kano, 1965). Additives other than coconut endosperm include banana homogenate (which enhances the growth of plantlets and seedlings), peptone (a byproduct of the digestion of meat protein by the enzyme pepsin), tryptone (a peptone produced by the action of trypsin), casein hydrolysate, taro (*Colocasia esculenta*) extract (all five are used to enhance the growth of explants), tomato juice (which must be filtered to remove seeds and can be used to stimulate the growth of plantlets and seedlings), and a number of other fruit homogenates and extracts. The reasons for the effects of these additives are not known. It is also not known whether the effects are due to the same or different factors. But, all this does not really matter from a practical point of view. If an additive can bring about the desired results, it should be used.

Solidifying (Gel-forming) Agents and Alternative Supporting Media

Agar was the principal gel-forming agent used in the seed and tissue culture of orchids. Commercial agar contains a wide variety of organic and inorganic impurities, and some of the cruder grades have as much as 6% ash content. For research purposes it is best to use purified grades with an ash content of 2% or below. United States Pharmacopeia (USP) purity grade contains a maximum 1% each of foreign organic and foreign insoluble matter. When using the purified grade of agar, 0.6–9 g l^{-1} are needed to solidify media, depending largely on the firmness of gel desired. Almost any agar, including kitchen grade, can be used for practical micropropagation (provided of course it is not toxic or inhibitory to the plants being propagated). In some cases kitchen grade may even be preferable simply because it is much less expensive.

More recently, gellan gum is used, available as Phytagel (trade named product sold by Sigma Chemical Co.) or Gelrite; it is a hydrocolloid produced by the microorganism *Sphingomonas elodea*. It is composed of glucuronic acid, rhamnose, and glucose. The gel it produces is clear and of high strength. To prevent clumping it should be added to solutions at room temperature with rapid stirring. The amounts which should be used are those recommended for each procedure. Typically, concentrations recommended for plant media are 1.5–2.5 g l^{-1}, but some media may require higher or lower levels. Gellan gum (Phytagel or Gelrite) requires the presence of divalent cations such as Ca^{2+} or Mg^{2+} for gelling to take place. Most micropropagation media contain sufficient amounts of these cations for gelling.

In the absence of experimental evidence, it is not clear at present if the solidifying agent makes a difference for some explants and tissues. This being the case it is best to use the solidifier recommended for each procedure. If a change is desired it is best to proceed with caution and only after preliminary tests.

A mineral inorganic product first discovered in the Hawaiian islands as part of formations created by volcanic activity, is the basis of rockwool (Fig. 4-13), a horticultural product made either from rock only or from a mixture of rocks, limestone,

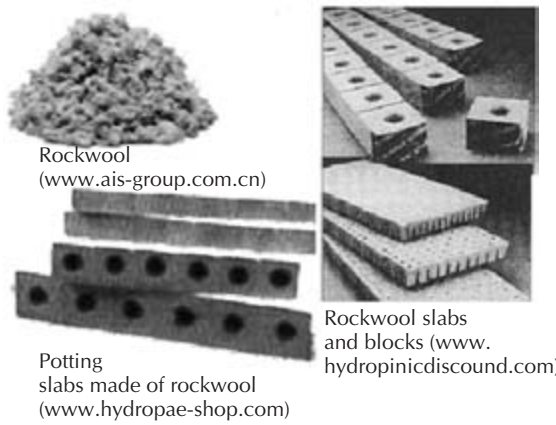


FIG. 4-13. **Rockwool.**

and coke. The components are melted at temperatures of around 1400°C (about 2500°F). This molten solution is poured over a spinning cylinder, flies away from it, elongates, and forms fibers as it cools. These fibers, called rockwool, can be granulated or pressed into sheets, cubes, or blocks. Granulated rockwool can be added to soil mixes as an amendment or used as support for older plantlets in culture (Hew and Yong, 1997).

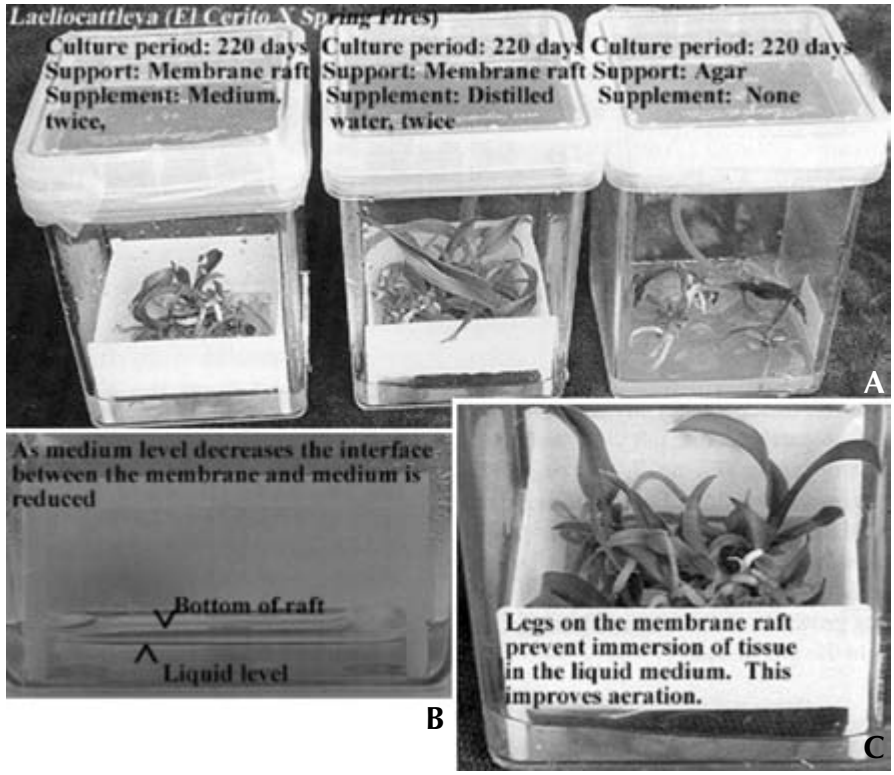
Root growth in *Phalaenopsis* was reported to be better in rockwool than on agar for reasons which are not clear (Hew and Yong, 1997). If the plants are grown in multi-blocks, transplanting to pots is less of a shock because the roots remain intact (Hew and Yong, 1997). However "more work is needed to evaluate the suitability of rockwool as an alternative method for . . . genera such as *Dendrobium*, *Aranda* and *Mokara*" (Hew and Yong, 1997).

Aeration improved growth when seedlings, protocorms, and explants of *Cattleya*, *Cymbidium*, *Dendrobium*, *Epidendrum*, and *Laeliocattleya* were cultured on microporous polypropylene membranes (as, for example, the Osmotek Life Raft™). The use of rafts also renders subculturing easier.

As mentioned and illustrated above (see Fig. 4-1), membrane rafts are a good support for the culture of explants, tissues, PLBs, and plantlets. Excellent research done since about 1990 by Dr. Jeffrey Adelberg and his associates at Clemson University has shown that proliferation on a membrane, rather than on agar, resulted in more harvestable plants. The plants were also larger (Fig. 4-14) and flowered earlier. Also, plants on membranes did not require regular transfers to fresh medium. Instead, adding water or medium could sustain and accelerate growth. Utilization and uptake of medium by the plants, as well as evaporation, tended to reduce or eliminate contact with the membrane and required periodic additions (Adelberg et al., 1991, 1992, 1997*b*, 1998; Adelberg and Darling, 1992; Young, 1994).

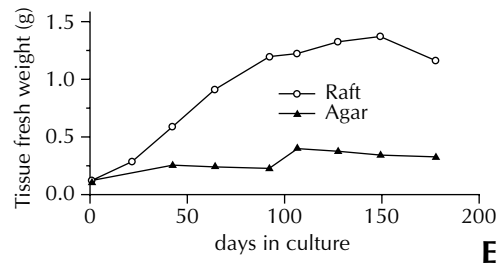
Adsorbents

Polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone resins have been suggested as additives to tissue culture media for the absorption of toxic metabolites such as polyphenol oxidates and other phenolics that may be produced by explants. Some of these substances are dark and easily visible and unsightly on agar- or gellan-gum-solidified media. Activated charcoal (AC), described in detail in Chapter 2, may also serve this purpose, among other beneficial effects. It also renders the dark spots invisible. The problem with both PVP and AC is that they can absorb culture medium components such as hormones and vitamins, thereby reducing their concentrations to subphysiological levels. Because of this, PVP and AC should be used with caution. Sometimes problems created by explant exudates can be eliminated or reduced by moving the tissues from a dark area on the medium to a clear spot. It may be necessary to do this a few times or even move explants to a new vessel before they cease to exude the offending substances.



Size range (g)	Number of plants Membrane	
	Float	Agar
+1.0	3.4	0.4
0.5–1.0	3.4	0.8
0.1–0.5	6.2	4.4

D



E

FIG. 4-14. **Utilization of membrane rafts for the culture and micropropagation of orchids. A. *Laeliocattleya* being cultured. B. As the medium level decreases, the interface between the membrane and medium is reduced. C. The legs on the membrane raft improve aeration. D. Number of plants of *Brassolaeliocattleya* Raye Holmes 'Mendenhall' per vessel produced during 16 months of culture. E. Fresh weight gains by *Laeliocattleya* Raye Holmes 'Mendenhall' on membrane rafts and on agar. (Adelberg et al., 1992.)**

Applications of Orchid Tissue Culture

It is not at all surprising that the culture of shoot tips, and subsequently of other explants of orchids, created considerable interest almost immediately (even if credit for the discovery went to the wrong person; see Chapter 1). The potential of these methods for mass clonal propagation and biotechnological applications was, and still is, immense. A large number of articles on the subject have been published over the years. Some researchers explained the procedures and described their background (for example, Murashige, 1962; Butcher and Ingram, 1976; Laetsch, 1967; Attawar, 1992; Ichihashi, 1997; Vij et al., 2000*a*); others have dealt with methodology (Teo, 1978; Warren, 1983; for reviews also see Arditti and Ernst, 1993; Dodds and Roberts, 1995); one embarrassingly bad pamphlet sought to exploit procedures which were novel at the time (Jasper, 1966) and was accorded the oblivion it richly deserved. There was also a self important report of an individual's personal experiences (Rutkowski, 1967). Some reviews were boastful but not very informative (Goh, 1996), not very useful (Goh, 1989), or telegraphic consisting of one (Sagawa and Kunisaki, 1982), six (Sagawa and Kunisaki, 1984; Griesbach, 1986), or seven (Tisserat and Jones, 1999) pages. Reviews have been published in Chinese and Malay (Chen, 1985), English (Morel, 1974; Arditti, 1977*b*; Holdgate, 1977; Rao, 1977; Teo, 1978; Arditti and Goh, 1981; Goh, 1983*b*; Griesbach, 1986; Hew and Yong, 1997), French (Bouriquet, 1986), German (Hahn, 1970; Fast, 1980*a*, 1980*b*), Japanese (for a review see Ichihashi, 1997), Russian (Cherevchenko et al., 1982; Cherevchenko and Kushnir, 1986), and many other languages. These and other publications have helped both amateur and professional growers to establish tissue culture laboratories, which revolutionized the industry.

Automation was introduced as the industry developed. A flow system first became available in 1982. It agitated cultures with air (Hew and Yong, 1997, 2004). This was followed by systems involving pumps and tubing (Fig. 4-15A; Tisserat and Vandercook, 1985) and computers (Hew and Yong, 1997). More elaborate systems followed (Fig. 4-15B, C; Okamoto, 1996; Adelberg et al., 1998; Paek et al., 2001). A commercial system (manufactured by Osmotek Ltd, www.osmotek.com) is also available (Fig. 4-15D). These systems can be useful for some laboratories and will undoubtedly become increasingly important, but they are not very common at present perhaps because much of the orchid micropropagation industry is in low wage countries.

Commercialization

By its very nature, micropropagation is a process well suited for commercialization. Because of that it is not surprising that commercial micropropagation laboratories exist in all parts of the world (Hew, 1994; Pamfil, 1996; Govil and Gupta, 1997, 2001; Ichihashi, 1997; Pathak et al., 2001). In 1997 the estimated global market for tissue culture was 15 billion US dollars (Govil and Gupta, 1997). Some of these laboratories are a one-person operation which started as a hobby (Parsons, 1990). The literature on commercialization of micropropagation is mostly descriptive rather than instructive.

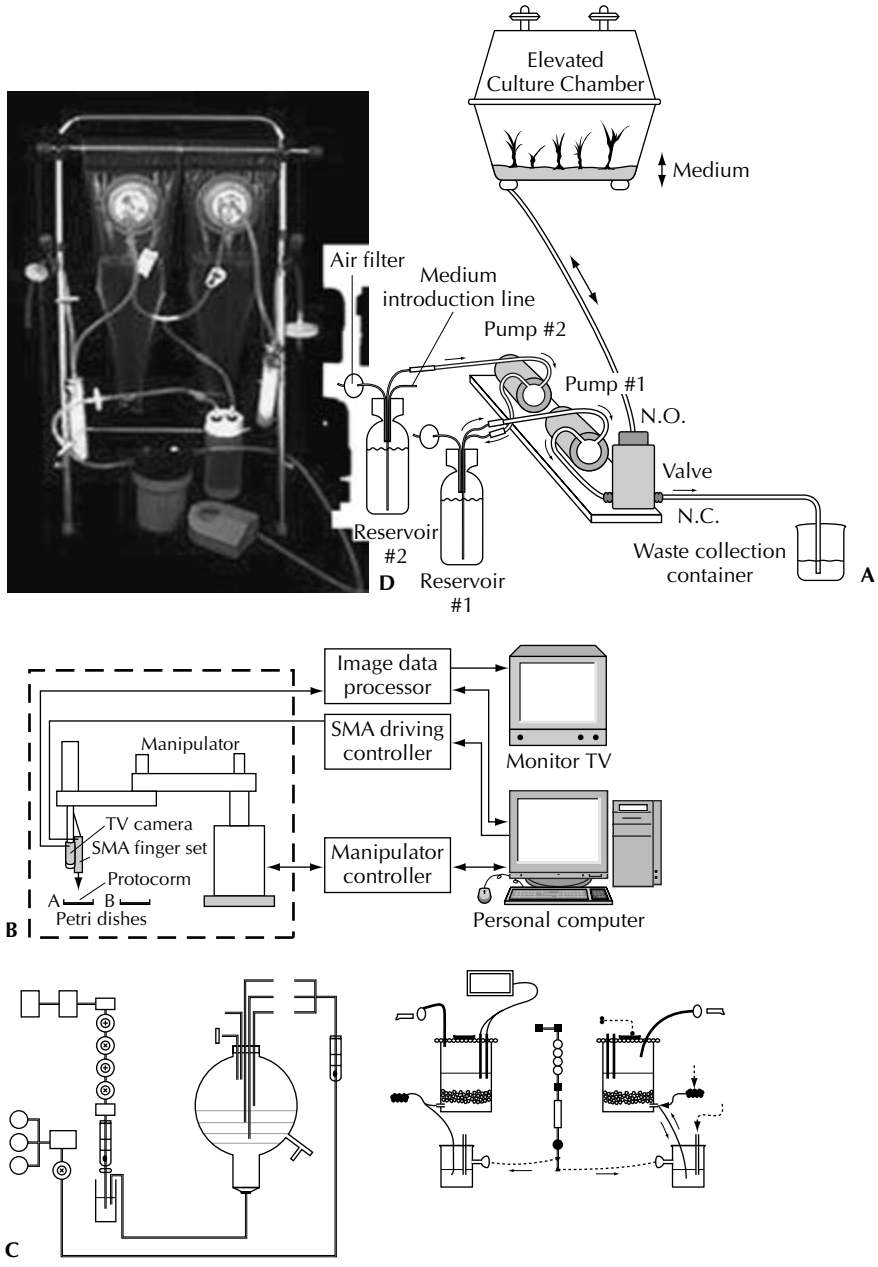


FIG. 4-15. **Automation for the micropropagation of orchids. A. Automated plant culture system. B. Robotic system for transplanting protocorms. C. Bioreactor. D. Osmotek LifeReactor™. (Sources: A, Tisserat and Vandercook, 1985; B, Okamoto, 1996; C, Paek et al., 2001; D, www.osmotek.com.)**

Conservation

Very rare species can be propagated in vitro and reintroduced into their natural habitat. This approach has the obvious disadvantages of genetic pauperization (i.e., reduction of genetic diversity) and introducing into nature a single clone or a few clones and its/their possible mutant(s). However, this is preferable to extinction. Further, orchids are usually very heterozygous, and heterogeneity could be re-established after a few years or several generations through natural pollination. Conservation projects utilizing in vitro seed germination have been initiated and/or exist in Australia (Dixon et al., 1991; Dixon, 1994), Belgium (Ronse, 1989), India (Pathak et al., 2001; Decruse et al., 2003*a*, 2003*b*), the UK (Fay, 1992; Ramsay, 1997), Singapore and Hong Kong (both under the direction of Dr. Tim Wing Yam of the Singapore Botanic Gardens), and the USA (Pence et al., 1997). The procedures used to culture plants for the purpose of conservation are no different from those utilized for standard commercial or hobby micropropagation. They can be found in Chapter 3.

Patents

In the United States, at least, plants can be patented only if they have been reproduced asexually and have then flowered. Micropropagation can be used for large-scale propagation of plants that have been or will be patented (Lecoufle, 1976). Several micropropagation procedures have been patented (Zhou and Tanaka, 2000; Oh and Kostenyuk, 2001; Chang, no date), but it is hard to imagine how such patents can be enforced. There was also an anecdotal report that an attempt was contemplated or made to patent an in vitro flowering procedure in a South East Asia country, or to sell it for an astronomical amount, but nothing seems to have come out of it, which is just as well. Patents and the process of obtaining them are legal matters and beyond the scope of this book.

Plant Improvement and Biotechnology

Orchids may have been the first crop plants to be improved in vitro through the doubling of chromosomes of seedlings (Rentoul, 1981). When tissue culture procedures became available, they were also used for the same purpose (Sanguthai and Sagawa, 1973; Binnie, 1979; Kam and Kamemoto, 1980; Sahavacharin, 1980; for procedures see Tanaka and Kamemoto, 1984). This too may have been another first for orchids, not only for ploidy changes but also for what is presently called genetic engineering. Not surprisingly the subject of genetic engineering and orchids has been discussed (Khaw and Ong, 1974/75; Kerr, 1983; Miller, 1983). Mutations, induced or accidental, have also been used to select improved clones (Vajrabhaya and Vajrabhaya, 1974; Sahavacharin, 1980; Teo, 1981).

One of the most interesting and impressive (and probably the first) biotechnology feats achieved with orchids has been the insertion of a bioluminescence gene in a *Dendrobium* by Professor Tet Fatt Chia of the Nanyang Technological University

in Singapore (Chia et al., 1994, 2000). For a period the plant could be seen on a World Wide Web site and it may still be there. Under illumination its appearance is that of a normal plant, but it glows in the dark. As so often happens with orchids, excessive competitiveness, ego, and jealousy led to intrigues and the spreading of a false rumor at one of the World Orchid Conferences that Professor Chia's genetically engineered plants were not stable. The truth is that the engineered plants are stable, very stable. The spreading of such false rumors, especially by a scientist who claims a standing among orchid scientists and growers, is unethical. Fortunately plants (especially ones which glow in the dark) speak louder than words and the false rumors were relegated to the trash heap of orchid history.

Chromosome doubling and bioengineering procedures are not covered in this book and this section was not updated for two reasons. One is the simple fact that even if performed in vitro, these techniques are not actually micropropagation. Descriptions of these techniques belong in a book on biotechnology. The second reason is that this book is already large enough.

Cryopreservation

Storing pollen in a refrigerator has been a common practice among orchid growers for at least 35 years. Orchid seeds have been stored in the cold for nearly 70 years. More recently, attempts have been made to store callus tissue of *Dendrobium* Multico White at 25, 10, 0, −5, and −78°C (Sivasubramaniam et al., 1987). The results indicate that callus can be stored easily at 10°C, but "a lot more work is needed to store orchid callus tissue at . . . temperatures . . . below 10°C" (Sivasubramaniam et al., 1987). Similar work was carried out in the laboratory of Professor Katsuhiko Kondo in Japan where methods were developed for the cryopreservation of *Cattleya loddigesii* var. *harrisoniana*, *Cattleya walkeriana*, *Dendrobium* Yukidaruma (Kondo et al., 2001*a*, 2001*b*), and *Vanda pumila* (Na and Kondo, 1995, 1996).

The methods used to produce tissues for cryopreservation are alluded to in Chapter 3. However, cryopreservation itself, even if it can be used to preserve tissues produced in vitro, is not micropropagation per se and is therefore not included in this book.

Physiological Studies

Explants, callus masses, PLBs, and plantlets produced through tissue culture methods have been used for studies of the effects of initial and subsequent pH on the growth of orchid plantlets (Piriyakanjanakul and Vajrabhaya, 1980), and the optimal composition of culture media (Ichihashi and Uehara, 1987). Other phenomena and processes can also be studied conveniently in vitro or with material produced by tissue culture.

Orchid roots are ageotropic and sometimes even negatively geotropic. This phenomenon was first studied at the famed Bogor Botanical Gardens in Indonesia in the early 20th century (Tischler, 1905; Wilkins, 1966; for reviews see Churchill et al.,

1972*b*; Arditti, 1977*b*). Aerial roots can often be seen to grow upward or sideways in nature, in vitro, and under cultivation. Such roots bend down eventually due to their own weight. The roots of *Grammatophyllum speciosum* are hard, point upward, have sharp thorn-like laterals, and form nest-like masses, called "trash baskets" (Dressler, 1981) that accumulate debris. The laterals are first noticeable 4–5 cm from the tips and always grow at a right angle to the main axis. Many large plants of *G. speciosum* grow on trees along one of the avenues in the Bogor Botanical Gardens. The view is spectacular and suggests that orchid roots could be used for studies of geotropism. Roots of *Brassia*, *Epidendrum*, *Oncidium*, and *Vanda* lack statoliths (Tischler, 1905), thereby offering a unique system for the study of the role of these organelles in nature, under horticultural conditions, and in vitro.

The roots of *Phalaenopsis* seedlings grown under illumination often point upward, whereas those of *Cymbidium* plantlets on dark medium grow downward. These phenomena suggest the use of orchid roots for studies of root phototropism and the relation between light and geotropism.

The roots of *Cattleya* and *Epidendrum* (Erickson, 1957), like those of many other orchids, are green and can fix carbon. The roots of "leafless" orchids fix carbon in a unique manner (Arditti, 1992). These roots undoubtedly perform not only all the functions of roots, but also those carried out by leaves and stems. For this reason they could prove to be a valuable system for certain physiological studies in culture.

The velamen of orchid roots (Pridgeon, 1987) is of considerable developmental, physiological, and ecological interest. Some orchid roots produce anthocyanins. Phytoalexins are present in the roots of many species following the establishment of mycorrhizae (Stoessl and Arditti, 1984). Orchid seedlings (and therefore probably also plantlets derived from explants) can establish mycorrhizae in vitro (Clements, 1982), a fact that may make seedlings useful for studies of this phenomenon. Other areas in which orchid roots in vitro may be a good research system are mineral uptake, thigmotropism, chemotropism, attachment to surfaces, and branching.

Leaf explants of both crassulacean acid metabolism (CAM) and C3 orchids have been cultured and used to produce callus and/or PLBs. All three (explants, callus masses, and PLBs) can be used for physiological and developmental research (for a review of recent research see Hew and Yong, 1997, 2004).

Phalaenopsis flower-stalk nodes can produce new flower stems when placed in culture (Intuwong et al., 1972*a*, 1972*b*). Explant-derived *Cymbidium* plantlets have been induced to flower in vitro by Xiong Wang at the Shanghai Plant Physiology Institute. The bud on a flower-stalk node of *Arachnis* Maggie Oei developed in vitro when placed in liquid stationary Knudson C medium by a worker at the Bogor Botanical Gardens. Seedlings of *Oncidium* and other orchids have been reported to flower in vitro at a very young age. Thus it seems that orchid seedlings and plantlets in vitro can be used for studies of flowering (for a review of recent and older research see Chia et al., 1999).

Several other areas for which orchids are suitable for in vitro research systems are: formation of somatic embryos, protoplasts, cell wall formation, comparisons between existing sexual hybrids and the same combinations obtained through protoplast fusion, development, mineral uptake, ontogeny, and other physiological phenomena. These topics will not be updated here due to space limitations (for reviews of work carried out since the first edition of this book see Arditti, 1994; Rasmussen, 1995; Arditti

and Pridgeon, 1997; Hew and Yong, 1997; Ichihashi, 1997; Chia et al., 1999; Kull and Arditti, 2002).

Negative Aspects of Micropropagation

Since micropropagation is a vegetative means of propagating plants, both horticulturists and scientists assumed at the outset that all offspring would be true to their parents. This assumption was reinforced by an early report regarding the blooming of "mericlones" that stated that "for each individual cultivar the flowers appeared exactly identical, and in hundreds of them no mutation was observed" (Lecoufle, 1967). This could have been the case or the statement may have been an exaggeration intended to allay the fears of potential customers. But several researchers (including myself) were also sanguine (Teo and Teo, 1974; Teo, 1975; Tanaka, 1987). However, as experience with micropropagation increased, it became evident that mutations do occur. Among the first workers to call attention to this were the Thai orchidologists Thavorn and Montakan Vajrabhaya (Vajrabhaya and Vajrabhaya, 1974; for a review see Vajrabhaya, 1977). Experience since then has confirmed that mutations do occur during tissue culture (Sahavacharin, 1980; Rentoul, 1981; Teo, 1981; Ichihashi, 1997).

Mutations are especially prevalent in cases where high concentrations of hormones are used to force proliferation and the production of a large plantlet yield. Excessive proliferation of plants through tissue culture also causes mutations. To prevent mutations: (1) proliferation should be brought about through culture in liquid medium with agitation or high levels of coconut water; (2) PLBs, tissues, or plantlets produced through tissue culture should be proliferated to a limited extent only; and (3) only a small number of plants should be produced from any one explant.

Phenotypically these mutations include changes in color, shape, malformed flowers, and stunted growth. In some overproliferated clones, individual flowers on a raceme may die and render the inflorescence commercially useless.

There is no way at present to predict the nature of mutations. It is also impossible to prevent propagators from overproliferating a clone. Buyers can protect themselves by purchasing plants produced in vitro only from known and reliable sources, and by demanding guarantees.

It is not uncommon for growers to have their most promising plants propagated by commercial micropropagation laboratories. Several of these laboratories set aside some of the plants and, if the clone proves popular, sell their stock. This can flood the market and reduce prices. It essentially deprives the original plant owner of market position and just profits. Assurances that this will not be done are only as good as the person who makes them. Patents offer limited protection against this practice even if they can be proven and enforced. Propagation of valuable clones in house is one means of preventing such robbery. Another would be for growers in specific geographic areas to establish joint, well-supervised laboratories for the propagation of clones owned by members.

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APPENDIX ONE

General Information on Supplies, Equipment, Terms, and Reagents

This appendix may be used by experienced workers familiar with orchids, tissue culture, and seed germination techniques. However, it may well be a major source of information for hobbyists, commercial growers, and/or investigators who have little or no such experience. In more isolated laboratories it could be the first or only source of information. Therefore, every effort has been made to include in it all necessary information. This list describes equipment and chemicals, in alphabetical order. The numbers in parentheses refer to supply sources in Appendix 2.

Agar. Agar may vary in purity, mineral content, organic substances, or the amount required to solidify a medium, depending on its source or batch. Bacto agar from Difco Laboratories (now part of Becton, Dickinson, and Company) was a commonly used brand in the United States for many years, but other brands have also been used with success. Agar purchased in food stores has been suitable or even preferable in some instances. In general, it may be wise to test agar from a new source before using it in important media. At present gellan gum (under brand names like Gelrite and Phytagel) is also used as a gelling agent. Some structural formulae and illustrations of agar and gellan gum can be found throughout Chapter 3. (Supply sources: 1, 2, 3, 7–13, 16, 19–21, 23, 24.)

Alcohol. A term that describes a large number of compounds. The alcohol recommended for use in this book is the one familiar to most people – ethanol or drinking alcohol. Ethanol must be used to make all alcohol stock solutions and should be employed as a sterilant since methyl alcohol (methanol, methylated spirits, wood alcohol) is toxic. The same is often true for denatured ethanol. In some countries ethanol, which is not taxed like an alcoholic beverage, can be purchased in pharmacies with a prescription. Special use permits may also be required for the purchase of untaxed ethanol. Isopropyl alcohol (isopropanol, rubbing alcohol) can be used as a sterilant for surfaces, but not as a solvent for components of media. (Supply sources: 1–4, 8, 11, 13, 19, 21, 23, 24.)

Amino acids. Amino acids are components of protein which are incorporated in several media; some are not heat-stable and should be filter-sterilized or dissolved in ethanol and added to media after autoclaving. However, amino acids are often added

before autoclaving to media that are still effective. This suggests that the amino acids in question are: (i) not really needed, (2) may not be destroyed (either partially or fully) by autoclaving, or (3) that the products of amino acid heat destruction may also meet the requirements of the explants. Some structural formulae can be seen in Chapter 4 (see Fig. 4-11) and through Chapter 3. (Supply sources: 1–4, 7–12, 16, 17, 19–21, 23, 24.)

Anticontaminants. These are compounds which can be added to cultures to prevent or combat contamination (see Anticontaminants in Chapter 2). Not all of the compounds listed in that chapter have been tested with orchids; the untested compounds must be used with caution. (Supply sources: 1–4, 7–12, 16, 17, 19–21, 23, 24.)

Ascorbic acid. This is the chemical name for vitamin C, which is sometimes used as an antioxidant. Vitamin C is not heat-stable, yet it is often added to culture media before autoclaving. (Supply sources: 1–4, 7–12, 16, 17, 19–21, 23, 24.)

Autoclaves. Autoclaves are sterilizers that develop high temperature and pressure. Many types (Fig. A1-1A, E) are available, the simplest being a pressure cooker. (Supply sources: 11, 19, 21, 24.)

Auxins. The auxins are a group of plant hormones. Indoleacetic acid (IAA) occurs naturally, but a number of synthetic auxins are available and are added to culture media. These include naphthaleneacetic acid (NAA), *para*-chlorophenoxyacetic acid (*p*CPA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), among others. The structural formulae of some auxins are shown in Chapter 4 (see Fig. 4-11) and through Chapter 3. The effects of, and plant responses to, different auxins can vary for reasons that are not known. (Supply sources: 1–4, 7–12, 16, 17, 19–21, 23, 24.)

Balances. Two types of balance are necessary for accurate weighing of most media components. The first is a simple swing or beam balance (Fig. A1-1G, H, J) for weighing several grams at a time. For quantities of around 1 mg, an analytical balance (Fig. A1-1K), although expensive, is necessary. If costs are prohibitive, a balance accurate to 10 mg may be used but will require making larger amounts of some stock solutions. (Supply sources: 11, 19, 21, 23.)

Beakers. Containers made of glass (Fig. A1-1B) or plastic can be used in the preparation of solutions. Glass jars, cups, or glasses can also be used. (Supply sources: 1–4, 6–12, 16, 17, 19, 21, 23.)

Blades, Cutting. *See* Microscalpels.

Bleach, Household. *See* Household Bleach.

Calcium Hypochlorite. This is used in a filtered solution of 7 g per 100 ml of water to surface-sterilize seeds or capsules. The solution must be used within a few hours.

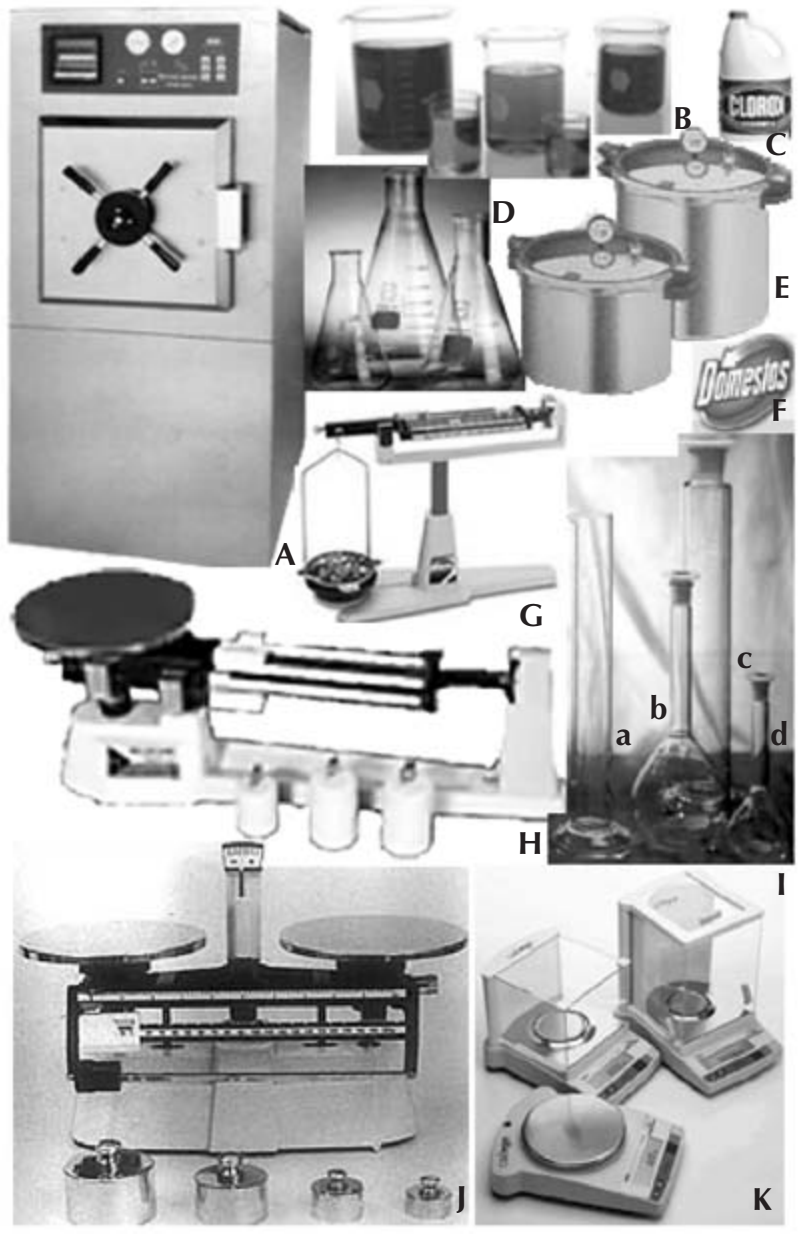


FIG. A1-1. **Laboratory equipment and glassware. A. Large autoclave. B. Beakers. C. Clorox bottle. D. Erlenmeyer flasks. E. Pressure-cooker-type autoclaves. F. Domestos. G, H, J. Swing and beam balances. I. Volumetric cylinders (a, c) and flasks (b, d). K. Analytical balances.**

A few drops of household detergent or baby shampoo should be added as a wetting agent. [Prolonged exposure to high concentrations of detergents (surfactants) can damage orchid tissues. However contact with low concentrations for short periods will not have harmful effects, especially if the tissues are rinsed with sterile distilled water after the sterilization.] (Supply sources: 1–4, 7–12, 16, 17, 19–21, 23, 24.)

Casein Hydrolysate. *See* Complex Additives.

Clorox. Clorox (Fig. A1-1C) is a brand of household bleach. Another brand is Domestos (Fig. A1-1F). There are additional brands throughout the world. (Supply source: 13.)

Coconut Milk. *See* Coconut Water.

Coconut Water. This is sometimes erroneously called coconut milk. It is liquid endosperm which enhances growth of cells, tissues, organs, or seedlings in vitro. Its exact composition and reasons for its effects are not known. Water from green coconut is preferable, but we have had reasonable success with liquid removed from relatively mature nuts purchased in local food stores. Unless otherwise specified, coconut water can be autoclaved. Coconut milk is a white milky liquid obtained by grating or pressing the solid endosperm of coconut. Coconut milk is not used in culture media. (Supply sources: frozen or dried, 2, 7, 8, 10, 16, 23; fresh, 13.)

Complex Additives. These are preparations like coconut water, casein hydrolysate, banana homogenate, peptone, tryptone, or yeast extract, which are added to media but whose exact composition is unknown. (Supply sources: 1–4, 7–12, 16, 17, 19–21.)

Culture Containers. *See* Culture Vessels.

Culture Flasks. *See* Culture Vessels.

Culture Tubes. *See* Culture Vessels.

Culture Vessels. At one time Erlenmeyer flasks, culture tubes, and a variety of jars and bottles were used as culture vessels. These are still being used at present. However, a number of specially designed glass and plastic culture vessels (Fig. A1-2) are also currently being used. Some are disposable, others are not. The use of disposable culture vessels may be more economical in countries where labor costs are high. Low cost disposable plastic culture vessels are preferable when in-vitro-produced plantlets or seedlings must be shipped or sold directly to consumers. Several of the plastic culture vessels are vented through openings covered with sterilizing filters. Changeover from one type of vessel to another may require an adjustment period and retraining of laboratory personnel. (Supply sources: 6, 7, 10, 11, 15, 16, 19, 21, 23, 26.)

Cylinder, Volumetric. *See* Volumetric Cylinders.

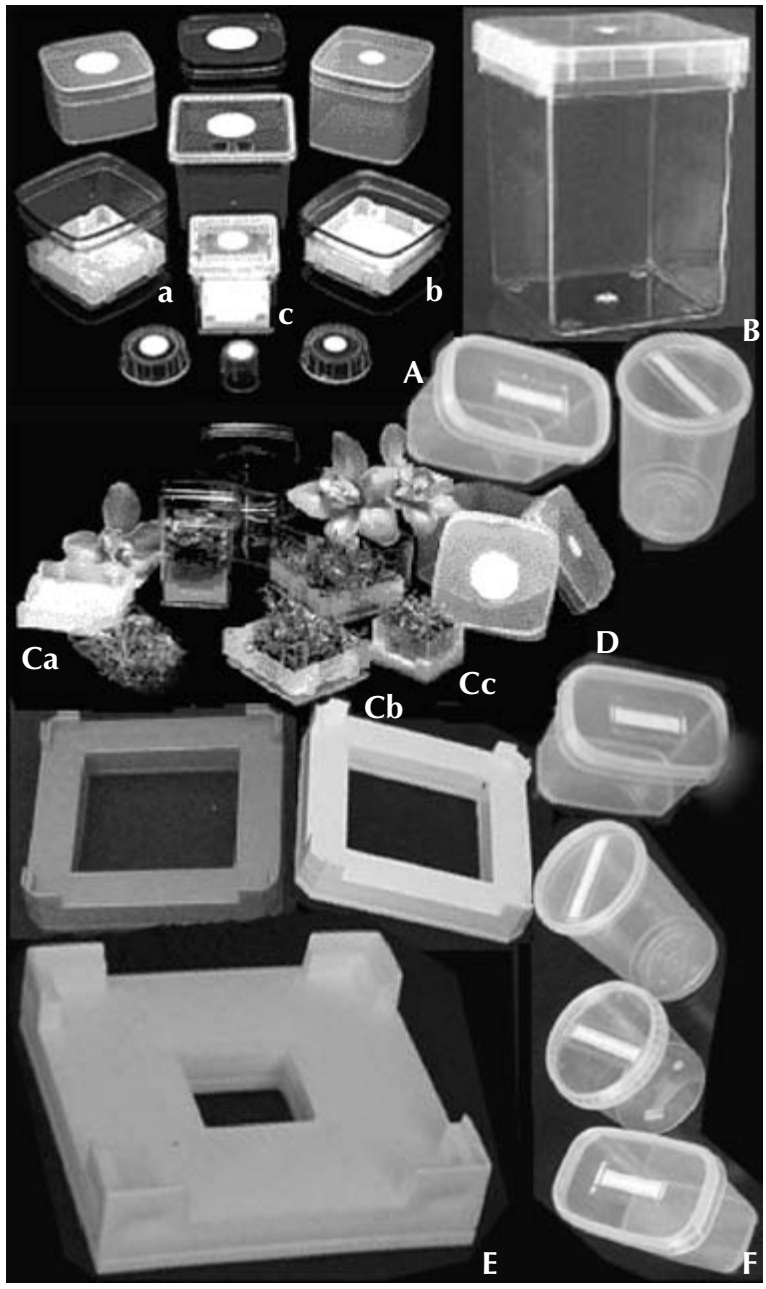


FIG. A1-2. **Culture vessels. A, C, D. Osmotek vented culture vessels, empty (A) and full (C, D). The vessels in Aa, Ab, Ac, Ca, Cb, and Cc contain lined Life Rafts™. All Osmotek culture vessels have round filter covered vents. B. Magenta culture vessels. E. Osmotek Life Rafts™. F. Duchefa culture vessels. All Duchefa culture vessels have rectangular vents.**

Cytokinins. Cytokinins are a group of hormones that regulate cell division and other plant functions. They may be used in culture media and include compounds such as benzyladenine (BA), 6-dimethyl-aminopurine (6-DMAP), kinetin, *N*-benzyl-9-(tetrahydro-2H-pyran-2-yl), an experimental compound SD 8339 (this may no longer be available), thidiazuron (TDZ), and the naturally occurring zeatin. Their formulae can be found in Chapter 4 (Fig. 4-11) and through Chapter 3. (Supply sources: 1–4, 7–12 16, 17, 19–21.)

Electrical equipment. Proper precautions should be exercised in the purchase of any electrical equipment, and the current, cycles, and voltage levels and their fluctuations should be considered. If voltage fluctuations are common and excessive, regulators are necessary. (Supply sources: 11, 13, 19, 21.)

Erlenmeyer Flasks. These are containers with relatively narrow necks and a broader base (Fig. A1-1D) that have been and still are used extensively for cultures and preparation of solutions. To withstand heating better they should be made of heat-resistant glass like Pyrex. (Supply sources: 4, 6, 7, 11, 13, 16, 19, 21, 23.)

Ethanol. *See* Alcohol.

Ethyl Alcohol. *See* Alcohol, Ethyl alcohol.

Filters, Paper. Paper filters are used to trap particles. *See also* Sterilizing Filters.

Flask, Erlenmeyer. *See* Erlenmeyer Flasks.

Flask, Volumetric. *See* Volumetric Flasks.

Graduated Cylinder. *See* Volumetric Cylinders.

Hormones (Abscisic Acid, Auxins, Cytokinins, Ethylene, Gibberellins). These substances are sparingly soluble or insoluble in water. They can be dissolved as soluble salts if they exist (e.g., potassium salts of auxins). Another approach is to dissolve them in ethyl alcohol (ethanol) plus a few drops of a dilute acid, if necessary (0.1N hydrochloric acid, for example), or a base (0.1N potassium hydroxide is suitable). Ethanolic solutions are preferable because they also sterilize the substances.

Most plant hormones are destroyed by autoclaving. Therefore they should be sterilized by filtration, a process that is somewhat complicated and may require relatively sophisticated or expensive equipment. A simpler approach is to dissolve them in ethyl alcohol (at least 70% in distilled water), which is a good sterilant as well as a suitable solvent. Stock solutions prepared for addition to media must be concentrated enough to allow for the addition of each hormone in 1 ml or less of stock solution per liter of medium. When such stock solutions are used, media are prepared as usual, but the hormones are omitted. They are added to the hot solution following autoclaving and mixed by swirling. The complete medium is then distributed into autoclaved culture vessels. Experience has shown that ethanol (up to 5 ml of 95% or 7 ml of 70% ethyl alchol per liter) will not harm orchid seedlings, explants,

or plantlets. In many cases hormones are added to media before autoclaving without any problems. (Supply sources: 1–4, 7–12, 16, 17, 19–21, 23, 24.)

Household Bleach. This is usually a solution containing 4.25–6% sodium hypochlorite. It is sold under a large number of brand names all over the world. Read the label for the active agent(s) and their concentration(s) in such bleaches to determine whether they are suitable and to calculate how to dilute them. (Supply source: 13.)

Inorganic Salts. These salts provide macro- and microelements in culture media. (Supply sources: 1–4, 7, 8, 10–12, 16–21, 23, 24.)

Inositol. Inositol is a polyol which is often added to culture media with presumed beneficial effects. It is also known as *myo*-inositol, *meso*-inositol, *i*-inositol, and a number of other names. Its structural formula can be seen in Chapter 4 (see Fig. 4-11). (Supply sources: 1–4, 7–12, 16, 17, 19–21, 23, 24.)

Isopropyl Alcohol (Isopropanol, Rubbing Alcohol). This is an alcohol which can be used to surface-sterilize tools and surfaces. It should not be used to dissolve media components. (Supply source: 13.)

Laminar Flow Hoods (or Cabinets). These appliances (Fig. A1-3) provide a sterile atmosphere for seed and tissue culture work. At one time they were used only by larger laboratories, and smaller laboratories used sterile boxes, some of them homemade. At present small desk-top laminar flow hoods are inexpensive enough to be within the reach of even small laboratories. (Supply sources: 5, 11, 19, 21, 25.)

Lamps, Fluorescent and Incandescent. A wide selection of lamps can be used to illuminate cultures. See the section on illumination in Chapter 2. Lamps can be ordered from many suppliers or purchased in local hardware, electrical, and lighting stores. (Supply source: 13.)

Methanol. Methanol is toxic if inhaled, ingested, or absorbed through the skin. It is best not to even have it in a laboratory. *See also* Alcohol. (Supply sources: 1–4, 7–12, 16, 17, 19–21, 23, 24.)

Microfilters. *See* Sterilizing Filters.

Microscalpels. Sharp scalpels (Fig. A1-4B–F) or single-edge razor blades (Fig. A1-4A) should be used for splitting fruits or excising tissues. They may be purchased from any biological supply house or can be made from single-edge razor blades (see Fig. A1-9A). Small hypodermic needles from disposable syringes are used by some workers as substitutes for microscalpels. (Supply sources: 7, 11, 13, 16, 19, 21, 23.)

Niacin (Nicotinic Acid). This is a vitamin added to some culture media. Its structure can be seen in Chapter 4 (see Fig. 4-11). (Supply sources: 1–4, 7–12, 16, 17, 19–21, 23, 24.)

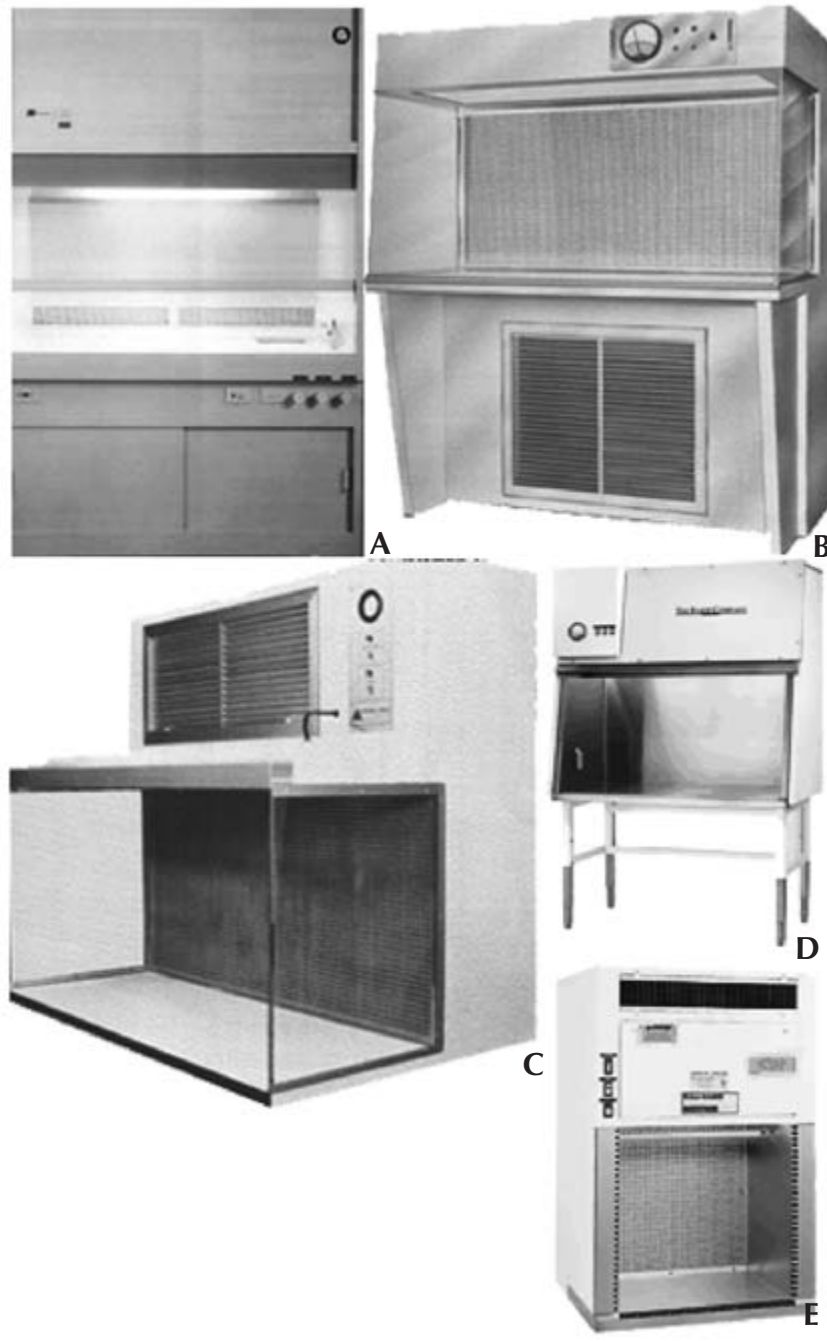


FIG. A1-3. **Laminar flow hoods. A, B, D. Stand-alone hoods. C, E. Table-top hoods. There are numerous models by many manufacturers.**

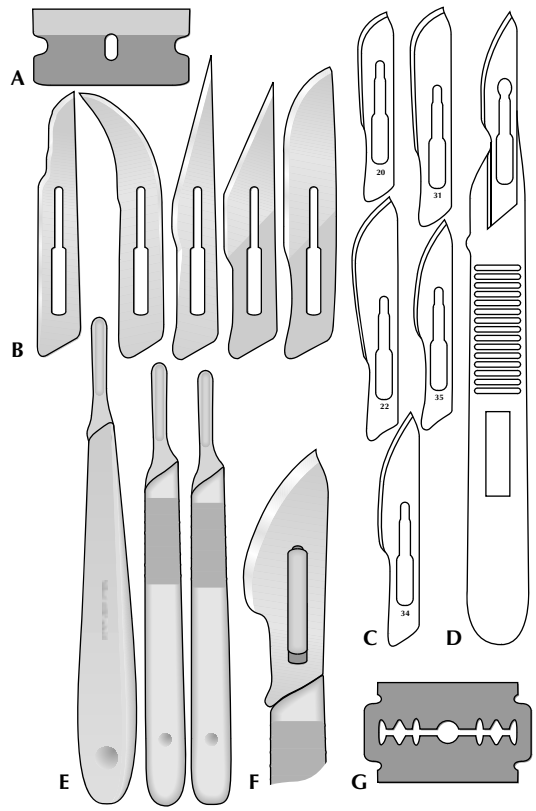


FIG. A1-4. **Cutting implements. A. One-sided razor blade. B–F. Scalpels and microscalpels. G. Two-sided razor blade.**

Nylon-mesh Filters. These are used for the filtration of protoplasts (Supply sources: www.pgcsci.com, www.marcson.com, www.coleparmer.com.)

Octadecyl-poly(ethoxy)ethanol. This is a surfactant with an average of seven ethylene oxide units, a molecular weight of 578.4, and a hydroxyl number of approximately 147. It induces proliferation when added to some culture media. This compound is not commercially available and may be hard to find. Related substances are possible substitutes. (Supply sources: 1–4, 7–12, 16, 17, 19–21, 23, 24.)

pH Meters. Such meters are electronic apparatus used to measure the reaction (basic or acidic) of a medium (Fig. A1-5). They are more accurate than pH paper. Some pH meters are portable and powered by batteries. (Supply sources: 11, 19, 21.)

pH Paper. These are strips of paper (Fig. A1-6) which can be used to measure the reaction (basic or acidic) of a medium. They are less accurate than a pH meter. (Supply sources: 11, 19, 21.)

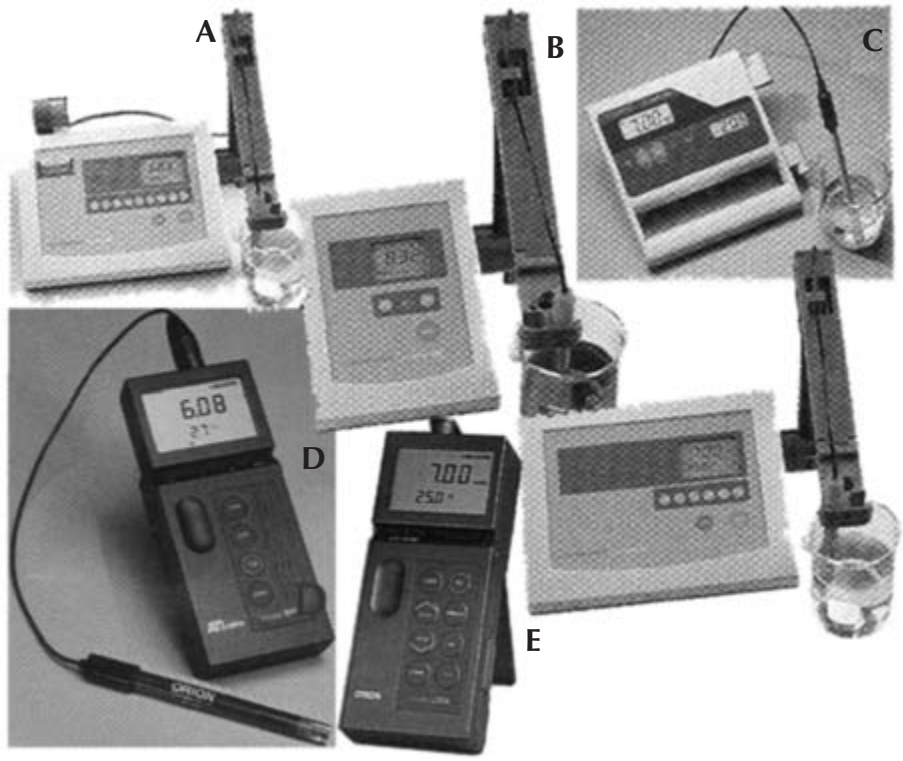


FIG. A1-5. **Desktop (A–C, F) and portable (D, E) pH meters (source: www.thomassci.com).**

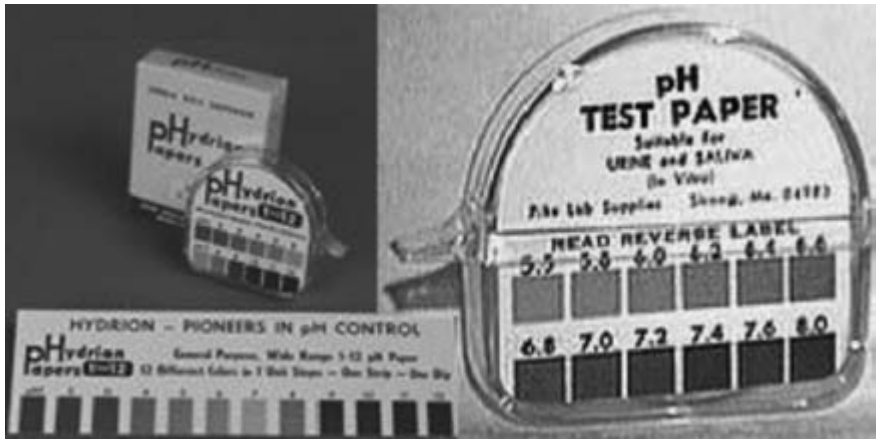


FIG. A1-6. **pH test paper.**

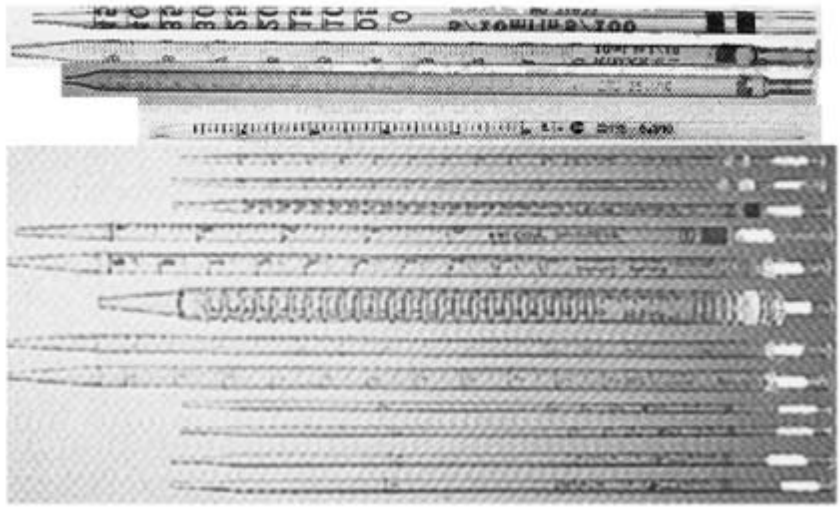


FIG. A1-7. **Pipettes.**

Pipettes. Glass or plastic pipettes (Fig. A1-7) are used to measure and dispense solutions. (Supply sources: 6, 11, 19, 21.)

Shakers. Different types of shakers (reciprocal, gyratory, and rotary) are available in a large variety of sizes (Fig. A1-8) from several suppliers. Simple shakers are also easy to construct. (Supply sources: 11, 19, 21.)

Sterile Box. A very adequate sterile enclosure (Fig. A1-9B) can be constructed from a cardboard box, Saran Wrap, and aluminum foil. Obtain a $90 \times 90 \times 90$ cm (approx $3 \times 3 \times 3$ ft, or similar) cardboard box and remove the top flaps. Draw a diagonal line along each side of the box (Fig. A1-9Ba), and cut along these lines to obtain a sloping surface (Fig. A1-9Bb). Line the inside of the box with aluminum foil and cover the top (sloping surface) with Saran Wrap. Cut two circular holes in the front (shorter) side so that a worker's hands can be inserted for work inside the box (Fig. A1-9Bc). Double-sided razor blades (see Fig. A1-4G) should not be used. (Supply sources: 7, 11, 13, 16, 17, 19, 21.)

Sterilizing Filters. Filters made of glass (Morton type) can be difficult to handle or clean, are expensive, and require a vacuum for effective filtration. Membrane filters (Millipore or other brand; Fig.A1-10A–C, E) are easier to handle and may be less expensive in the long run because the non-disposable units (Fig. A1-10C) are easier to wash and the disposable ones (Fig. 2-21E) do not have to be washed. However, membrane filters are not cheap, require vacuum pumps, and need more elaborate set ups. They may be better suited for larger and better-equipped laboratories. Disposable sterilizing filters which can be attached to syringes (Fig. A1-10D, F, G), are inexpensive, and are easily available. Metal enclosures for glass syringes are advisable to prevent injury in case of breakage, but they can be expensive. Plastic syringes

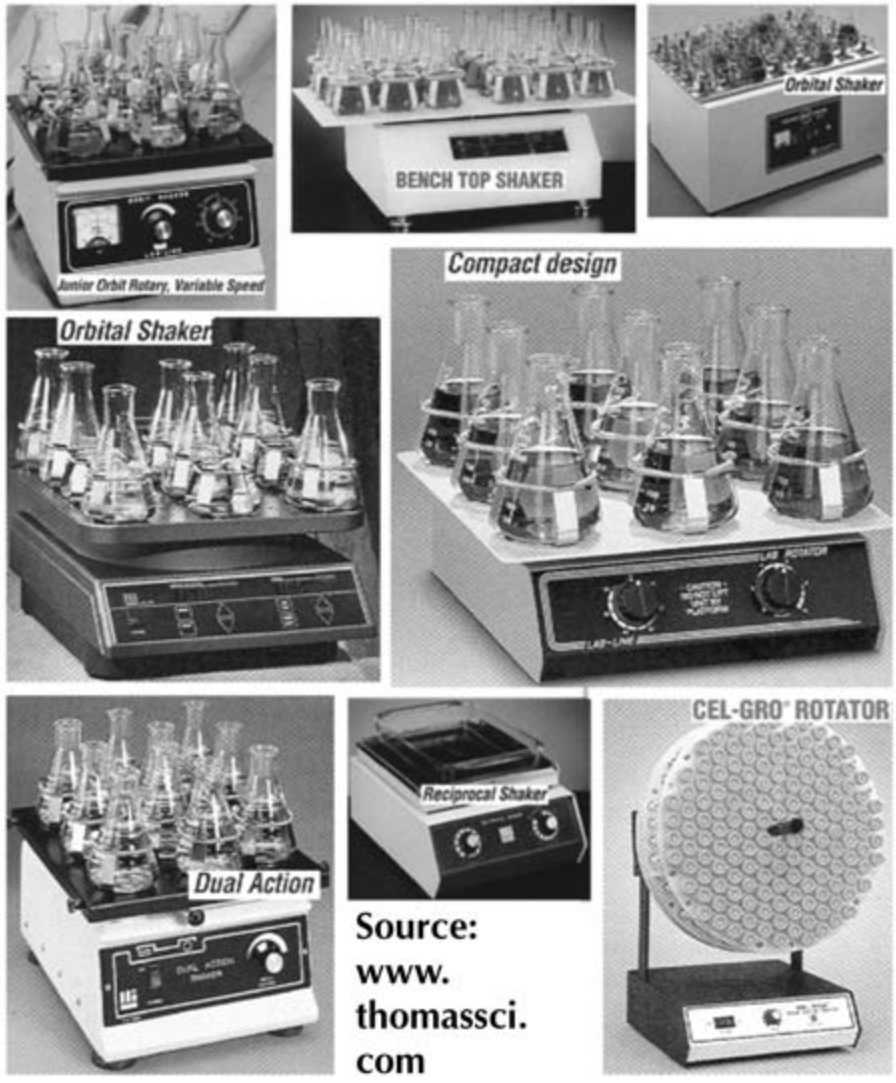


FIG. A1-8. **Shakers.**

can also be used and are preferable because they are less likely to shatter and injure the operators. (Supply sources: 11, 12, 14, 16, 19, 21, 24.)

Sterilizing Lamp. Alcohol lamps or burners (Fig. A1-11) are used to sterilize tools by flaming while working in a laminar flow hood or a sterile box. Such lamps can be purchased from supply houses or made by placing a cotton wick inside a metal container (of the kind used to hold rubber cement), through a hole made in the cover of a small jar. A small bottle can also be used. The fuel should be alcohol, and not

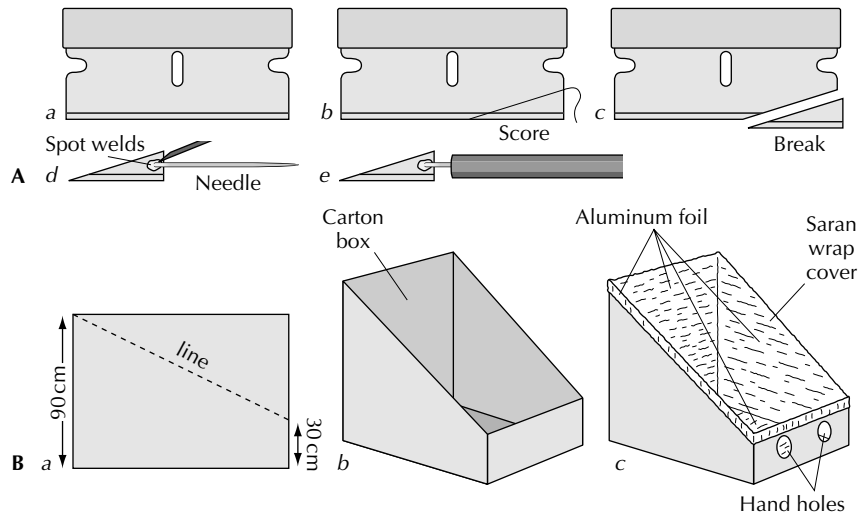


FIG. A1-9. **Making a sterile box and a simple scalpel. A. To make a microscalpel from a single-edge razor blade (a), you need a needle and a pencil, section of a branch, or piece of dowel. Score the blade (b), break off the corner with a pair of pliers (c), and weld this on to the unsharp (ear) edge of the needle (d). Then insert the entire length of the needle into the pencil, dowel, or section of branch. This simple, yet very effective, microscalpel was invented by the late Professor Ernest A. Ball, one of the first two botanists to culture a shoot tip (the other was Professor Shih wei Loo). To sterilize the scalpel while working, dip it into alcohol and a flame or dip it into household bleach. B. Construction of a sterile enclosure from a cardboard box, aluminum foil, Saran Wrap, and two plastic bags. Draw the lines as indicated (a) where the box is to be cut (b). After cutting, line the box with aluminum foil and cover with Saran Wrap. Cut two round holes in the front (c) and attach to them plastic bags whose bottoms have been cut to make them into tubes. These tubes can be used as "tunnels" for the placing of culture vessels, plant material, sterilants, and tools into the box. When working in the box, fasten the open end of the bags to the hands below the elbow to insure sterility. Sterilize the inside of the box by spraying with alcohol or domestic bleach. If the Saran Wrap becomes foggy due to condensation, wipe with paper towel. A "door" can be cut on one side of the box. Should this be done care must be taken to close the door while working in the box.**

gasoline (petrol), ether (which can cause an explosion), paraffin oil, or kerosene (because they produce dark soot). Candles also produce soot and should not be used. (Supply sources: 11, 19, 21.)

Stock Solutions. Weighing components of a medium can be time-consuming and subject to errors. Therefore more concentrated solutions of some media components are prepared in many laboratories. These are called stock solutions. For example, if a recipe calls for 1 gram per liter (1 g l^{-1}) of magnesium sulfate, a stock solution containing 10 g l^{-1} can be prepared. Then, in making the medium, 100 ml (one-tenth of a liter and therefore one-tenth of the magnesium sulfate) of the stock solution is used. If a stock solution of 100 g l^{-1} is prepared, only 10 ml of it should be used to make the medium. Using stock solutions is faster than weighing 1 g every time and also more accurate. Stock solutions should be stored under appropriate conditions.

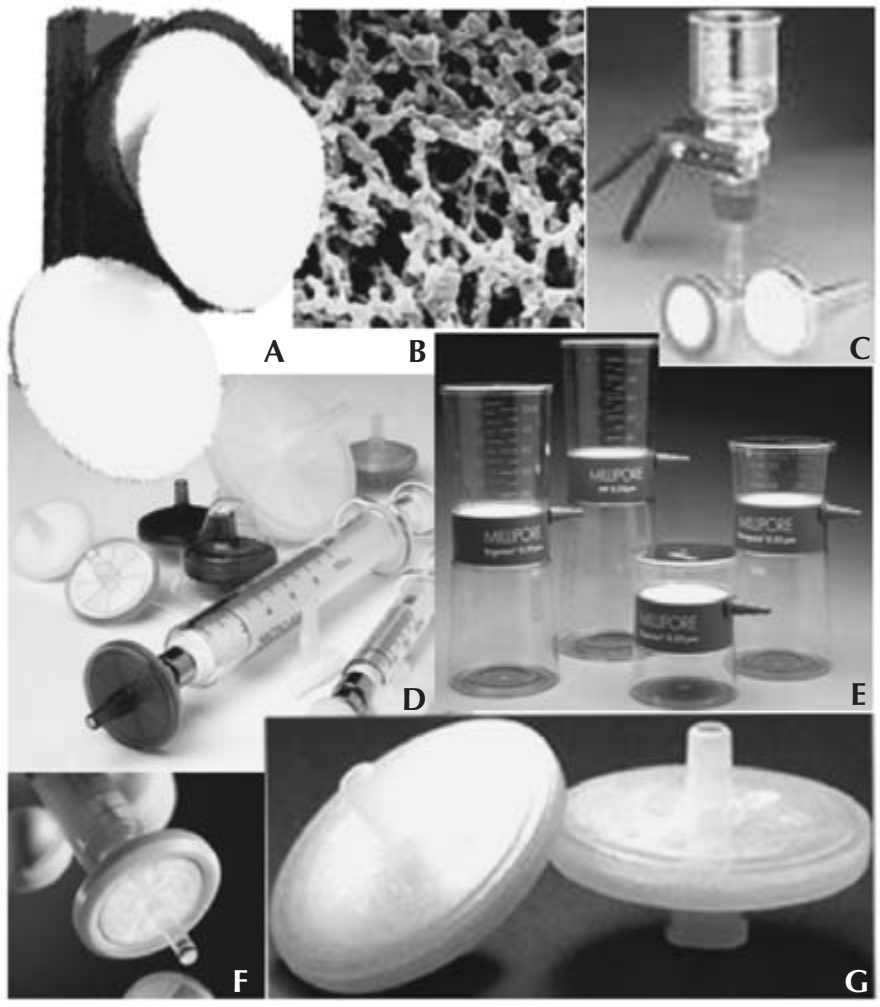


FIG. A1-10. **Sterilizing filters. A. Membrane sterilizing filters. B. Electron photograph of a sterilizing filter. C. Reusable glass filter holder and clamp. D, F, G. Plastic filter holders which attach to syringes. E. Disposable non-reusable filter holders with filters in them. These filters and the ones in (C) require a vacuum pump for suction.**

Solutions of nitrogen, even those containing inorganic salts, can become contaminated with time. Therefore it is best to prepare them fresh every few months and discard those that are no longer completely clear and/or contain a precipitate, especially one that appears to be slimy. Storage in a freezer can prolong the life of such solutions by eliminating contamination.

Solutions of organic substances in water must be kept frozen. Alcoholic solutions should also be frozen because some substances may not be stable, even in a refrigerator. Organic substances which are affected by light should be kept in the dark. Autoclaved media should not be stored for excessively long periods.

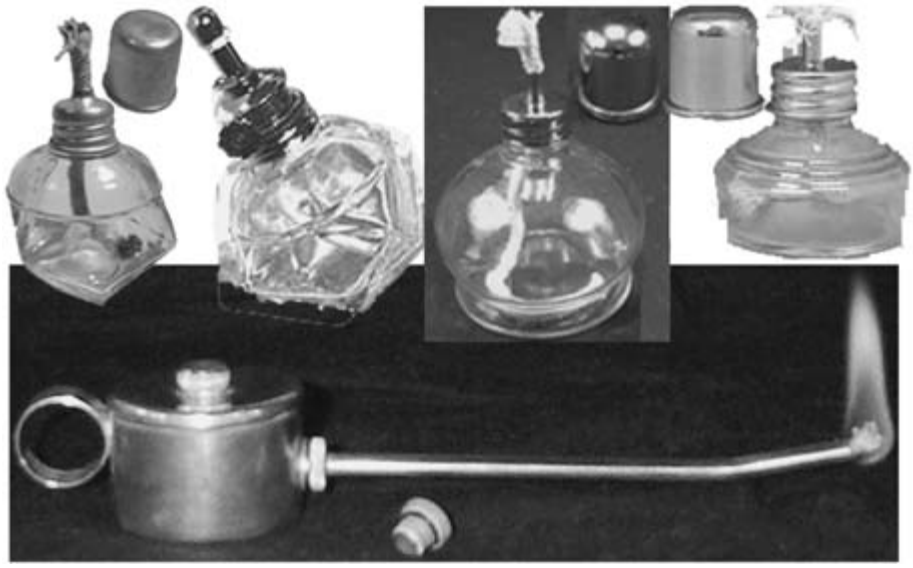


FIG. A1-11. **Alcohol lamps or burners.**

All stock solutions should be labeled with the information in the example below (self-adhesive labels can be printed in advance). A pencil or indelible ink must be used when writing on such labels. Ball point writing fluid or regular inks will smudge.

1 Name of compound: Potassium phosphate
2 Formula of compound: KH_2PO_4
3 Concentration as weight or volume per liter of stock solution: 150 g
4 Date on which the solution was made: November 23, 2003
5 Where to store stock solution: A shelf
6 Can compound be autoclaved: Yes
7 Name of the person who made the solution: Jonathan O. Arditti

If one stock solution of a substance is to be used for the preparation of several media, it is a good idea to have on the bottle a second label with a list of how much to use for each medium, as for example:

Medium A: Use 10 ml	Medium D: Use 7 ml	Medium G: Use 3 ml
Medium B: Use 10 ml	Medium E: Use 5 ml	Medium H: Use 0 ml
Medium C: Use 12 ml	Medium F: Use 8 ml	Medium I: Use 15 ml

A separate stock solution should be prepared for each macroelement. Concentrations of macroelement stock solutions should be 100 times the amount used in the medium (100×) if the solubility of the compound will allow it.

Microelements can be combined in one stock solution, usually at a 1000× concentration. Since the composition of microelements may vary with the medium, it may be necessary to prepare more than one stock solution. If this is done, the

bottle must be labeled very clearly with the name of the medium for which the stock solution is to be used.

Surfactants. These agents (surfactants, surface active agents, wetting agents, detergents, baby shampoo) are used to decrease the surface tension of liquids or solutions and increase their wetting power. Exposing orchid seedlings, tissues, and plantlets to high concentrations of these agents for long periods can cause damage. However, contrary to some overcautious advice which does not seem to have any basis in experimental evidence, short exposures to low levels of most mild household and laboratory detergents (like Tween 20 for example) or baby shampoos, followed by a rinse, will not cause any harm to tissues. (Supply sources: 1–4, 7–13, 16, 17, 19–21.)

Surgical Gloves. Lightweight surgical gloves for sterile work should be used to protect workers from alcohol, which can dry the skin, and other agents that may cause damage or be toxic. If it were not for these reasons, well-washed hands would cause no more surface contamination than gloved ones. Household gloves should not be used because they are very thick and can reduce manual dexterity. (Supply sources: 11, 13, 19, 21.)

Vials and Tubes. Various test tubes and vials can be used as culture vessels. (Supply sources: 6, 7, 11, 16, 19, 21.)

Vitamins. These substances may be heat-labile. Therefore they should generally be handled like hormones (even if their mode of action is different) in the preparation of stock solutions or the sterilization of media. They are added to culture media because some tissues and explants may require them. (Supply sources: 1–4, 7–12, 16, 17, 19–21.) *See also* Sterilization, Filtration, and Solvents.

Volumetric Cylinders. Made of glass or plastic and graduated to contain specific volumes (see Fig. A1-1Ia, c), volumetric cylinders are a necessity. Their size designation is based on the maximum volume they can contain. A seed germination or micropropagation laboratory should have volumetric cylinders in capacities of 10, 25, 50, 100, 250, 500, and 1000 ml. Always use the next largest cylinder to measure a particular volume (for example, use a 50-ml cylinder to measure 30 ml, not a 10-, 20-, or 100-ml cylinder). Plastic cylinders are less apt to break. Cylinders with stoppers can be used like volumetric flasks, whereas those with lips are for measuring and pouring; both are graduated. (Supply sources: 4, 6, 7, 11, 13, 14, 16, 19, 21, 23.)

Volumetric Flasks. Volumetric flasks are made of glass or plastic and are marked to contain specific volumes (Fig. A1-1Ib, d). They are designated by the volume they contain. A micropropagation and/or seed culture laboratory should have volumetric flasks in capacities of 5, 10, 25, 50, 100, 250, 500, and 1000 ml. To ensure accuracy, use volumetric flasks rather than beakers or flasks to make dilutions and prepare media. Volumetric flasks are expensive and should not be used as storage containers. Also, they must not be autoclaved because the high temperature and pressure may change them enough to impair their accuracy.

When using a volumetric flask to make dilutions or prepare solutions, pour the solvent into it (approximately 20–50% of its total volume), and then introduce the compound to be dissolved or solution to be diluted. Agitate by gently inverting the flask back and forth several times (after the first few inversions hold the flask with the opening pointing upward and away from individuals or equipment and gently and carefully remove the stopper to release pressure) and add solvent (water, alcohol, etc.) up to the mark. Then shake again and pour into a bottle. Follow the instructions in the tables in Chapter 3 when using a volumetric flask to prepare media. Always add acid (for example HCl, H_2SO_4 acetic) or alkali (bases like NaOH, KOH) into water (remember A comes before W) to prevent boiling, sputtering, or overheating. (Supply sources: 4, 6, 11, 13, 14, 16, 19, 21, 23.)

Wetting Agents. See Surfactants (1–4, 8, 11–13).

APPENDIX TWO

Sources of Supplies and Equipment

All chemicals and apparatus mentioned in this list are available from many suppliers in the United States and other countries (with the advent of the World Wide Web and the expansion of international trade many companies have web sites and/or branches in different parts of the world), but because an occasional item may be difficult to obtain locally, a list of suppliers is provided here. The list was compiled from a variety of sources. Listings are coded by numbers that appear in parentheses at the end of the description in each Appendix 1 entry. In addition, the general categories of laboratory equipment, reagents, chemicals, and other supplies offered by each source are summarized. There are, no doubt, other equally adequate sources in the United States and abroad; the mention of some and omission of others should not be interpreted as endorsements, approval, or criticism, or lack of them. I do not have any financial or other interest in any of the listed sources. None of the listed sources has provided me with any incentives to list them and no one has asked not to be listed.

With few or no exceptions suppliers have web sites which provide a rapid, simple, and convenient means of ordering supplies and equipment. Most suppliers are ethical, honest, and strive to serve their customers promptly, courteously, and well. However, there are exceptions and therefore caution is advisable when dealing with new or little known sources. And one should never, ever deal with suppliers who send or advertize through unsolicited faxes and e-mail messages (the much hated "spam"). Many suppliers who have web sites also issue catalogs. Those who prefer to use catalogs should request them well in advance of the time when supplies will be needed. If not ordering from a web site, orders should be sent by fax (many suppliers have in-country toll-free numbers) or by airmail from outside the United States. Airmail or shipping company (Federal Express, DHL, UPS, and similar) delivery should be requested for smaller items. In some countries the importation of equipment, apparatus, and chemicals requires special permits, which should be secured well in advance.

Some chemicals should be stored under refrigeration, and allowing them to linger in a postal warehouse may cause damage. Therefore it is advisable to alert postal authorities or delivery companies of their arrival and to make arrangements to pick them up promptly. A final word: the author and/or the publisher can neither

undertake to supply equipment, chemicals, and reagents nor act as intermediaries between suppliers and buyers.

A complaint made about this list in the first edition was that it was limited almost entirely to American companies. This was so because the authors are American who live and function in the USA, and are not familiar with suppliers elsewhere. The same is true for the author of this edition. Many American companies have branches in numerous countries. However, due to the advent of the World Wide Web this list also includes a number of foreign companies. Those who do not wish to order from American companies or their branches in other countries can easily find local suppliers.

An important point to keep in mind is that companies move, change names, are sold, go out of business, and consolidate. Therefore the accuracy of this list may change during the life of this book. At first an attempt was made to maintain a numbered alphabetical list. This proved to be impossible as new sources came to light.

1 Acrõs Organics-Fisher Scientific 2002–2003 catalog has 2448 pages which lists organics and fine chemicals. It also includes a considerable amount of helpful information. The organics catalog is also published on a CD ROM. Their address is Fisher Scientific, 2000 Park Lane Dr., Pittsburg, PA 15275-9952, USA. Telephone: orders, 800 766 7000; technical service, 800 227 6701. Fax: 800 926 1166. URLs: www.fishersci.com and www.acros.com.

2 Aldrich Chemical Co. is now part of Sigma-Aldrich, 3050 Spruce St., St. Louis, MO 63103, USA. Telephone: orders, 800 325 3010; technical service, 800 325 5832. Fax: 800 325 5052. URL: www.sigmaaldrich.com; technical service, www.sigmaaldrich.com/techinfo. E-mail: bigred@sial.com. This company carries one of the largest selections of chemicals in the world including: inorganic chemicals, organic chemicals, biochemicals, reagents, hormones, amino acids, and vitamins. Sigma-Aldrich has branches in many parts of the world. They are listed in their web site together with representatives and associated companies. Sigma and Aldrich issue separate catalogs which can be requested on their web site.

3 Anachemia is a Canadian laboratory supply house which offers chemicals and reagents. Their address is Anachemia Canada Inc., 255 Norman, Lachine, Canada H8R 1A3. Telephone: 514 489 5711. Fax: 514 363 5281. URL: www.anachemia.com. E-mail: info@anachemia.com, sales@anachemia.com, and techhelp@anachemia.com.

4 J. T. Baker Chemical Co. is now Mallinckrodt Baker, Inc., 222 Red School Lane, Phillipsburg, NJ 08865, USA. Telephone: 908 859 2151 and 800 582 2537 (1 800 JTBAKER). Fax: 908 859 6905. URL: www.jtbaker.com. E-mail: infombi@mkg.com and techservice@mkg.com. They carry a large variety of chemicals, mostly inorganics and some organic chemicals, hormones, amino acids, and vitamins. Mallinkrodt Baker has branches in many parts of the world.

5 The Baker Company, Inc., P. O. Drawer E, Sanford Airport, Sanford, ME 0407, USA manufactures and sells laminar-flow hoods. Telephone: 800 992 2537 and 207 324 8773. Fax: 207 324 3869. URL: www.bakercompany.com. E-mail: bakerco@bakerco.com.

6 The Bellco Glass Company is now part of the Schott Corporation, 3 Odell Plaza, Yonkers, NY 10701, USA. Telephone: 914 968 1400. Fax: 914 968 8585. URL: www.us.schott.com. They produce and sell a large variety of glassware as well as some laboratory equipment. Their web site lists a large number of dealers.

7 Caisson Laboratories, Inc., 5 West Center, Sugar City, ID 83440, USA or P. O. Box 337, Rexburg, ID 83440, USA produce and/or sell culture media, chemicals, containers, and equipment. Telephone: 208 656 0880 and 877 840 0500. Fax: 208 656 0888. URL: www.caissonalbs.com. E-mail: custserv@caissonlabs.com, sales@caissonlabs.com, and custserv@caissonlabs.com.

8 CalBiochem, EMD Biosciences, Inc., 10394 Pacific Center Court, San Diego, CA 9212, USA. Telephone: 800 854 3417 and 858 453 3552. Fax: 800 776 0999, 866 642 0301, and 858 450 9600. URL: www.emdbiosciences.com. E-mail: customer.service@emdbiosciences.com; technical support, technical@calbiochem.com. Their web site lists branches, dealers, and associated companies in 55 countries. CalBiochem is now a subsidiary of Merck Biosciences. They carry a wide selection of biochemicals (organic chemicals, hormones, amino acids, and vitamins), antibiotics, and other supplies.

9 Difco Laboratories was a major supplier of agar, culture media, and medium components for a long time. They are now part of BD (Becton, Dickinson, and Company), 1 Becton Dr., Franklin Lakes, NJ 07417, USA, and still carry some supplies and equipment that can be of use to orchid micropropagators, but can no longer be viewed as a major source. Telephone: technical assistance, 800 638 8663 (selection 2); customer servive, 800 675 0908; worldwide, 410 316 4000. URL: www.bd.com. E-mail: Technical_Services@bd.com

10 Duchefa Biochemie B. V., A. Hofmanweg 71, 2031 BH Haarlem, the Netherlands or P. O. Box 809, 2003 RV Haarlem, the Netherlands produces and/or sells biochemicals, plant cell and tissue culture media, and supplies and plant molecular biochemicals. Telephone: +31 (0)23 531 90 93. Fax: +31 (0)23 531 80 27. URL: www.duchefa.com. The web site lists a number of world wide locations. Their catalog is well illustrated and very informative.

11 Fisher Scientific, 2000 Park Lane Dr., Pittsburg, PA 15275-9952, USA, with branches in Canada and China, sells just about all supplies, chemicals, equipment, and apparatus a laboratory may need. The main section of their catalog consists of 1946 pages. There are also 416 pages of chemicals, several detailed indexes, and a considerable amount of valuable information. They also have a chemicals-only catalog. Telephone: 412 490 8300 and 800 766 7000. Fax: 800 926 1166. URL: www.fishersci.com.

12 Fluka Chemie AG, Industriel Strasse 25, CH-9471 Buchs, Switzerland, P. O. Box 14508, St. Louis, MO 63178-9916, USA, or P. O. Box 2060, Milwaukee, WI 53201, USA, sells laboratory chemicals, analytical reagents, and some media. Telephone: Switzerland, +41 81 755 2511; USA, 414 273 3859 and 800 558 9160. Fax: Switzerland, +41 81 756 5449; USA, 414 273 4979 and 800 962 9591; USA technical service, 800 493 7262 and 800 HYDRANAL; USA customer service, 800 358 5287; USA general service, 800 803 5832. URL: www.fluka.com (will open the Sigma-Aldrich web site). E-mail: fluka@sial.com. Fluka has worldwide branches.

13 Local stores. Many items can be purchased in local retail stores.

14 Millipore Company, 290 Concord Rd., Billerica, MA 01821, USA, sells sterilizing filters and other equipment. Telephone: 800 645 5476. URL: www.millipore.com. The web site can be accessed in several languages.

15 Osmotek Ltd., P. O. Box 550, Rehovot 76120, Israel, sells well-designed culture vessels and plant tissue culture micropropagation systems. Telephone:

+972 8 931 5556. Fax: +972 8 731 5177. URL: www.osmotek.com. E-mail: osmotek@attglobal.net. They have a free CD with excellent training movies and interesting information. This company may no longer exist.

16 Phytotechnology Laboratories, 7895 Mastin Dr., Overland Park, KS 66204-2312, USA, sells media, medium components, equipment, and supplies. Telephone and fax: 888 749 8682 and 913 341 5343. URL: phytotechlabs.com. E-mail: sales, sales@phytotechlabs.com; technical information, info@phytotechlabs.com.

17 Plant Media, www.plantmedia.com, sells culture media, chemicals, glassware, and accessories.

18 Sigma Chemical Corporation, *see* Aldrich Chemical Company (2).

19 Thomas Scientific is a 100-year-old company at 99 High Hill Rd., I-295, P. O. Box 99, Swedesboro, NJ 08085, USA. Their catalog (2000+ pages) contains everything a laboratory may need. Telephone: main number, 856 467 2000; customer service, 800 345 2100; export sales (from US telephones only), 800 524 0018. Fax: worldwide, 856 467 3087; from US only, 800 345 5232; international sales, 856 467 0512. URL: www.thomassci.com. E-mail: general, value@thomassci.com; international sales, global@thomassci.com.

20 Tissue Quick Plant Laboratories, www.tissuequickplantlabs.com, sells tissue culture and seed germination media and supplies.

21 VWR (at one time Van Waters and Rogers), 1310 Goshen Parkway, West Chester, PA 19380, USA, also has a number of global sites and web sites in several languages. Like Thomas Scientific and Fisher Scientific they sell all chemicals, supplies, apparatus, and equipment laboratories may need. Telephone: 800 932 5000 and 800 320 4357. URL: www.vwr.com. E-mail: solutions@vwr.com.

22 Whatman Inc. is a name synonymous with high quality filter and chromatography paper. Their corporate address is Whatman plc, Whatman House, St. Leonard's Road, 20/20 Maidstone, Kent ME16 0LS, UK. One US address is 9 Bridewell Place, Clifton, NJ 07014. Telephone: UK, +44 (0)1622 676670; USA main, 973 773 5800, 800 441 6555; USA customer service, 800 631 7290; USA technical service, 800 922 0361. Fax: UK, +44 (0)1622 677011; USA main, 973 472 6949. URL: www.whatman.com. E-mail: UK, information@whatman.com; USA, info@whatman.com. The web site has an interesting history of the company.

23 Austratech, 19 Blaxland Court, Mooroolbark, Victoria 3138, Australia. Telephone: +61 3 9737 1416. Fax: +61 3 9737 1418. URL: www.austratech.com.au. E-mail: sales@austratech.com.au. They carry chemicals, reagents, media components, and equipment.

24 Wako Chemicals is a Japanese company with offices in Germany and the USA. They sell a wide selection of chemicals and reagents. URL: Japan, www.wako-chem.co.jp; USA, www.wakousa.com; Germany, www.wako-chemicals.de; catalog, www.search.wako-chem.com.

25 AirClean Systems, Raleigh, NC 27604, USA, produce laminar flow hoods. Telephone: 919 255-3220 and 800 849-0472. Fax: 919 255 6120. URL: www.aircleansystems.com. E-mail: info@aircleansystems.com.

26 Unicorn Imp. & Mfg. Corporation, P. O. Box 272, Commerce, TX 75429, USA, sells plastic culture vessels. Telephone: USA, 800 888 0811; worldwide, 903 886 8282. Fax: 903 886 8878. URL: www.unicornbags.com.

APPENDIX THREE

Some Sites of Interest on the World Wide Web

An enormous amount of information on orchids and other subjects of interest is available on the World Wide Web (www). The number of web sites is very large and listing all of them is a near impossibility. Even if a list of all sites could be compiled, it would not remain accurate for long. Sites are constantly added, removed, or changed. The list below is intended as both a source of information and a starting point. Additional web sites can be found with a search engine.

- Agritech Publications, the Plant Tissue Culture Book Store, http://agritechpublications.com
- American Orchid Society, www.aos.org
- Australian Orchid Council, www.orchidsaustralia.com
- Botanical Society of America, www.botany.org
- Brazilian orchids, http://delfinadearaujo.com
- *Bulbophyllum* and *Cirrhopetalum* photographs page, www.edit.ne.jp/~kohsaka/hhtml/haru1100.htm
- Canadian Orchid Congress, www.canadianorchidcongress.ca
- Chemicals, structure, and formulae, http://chem.sis.nlm.nih.gov/chemidplus
- CITES, web page of the Secretariat for CITES, www.cites.org
- Florida native orchids, www.fl-orchid.com
- Germany, German Orchid Society, http://ourworld.compuserve.com/Homepages/rolfachim_reichart/dog.htm
- Hybrids, registration (information about registration), www.rhs.org.uk/research.registration_orchids.asp
- Index of Plant Names, www.ipni
- Indonesian orchids, www.geocities.com/RainForest/Vines/9686
- International Code of Botanical Nomenclature (St. Louis Code), with the official text in English, www.bgbm.fu-berlin.de/iapt/nomenclature/code/SaintLouis/0001ICSLContents.htm
- Japan, All Japan Orchid Society, www.orchid.or.jp/orchid/people/tanaka/index.htm
- Jay's Internet Orchid Species Encyclopedia, www.orchidspecies.com
- Kitchen Culture Kits, Inc. (tissue culture kits, supplies sales, and informative articles), www.kitchenculturekit.com/index.htm

- Lotte and Thomas Orchids (an interesting and informative orchid propagation site), www.orchideenvermehrung.at/english
- Malaysia, Orchid Society of Malaysia, soonpeng@tm.net.my
- Media recipes, http://members.cox.net/lmlauman/osp/html/mcsg_database.html
- Miltonias and related orchids, http://miltoniopsis.com
- Missouri Botanical Garden Vascular W3 Tropicos Database, http://mobot.mobot.org/W3T/Search/vast.html
- Nero Wolfe's Orchids, www.concentric.net/~Kgunby
- New Zealand, www.orchidsonline.com.au/NZOrchidSocieties.html.
- North American orchids, www.flmnh.ufl.edu1/naorchid
- North American terrestrial orchids, www.wfnirvana.com/orchids/orchids.htm
- Orchid dealers, lecturers, sales, and general site, www.orchidmall.com
- Orchid genera and abbreviations, www.notsogreenthumb.org/orchids/genera/gebnera1.htm
- Orchid history, www.r-rigby.demon.co.uk/histo6.html
- Orchid House, http://retires.uwaterloo.ca/~jerry/orchids
- Orchid information and photographs index, www.orchidsonline.com/au/photo.htm
- Orchid Mall, www.orchidmall.com
- Orchid Mania, www.orchids.org
- Orchid media recipes, http://members.cox.net/lmlauman/osp/html/mcsg_database.html
- Orchid names, www.rhs.org.uk/research/registerpages/orchidsearch.asp
- Orchid nomenclature, Brisbane Orchid Society, www.users.bigpond.com/gmcorbin/bos/nomencl.html
- Orchid parentages, www.rhs.org.uk/research/registerpages/orchid_parentage.asp
- Orchid photographs database, http://los.lon.imag.net/picref.asp
- Orchid photographs page, www.orchidworks.com
- Orchid Registrar, Royal Horticultural Society, www.rhs.org.uk
- Orchid Research Newsletter, Royal Botanic Gardens, Kew, www.rbgkew.org.uk/herbarium/orchid
- Orchid Review, Royal Horticultural Society, www.rhs.org.uk
- Orchid Safari, www.geocities.com/~marylois
- Orchid Seed Bank Project (a site which contains culture media, methods, discussions, and seed sources), http://members.cox.net/lmlauman/osp/index.html
- Orchid sites, list of, http://lab.troymeyers.com/orchidculture/FMPro?-db=favoritelinksgroup&-lay=cgi921&-format=favorites.htm&-sortfield=s&-findall
- Orchid Spring, a serious and informative discussion group, www.orchidspring.com
- Orchid Wire, resource directory managed by Wendell Kozak, www.orchidwire.com
- Orchids on the World Wide Web (a web site which lists other sites), http://retirees.uwaterloo.ca/~jerry/orchids/osites.html
- Orhidei, Russian orchid web site, www.allaboutorchids.r52.ru
- Papua New Guinea orchid pictures (nearly 2000 as this is being written), an excellent site (one of the very best) maintained by Wolfgang H. Bandisch, http://gallery.orchidspng.com/index.php

- Peruvian orchids, www.geocities.com/RainForest/6514
- Pest problems in the Pacific and South East Asia, www.pestnet.org
- Pesticides and toxicology, http://ace.orst.edu/info/extoxnet
- Phylogenetic systematics, www.ucmp.berkeley.edu/clad/clad4.html
- Plant hormones, www.plant-hormones.info
- Plant tissue culture information, http://aggie-horticulture.tamu.edu/tisscult/tcintro.html
- Plant Tissue Culture Information Exchange at Texas A&M University, http://aggie-horticulture.tamu.edu/tisscult/database
- Plant Tissue Culture Research at the University of Minnesota (an excellent and informative discussion group), owner-plant-tc@lists.umn.edu
- Plant World Explorations, http://members.ozemail.com.au~mhepel
- Provisional Checklist of Orchidaceae, Monocot Checklist Project, Royal Botanical Gardens, Kew, www.rbgkew.org.uk/data/monocots/orchid_intro.html
- Royal Botanic Gardens, Kew, Orchid Research Newsletter (does not specialize in micropropagation), www.rbgkew.org.uk/herbarium/orchid
- Sigma-Aldrich media expert (site with information on culture media of all types), www.sigmaaldrich.com/Area_of_Interest/Life_Science/Cell_Culture/Helpful_Resources/Media_Expert.html
- Singapore, Orchid Society of South East Asia, www.mediav.com/sg/ossea
- Tissue culture media recipes, http://members.cox.net/lmlauman/osp/html/mcsg_database.html
- Tissue culture protocols, www.sigmaaldrich.com/Area_of_Interest/Life_Science/Plant_Biotechnology/Tissue_Culture_Protocols.html
- United States Department of Agriculture (USDA), www.usda.gov
- Units of measurement (a site which contains descriptions and conversion factors for an amazing number of units), www.unc.edu/%7Erowlett/units/index.html

I thank Wolfgang H. Bandisch, Stephen Kemp, Hideka Kobayashi (who also provided extensive lists and many copies of literature on orchid micropropagation), Jose A. Izquierdo-Rivera, and the Orchid Spring discussion board (managed by Wolfgang H. Bandisch) among others for information about some of the entries on this list.

APPENDIX FOUR

Light

Illumination is an extremely important factor in micropropagation. Light was discussed in Chapter 2 at some length. However the additional information in this appendix may be useful.

Measuring Light

Light is the source of energy for plant growth. It consists of different wavelengths (colors). The distribution and intensity of these wavelengths produced by a source of illumination is called the emission spectrum. In nature plants grow under natural sunlight. Under culture room conditions explants, seedlings, and plantlets are illuminated by artificial light sources which are designed to simulate the sun or at least emit a spectrum that approximates natural sunlight.

Light measuring instruments or sensors differ in their sensitivity to the energy spectrum. To measure light intensity in terms of the wavelengths required by plants, it is necessary to determine the photosynthetically active radiation (PAR) produced by a source. PAR light meters measure energy in the 400–700 nm range. They measure light in micromoles (or microEinsteins) per second per square meter ($\mu mol\ s^{-1}\ m^{-2}$).

Meters that measure foot candles are not appropriate because they measure light intensity in terms of human vision. Photographic light meters are also not appropriate because they measure light intensity as it affects photographic film (or digital camera sensitivity). However, it is possible to obtain approximations by using photographic light meters (stand alone or built into cameras) by making measurements with them and referring to the appropriate tables in Chapter 2. As a rule, sufficient illumination is produced by two cool white 40-W fluorescent tubes plus a pair of 25-W incandescent bulbs placed approximately 30 cm above the cultures.

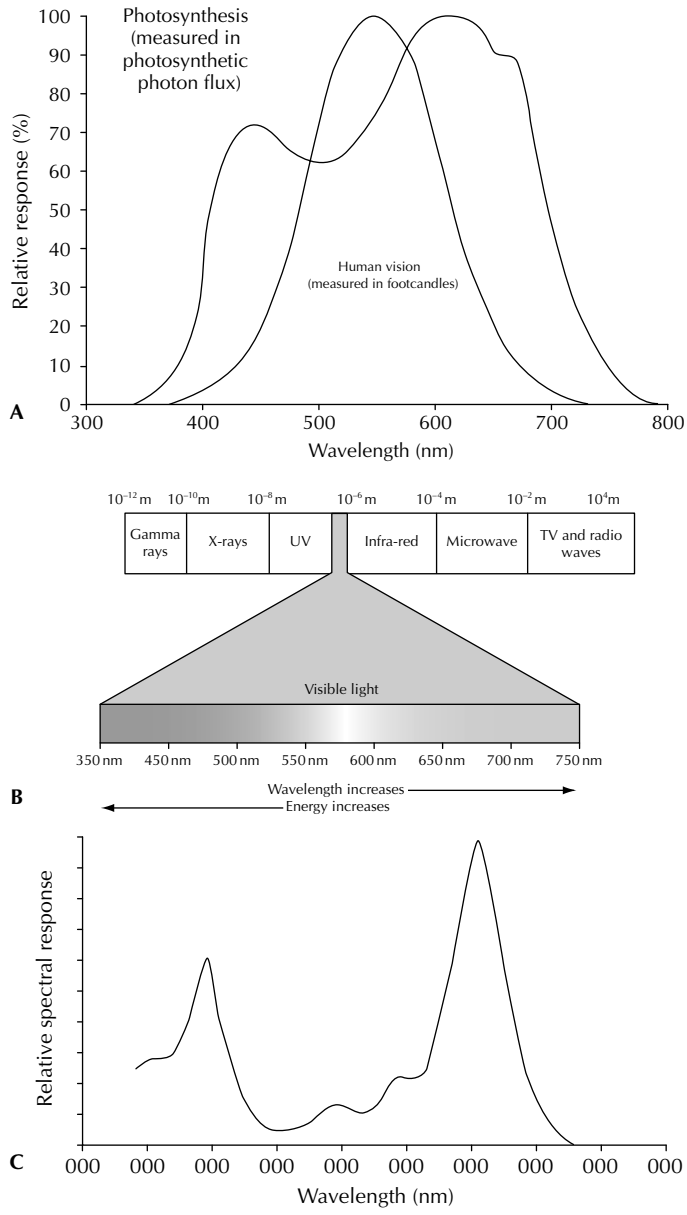


FIG. A4-1. **Light and action spectra. A. Photosynthesis and human vision. B. Electromagnetic and light spectrum. C. Action spectrum of chlorophyll synthesis.**

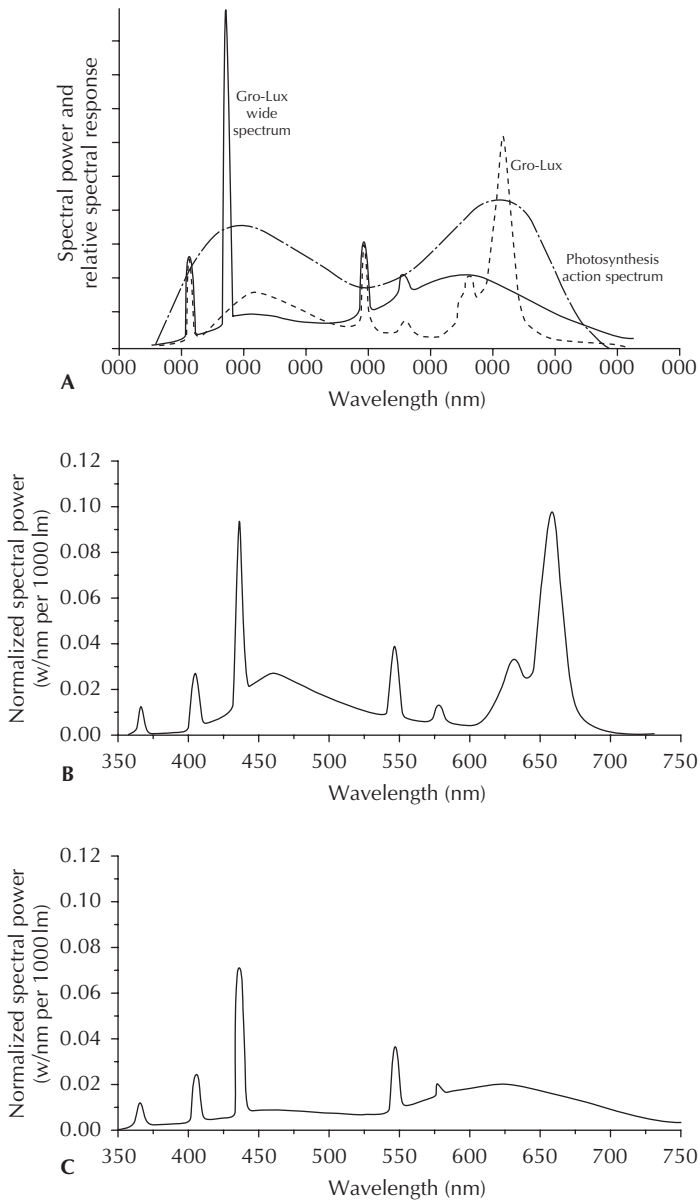


FIG. A4-2. **Emission spectra of Gro Lux lamps and photosynthesis. A. The emission spectra of Gro Lux lamps and Wide Spectrum Gro Lux lamps versus the light spectrum of photosynthesis. B. Emission spectrum of Gro Lux lamps. C. Emission spectrum of Wide Spectrum Gro Lux (GRO/WS) lamps.**

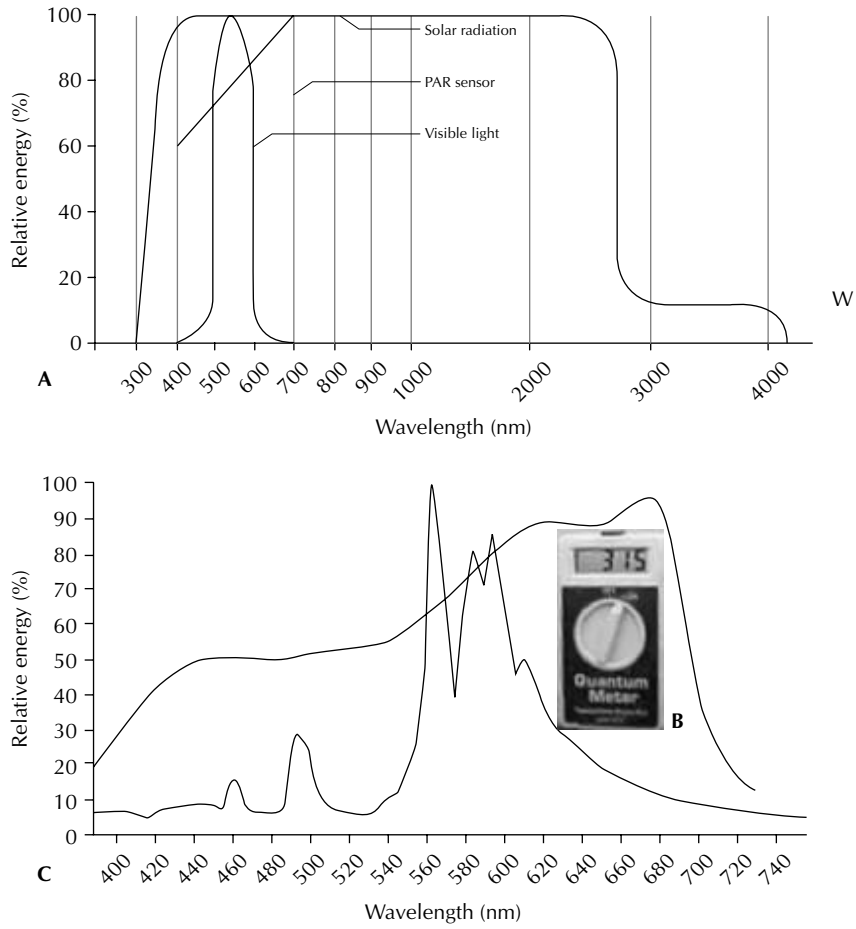


FIG. A4-3. **Light spectrum and measurements. A. Measurements of the same light source with different meters. B. A light meter that measures photosynthetically active radiation (PAR). C. Emission spectrum of a high pressure lamp versus a plant sensitivity curve.**

Wave Length and the Color of Light

TABLE A4-1. **Wave length and the color of light**[a]

Color	Wavelength, nm	
	Representative	Limits
Ultraviolet		< 400
Limit of sun UV at earth's surface		292
Visible spectrum		
Violet	410	400–424
Blue	470	424–491.2
Green	520	491.2–575
Maximum human acuity	556	
Yellow	580	575–585
Orange	600	585–647
Red	650	647–700
Infrared		> 700

[a]Plant physiologists sometimes refer to the color or spectrum of light as light quality.

Light Radiation Conversions

Plants use light energy between 400 and 700 nm. This region is called photosynthetically active radiation and is generally referred to as PAR. Illumination for plant growth, which is called "irradiance," is sometimes measured as watts of PAR per square meter ($W\ m^{-2}$). The preferred method of measuring light for plant growth utilizes the quantum flux unit in the PAR region called "photons." The units are micromoles per square meter per second ($\mu mol\ m^{-2}\ s^{-1}$), where 1 mol of photons = 6.022×1023 photons. This is an appropriate measurement because it measures directly the light energy that impacts the plants and is available for photosynthesis. Unfortunately, lamp manufacturers usually rate their products in lumens, a unit of light intensity that measures the portion of the spectrum which is visible to humans. Lighting levels are often measured or reported in lumens per square meter (lux, lx) or per square foot (foot-candles, ft-c). This is unfortunate because the spectral sensitivities of the human eye and of plants are quite different. A simple and direct method for interconverting the different units does not exist. Conversion is possible only through the evaluation of the entire range of spectral characteristics of a specific light source.

An excellent calculator and conversion table for radiation in the 400–700 nm range produced by different lamps is available on www.egc.com/illumination.html.

APPENDIX FIVE

Formulary

This formulary contains information about some chemicals and reagents which are routinely used in micropropagation laboratories.

Abscisic Acid. Abscisic is not used in orchid micropropagation. If needed it should be dissolved in 70% ethanol.

Acids and Bases. The molarities, densities, formulae, and other information for some acids and bases are given in Table A5-1.

Ammonium Hydroxide. Solutions of ammonium hydroxide can be used as a solvent and to raise pH (Table A5-2).

Auxins. Auxins can be dissolved in 0.1- or 1-*M* ammonium or potassium hydroxide as well as in 95 or 70% ethyl alcohol (ethanol, drinking alcohol). It is best to dissolve them in 70% ethanol because the alcohol is also a sterilant. Auxin solutions should be stored in a freezer between uses. Prolonged storage of solutions is not advisable.

TABLE A5-1. **Characteristics of some acids and bases: molarities, densities, formulae and other information.**

Acid or base	Molecular weight	Approximate density		Molarity of commercial product	Volume (ml) for 1000 ml of 1 normal solution (1N)[a]	Normality of concentrated reagent	Approximate percent in concentrated reagent
		g ml^{-1}	g l^{-1}				
Acids							
Hydrochloric, HCl	36.47	1.19	445	12.2	82.0	11.6	37.0
Nitric, HNO_3	63.02	1.41	989	15.7	63.8	16.0	70.0
Phosphoric, H_3PO_4	98.04	1.70	1462	14.9	22.4	44.7	85.0
Sulfuric, H_2SO_4	98.08	1.84	1742	17.8	28.2	36.0	96.0
Bases							
Ammonium hydroxide, NH_4OH	17.03 as NH_3	0.90	252	14.8	67.6	15.0	58.0 (29.0 as NH_3)
Potassium hydroxide, KOH	56.11	1.54	800	14.3	70.2		
Sodium hydroxide, NaOH	40.01	1.63	763	19.1	52.5		
Sodium hydroxide, saturated	40.01			19.1	52.5		

[a]Concentrations of commercial solutions should always be checked before use. Concentrated acids and bases are dangerous and must be used with care.

TABLE A5-2. **Preparation of ammonium hydroxide solutions**[a]

Desired molarity	Volume of reagent grade NH_4OH, ml	Distilled water to, ml[a]
15	Use as is, reagent concentration is 14.8 molar	
10	676	1000
5	338	1000
2.5	169	1000
1	67.6	1000
0.1	6.8	1000
0.01	0.7	1000

[a]Pour 500 ml of water in a 1-l volumetric flask, add the NH_4OH slowly, add 200 ml of distilled water, place stopper in opening, mix by inverting flask a few times, remove stopper carefully to release pressure, adjust volume to 1 l, replace stopper, and mix again several times. Store in a plastic bottle.

Benzyladenine. Benzyladenine (6-benzylaminopurine, N^6-benzylaminopurine, N^6-benzylaminopurine, BA, or BAP) can be dissolved in 0.1-*M* hydrochloric acid or 95 or 70% ethanol. It is best to dissolve it and other cytokinins in 70% ethanol because the alcohol is also a sterilant. Solutions of BA and other cytokinins should be stored in a freezer between uses. Prolonged storage of solutions is not advisable.

Buffer. *See* Potassium Phosphate Buffer.

Calcium Hypochlorite. As a saturated solution, this can be used to surface-sterilize seeds, plants, organs, and tissues. The use of this solution was formulated by J. K. Wilson, who was a student in Lewis Knudson's laboratory. Wilson published his work in the *American Journal of Botany* in 1915. According to Giltner J. Knudson, Lewis Knudson's youngest son, Wilson abandoned botany and science to become a tennis teacher and professional player. This sterilant is prepared by dissolving 10 g of calcium hypochlorite in 140 ml (or 7 g 100 ml^{-1}) of water, stirring vigorously, allowing the solution to stand for 3–5 min until the precipitate settles, stirring again, and decanting the yellowish supernatant for use. The solution must be used within 12 h. It should be discarded after that. A few drops of Tween 20, mild household detergent, or baby shampoo should be added as a wetting agent.

Clorox. Clorox is often used as surface sterilant/decontaminant for plants, organs, and tissues that serve as sources of explants, and work surfaces. Concentrations are sometimes given as percent (v/v) Clorox or levels of sodium hydroxide. Table A5-3 contains information regarding the preparation of both kinds of dilutions. In the USA, Clorox contains 6% sodium hypochlorite at present. Previously the concentration was 5.25% sodium hypochlorite. Other domestic bleaches may contain similar or slightly different levels of the active ingredient (± 0.25–1%). Small differences (0.25%) are insignificant and can be ignored. Larger differences (more than 0.25%) must be taken into consideration when dilutions are made. Clorox or other domestic bleaches should not be used to wash hands.

Cytokinins. Cytokinins are hormones which enhance bud and shoot formation and development. They include benzyladenine, dimethylaminopurine, isopentenyl adenosine, kinetin, thidiazuron, and zeatin.

TABLE A5-3. **Clorox (5.25% active principle) dilutions**[a]

Desired percentage				
	Sodium hypochlorite			
Clorox	5.25%	6.0%	Volume of Clorox, ml	Distilled water to, ml[a]
100		6.00	Use as is	None
100	5.25		Use as is	None
50	2.63	3.00	500	1000
25	1.31	1.50	250	1000
10	0.53	0.60	100	1000
5	0.27	0.30	50	1000
2.5	0.13	0.15	25	1000
1	0.05	0.06	10	1000
0.25	0.01	0.015	2.5	1000
0.1	0.005	0.006	1	1000

[a]Pour 500 ml of water in a 1-l volumetric flask, add the Clorox, add 200 ml of distilled water, place stopper in opening, mix by inverting the flask a few times, remove stopper carefully to release pressure, adjust volume to 1 l, replace stopper, and mix again a few times. Use solutions shortly after preparation and discard unused leftovers. Tissues which are surface-sterilized with these solutions will eventually be washed with sterile distilled water. Therefore Clorox solutions can be prepared with tap or clean rain water if distilled water is in short supply, or in an emergency.

Dimethylaminopurine. This synthetic cytokinin should be dissolved in 0.01- or 0.1-*M* hydrochloric acid or sodium hydroxide in water or 70% ethanol. The latter is preferable because it is also a sterilant.

Ethyl Alcohol. Ethyl alcohol (ethanol, alcohol, drinking alcohol) is very useful in the laboratory as a solvent and sterilant. Only non-denatured ethanol should be used because the denaturing agent can be toxic to workers, tissues, and explants. Laboratory grade ethanol is available in 97 and 95% purity (the rest being water). The 97% grade is known as absolute ethanol. It is more expensive than the 95% grade and may contain a small amount of benzene. There is no reason to use it, particularly as laboratory dilutions are made with water. Also, this grade can absorb moisture from the atmosphere with time. This will reduce ethanol levels. The 95% grade is more economical in cost and preferable to absolute ethanol for most purposes. In fact the most suitable ethanol dilution for use as a solvent, as a sterilant for tissues and organs, and for swabbing surfaces is 70% in distilled water. This is more effective than higher concentrations. This solution can also be sprayed on work surfaces to ensure sterility. Instruments can be placed in it between uses or dipped and shaken to remove it and/or flamed. Table A5-4 provides instructions for making a number of dilutions. Ethanol (97 or 95%) and ethanol dilutions should be stored in glass or plastic bottles. Bottles containing 97% ethanol should be capped very tightly.

Methyl alcohol (methanol) is toxic to workers and plants and must not be used. Isopropyl (rubbing alcohol) can be used to sterilize tools and surfaces and in alcohol lamps, but it must not be employed as a solvent for compounds which may be added to culture media.

GA. *See* Gibberellins.

GA_3. *See* Giberellins.

TABLE A5-4. **Ethanol dilutions**[a]

Desired		Grade of ethanol, ml[a]		
Concentration, %	Volume, ml	97%	95%	Distilled water to, ml[b]
10	1000	103	105	1000
20	1000	206	210	1000
30	1000	309	315	1000
40	1000	412	420	1000
50	1000	515	525	1000
60	1000	618	630	1000
70	1000	721	735	1000
80	1000	824	840	1000
90	1000	927	945	1000
95	1000	Use 95% laboratory grade as is		
97	1000	Use 97% laboratory grade as is		

[a]Pour 500 ml of water in a 1-l volumetric flask, add the ethanol, add 200 ml of water, place stopper in opening, mix by inverting the flask a few times, remove stopper carefully to release pressure, adjust volume to 1 l, replace the stopper, and mix again a few times. Store in a glass or plastic bottle.
[b]Rounded off.

Gibberellins. These are used to a very limited extent in orchid micropropagation, if at all. They can be dissolved in 0.1-*M* potassium hydroxide, 0.1-*M* sodium hydroxide, or 70% ethanol. A small amount of base can facilitate their solubility in alcohol. It is best to dissolve them in 70% ethanol because the alcohol is also a sterilant.

Hoagland's solution. Hoagland's solution is not used as a culture medium for orchid micropropagation, but there are occasional inquiries about it. There are two versions of this solution (Table A5-5).

Hydrochloric Acid. Hydrochloric acid (HCl) can be used as a solvent and to lower pH in different concentrations (Table A5-6).

Indoleacetic Acid. Indoleacetic acid (IAA), a naturally occurring auxin, should be dissolved in 70% ethanol and stored in a freezer. Prolonged storage is not advisable.

Indolebutyric Acid. Indolebutyric acid (IBA), a synthetic auxin, should be dissolved in 70% ethanol and stored in a freezer. Prolonged storage is not advisable.

Indolepropionic Acid. Indolepropionic acid (IPA), a synthetic auxin, should be dissolved in 70% ethanol and stored in a freezer. Prolonged storage is not advisable.

Kinetin. This synthetic cytokinin should be dissolved in 0.01- or 0.1-*M* hydrochloric acid or sodium hydroxide in water or 70% ethanol. The latter is preferable because it is also a sterilant.

Lanolin Pastes. Lanolin pastes can be prepared by dissolving the relevant hormone in an appropriate solvent and adding it to lanolin that has been heated to liquefaction in a water bath. It is safe to assume that 1 g of lanolin is equivalent to 1 ml of water or solvent.

TABLE A5-5. **Preparation of Hoagland's solution**[a,b]

Salt	Weight, g	Distilled water to, ml[a]	Volume of stock solution to use in preparation of nutrient, ml[b]
Version 1			
KH_2PO_4	136.09	1000	1
KNO_3	101.10	1000	5
$Ca(NO_3)_2$	164.10	1000	5
$MgSO_4$	120.39	1000	2
Version 2			
$NH_4H_2PO_4$	115.04	1000	1
KNO_3	101.10	1000	6
$Ca(NO_3)_2$	164.10	1000	4
$MgSO_4$	120.39	1000	2
Iron			
Iron tartrate[c]	5	1000	1
Micronutrients[d]			
H_3BO_3	2.86		
$MnCl_2 \cdot 4H_2O$	1.81		
$ZnSO_4 \cdot 7H_2O$	0.22	1000	1
$CuSO_4 \cdot 5H_2O$	0.08		
$H_2MoO_4 \cdot H_2O$	0.02		

[a]A separate stock solution should be prepared for each salt. Pour 500 ml of distilled water in a volumetric flask, add salt, shake to dissolve, and adjust volume to 1000 ml. Nitrogen-containing solutions can become contaminated and should be frozen.

[b]Pour 500 ml of distilled water into a volumetric flask, add the major element stock solutions, iron, and the micronutrients, shake to mix, and adjust volume to 1000 ml.

[c]This salt can be replaced with chelated iron.

[d]Add all five salts to the same 1 l of stock solution and prepare as in footnote *a* above.

TABLE A5-6. **Dilutions of hydrochloric acid**[a]

	Desired concentration, molarity[a]									
Component	0.01	0.1	1	2	2.5	4	5	6	8	10
HCl, ml	0.86	8.6	86	172	215	344	430	516	688	860
Solvent (water, ethanol, other) to, ml	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

[a]Pour 500 ml of water in a 1-l volumetric flask, add the hydrochloric acid, add 200 ml distilled water, place stopper in opening, mix by inverting the flask a few times, remove stopper carefully to release pressure, adjust volume to 1 l, replace stopper, and mix again a few times. Store in a glass or plastic bottle.

Naphthaleneacetic Acid. Naphthaleneacetic acid (NAA) should be dissolved in 70% ethanol and stored in a freezer. Prolonged storage is not advisable.

Nitric Acid. Nitric acid (HNO_3) can be used as a solvent and to reduce pH in different dilutions (Table A5-7).

Potassium Hydroxide. Potassium hydroxide (KOH) can be used as a solvent and to raise the pH of solutions (Table A5-8).

Potassium Phosphate. Potassium phosphate solutions are prepared by dissolving the indicated amount of salt in 1 l of distilled water (Table A5-9).

TABLE A5-7. **Dilutions of nitric acid**[a]

Desired			
Molarity	Normality	Concentrated HNO_3, ml	Distilled water to, ml[a]
15	15	955	1000
10	10	637	1000
5	5	318	1000
3	3	191	1000
2	2	127.2	1000
1	1	63.6	1000
0.1	0.1	6.36	1000
0.01	0.01	0.64	1000

[a]Pour 500 ml of water in a 1-l volumetric flask, add the nitric acid, add 200 ml distilled water, place stopper in opening, mix by inverting the flask a few times, remove stopper carefully to release pressure, adjust volume to 1 l, replace stopper, and mix again a few times. Store in a glass or plastic bottle. Always add acid to water.

TABLE A5-8. **Preparation of potassium hydroxide solutions**[a]

Desired molarity or normality	KOH, g	Distilled water to, ml[a]
0.01	0.56	1000
0.1	5.61	1000
1.0	56.11	1000
2	112.22	1000
5	280.55	1000

[a]Pour 500 ml of distilled water in a 1-l volumetric flask, drop the pellets in a few at a time, add 200 ml distilled water, place stopper in opening, mix by inverting the flask a few times, remove stopper carefully to release pressure, adjust volume to 1 l, replace stopper, and mix again a few times. Store in a plastic bottle.

TABLE A5-9. **Preparation of potassium phosphate solutions**[a]

	Weight, g		
Desired molarity	K_2HPO_4	KH_2PO_4	Distilled water to, ml[a]
0.01	1.74	1.36	1000
0.1	17.4	13.6	1000
1.0	174	136	1000

[a]Pour 500 ml of distilled water in a 1-l volumetric flask, add the salt, add 200 ml distilled water, place stopper in opening, mix by inverting the flask a few times, remove stopper carefully to release pressure, adjust volume to 1 l, replace stopper, and mix again a few times. Store in a glass or plastic bottle.

Potassium Phosphate Buffer. This buffer is not used extensively in micropropagation, but may become necessary under certain circumstances. The concentration of the buffer is determined by the molarity of the potassium phosphate solutions; its pH depends on the K_2HPO_4/KH_2PO_4 ratio (Table A5-10).

Sodium Hydroxide. Sodium hydroxide (NaOH) can be used as a solvent and to raise the pH of solutions (Table A5-11).

Sulfuric Acid. This can be used as a solvent and to raise the pH of solutions (Table A5-12).

TABLE A5-10. **Preparation of potassium phosphate buffers**[a]

Volume, ml			Volume, ml			Volume, ml		
K_2HPO_4	KH_2PO_4	pH of mixture	K_2HPO_4	KH_2PO_4	pH of mixture	K_2HPO_4	KH_2PO_4	pH of mixture
10.00		8.3	7.00	3.00	7.2	1.00	9.00	5.9
9.90	0.10	8.2	6.00	4.00	7.0	0.50	9.50	5.6
9.75	0.25	8.0	5.00	5.00	6.8	0.25	9.75	5.3
9.50	0.50	7.9	4.00	6.00	6.6	0.10	9.90	5.0
9.00	1.00	7.7	3.00	7.00	6.5		10.00	4.5
8.00	2.00	7.4	2.00	8.00	6.2			

[a]A separate solution should be made for each salt. Pour 500 ml of water in a 1-l volumetric flask, add the salt, add 200 ml distilled water, place stopper in opening, mix by inverting the flask a few times, remove stopper carefully to release pressure, adjust to 1 l, replace stopper, and mix again a few times. Store each solution separately in a glass or plastic bottle. To make 10 ml of buffer, mix solutions as indicated in table. The German orchid expert Hans Burgeff was the first to suggest the use of K_2HPO_4/KH_2PO_4 buffer in orchid media. K_2HPO_4 contains twice as much potassium as KH_2PO_4, the same amount of PO_4, and an equal level of H.

TABLE A5-11. **Preparation of sodium hydroxide solutions**[a]

Desired molarity	NaOH, g	Distilled water to, ml[a]
0.1	4.00	1000
1.0	40.00	1000
2	80.00	1000
5	200.00	1000

[a]Pour 500 ml of distilled water in a 1-l volumetric flask, add the pellets a few at a time, add 200 ml distilled water, place stopper in opening, mix by inverting the flask a few times, remove stopper carefully to release pressure, adjust volume to 1 l, replace stopper, and mix again a few times. Store in a plastic bottle.

TABLE A5-12. **Sulfuric acid dilutions**[a]

Desired			
Molarity	Normality	H_2SO_4, ml	Distilled water to, ml
0.01	0.02	0.56	1000
0.1	0.2	5.64	1000
1.0	2.0	56.4	1000
2	4.0	112.8	1000
5	10.0	282	1000

[a]Pour 500 ml of distilled water in a 1-l volumetric flask, add the acid slowly, add 200 ml distilled water, place stopper in opening, mix by inverting the flask a few times, remove stopper carefully to release pressure, adjust volume to 1 l, replace stopper, and mix again a few times. Store in a glass or plastic bottle. Always add acid to water (mnemonic: A comes before W in the alphabet).

APPENDIX SIX

Atomic Weights, Concentrations, Exponents, Greek and Roman Letters, Ions, Measurements, Molecular Weights, Prefixes, Valences, Units, and Solutions

A large variety of units, numbers, mathematical expressions, concentrations, designations, and other values are used in micropropagation. Some of them are listed, defined, and/or explained in this appendix.

Area

- 1 square meter (m^2) is equivalent to 10,000 square centimeters (1×10^4 cm^2) or 1,000,000 square millimeters (1×10^6 mm^2)

Circles

- Diameter × 3.14 = circumference
- Diameter^2 × 0.79 = area

Concentrations

Amounts per unit volume or weight should be written exponentially. Examples:

- 5 mg per liter or 5 mg/liter should be written as 5 mg l^{-1}
- 10 g per kg or 10 g/kg should be written as 10 g kg^{-1}
- 1 ml per liter or 1 ml/l should be written as 1 ml l^{-1}
- 3 millimoles per liter should be written as 3 mmol l^{-1} or a 3-m*M* solution (there is a difference between *M* which means molar as in "a three molar solution of

sucrose" and mole as in "dissolve 3 moles of sucrose in 500 ml of water." The term *M* (molar) refers to concentration, whereas moles should be applied to amount. It is incorrect to write "dissolve 3 *M* sucrose in 500 ml of water")
- 6 flasks per square meter or 6 flasks/m^2 should be written as 6 flasks m^{-2}

Current practice is to favor the exponential form.

Cylinder

- (Diameter$^2 \times 3.14) \times$ length = surface

Liquid Measure (not metric)

- 2 pints equal 1 quart

Energy

- 1 calorie (or calory) or gram calorie (c) is the amount of heat needed to raise the temperature of 1 g of water by 1°C, from 14.5 to 15.5°C
- 1 erg is equivalent to 10^{-7} joule (J)
- 1 joule (J) is m^2 kg s^{-2}
- 1 kilocalorie (kc) is equivalent to 1000 calories or 10^3 calories
- 1 watt (W) is J s^{-1}; it is equivalent to 1.3×10^{-3} horse power (hp)

Exponents

Very large or very small numbers can be written as exponents. Examples:

- 0.3 can be written as 3×10^{-1} and 0.000005.5 can be expressed as 5.5×10^{-6}
- 10,000 can be presented as 1×10^4

Some Greek Letters

α, alpha as in α-D-glucose (the glucose residue in starch)
β, beta as in β-D-glucose (the glucose residue in cellulose)
γ, gamma (as in gamma radiation)
Δ, δ, capital and lower case delta (Δ is used to designate difference)
μ, mu, which is used to abbreviate micro as in μmole
π, pi (3.14)
Σ, σ, capital and lower case sigma (Σ, summation of; σ, standard deviation)

Ions of Essential Elements, their Names, Weights, and Equivalent Weights

Ion	Name	Weight	Equivalent weight
Anions			
Cl^-	Chloride ion	35.46	35.46
NO_3^-	Nitrate ion	62.01	62.01
HPO_4^{2-}	Phosphate ion	97.99	48.99
$H_2PO_4^{2-}$	Phosphate ion	98.99	49.49
SO_4^{2-}	Sulfate ion	96.06	48.03
Cations			
Ca^{2+}	Calcium ion	40.08	20.04
Mg^{2+}	Magnesium ion	24.30	12.15
Na^+	Sodium ion	22.99	22.99
NH_4^+	Ammonium ion	17.03	17.03
K^+	Potassium ion	39.10	39.10

Length

- 1 centimeter (cm) consists of 10 millimeters (mm); it is 10^{-2} m and equivalent to 0.39 inches (in)
- 1 micrometer (μm) consists of 1000 (10^3) nanometers (nm); it is 1^{-6} m or 1^{-3} mm
- 1 millimeter (mm) consists of 1000 (10^3) micrometers (μm); it is 1^{-3} m and equivalent to 0.04 inches (in)
- 1 nanometer (nm) is 1^{-9} m, 1^{-6} mm, or 1^{-3} μm
- 1 angstrom (Å, no longer used) is 10^{-8} cm

Light

- The candela (cd) is a unit of luminous intensity based on the human eye. Therefore it is not an appropriate unit to measure light intensity as it relates to plant growth
- 1 foot candle (ft-c) = 10.76 lux (lx) = 10.76 meter candles = 0.0011 lumens cm^{-2}
- Irradiance (radiant energy flux received on a unit of plane surface) as energy is given in W m^{-2} or watts per square meter, which is also J s^{-1} m^{-2}, but in the case of plant growth it is important to note the light quality (color)
- Irradiance as moles of photons or moles per square meter per second is mol m^{-2} s^{-1}
- Spectral irradiance as moles per square meter per second per nanometer is mol m^{-2} s^{-1} nm^{-1}
- 1 lux is equivalent to 0.09 foot candles (ft-c). The lux is based on the human eye and therefore it is not an appropriate unit to measure light intensity as it relates to plant growth
- Photosynthetically active radiation (PAR) includes wavelengths between 400 and 700 nm. It is measured as W m^{-2}

Macro- and Microelements, their Atomic Weights and Most Common Valences

Element	Symbol	Atomic weight	Most common valences
Boron	B	10.82	3
Calcium	Ca	40.08	2
Carbon	C	12.01	–4 to +4
Chlorine	Cl	35.46	–1
Cobalt	Co	58.94	2
Copper	Cu	63.54	1, 2
Hydrogen	H	1.01	1
Iron	Fe	55.85	2, 3
Magnesium	Mg	24.31	2
Manganese	Mn	54.94	2, 4, 7
Molybdenum	Mo	95.94	3, 4, 6
Nickel	Ni	58.69	2
Nitrogen	N	14.01	3, 5
Oxygen	O	16.00	–2
Phosphorus	P	30.98	5
Potassium	K	39.10	1
Sulfur	S	32.06	4, 6
Zinc	Zn	65.37	2

Moles

- Mole, an amount in grams equal to the molecular weight of a substance (1000 millimoles)
- Millimole (1000 micromoles or 1000 μmol)
- Molar or *M*, number of moles in a solvent brought up to 1 liter (i.e., the amount of solute plus enough solvent to bring the total volume of the solution to 1 liter or 1000 ml)
- Molal, number of moles per liter (i.e., the amount of solute per 1 liter or solute plus 1000 ml of solvent)
- Moles per liter (mol l^{-1}), amount of solute expressed as moles in solvent brought up to 1 liter (i.e., the amount of solute plus enough solvent to bring the total volume of the solution to 1 liter or 1000 ml)

Plant Growth Regulators, Molecular Weights, Weight/µmole Conversions, Solubility, and Sterilization[a]

	Growth regulators, molecular weights and weight/µmole equivalents[b]																				
Amount, mg l^{-1}	ABA 264.3	Anc 256.3	ADS 184.2	AD 135.1	BA 225.3	*t*CA 148.2	DMSO 203.2	DZe 221.3	2,4-D 221	KGA 384.5	GA_3 346.4	IAA 175.2	IBA 203.2	KIBA 241.3	IPA 189.2	KIN 215.2	NAA 186.2	TDZ 220.2	2,4,5-T 255.5	ZT 219.2	ZTR 351.5
0.1	0.38	0.39	0.54	0.74	0.44	0.68	0.49	0.45	0.45	0.26	0.29	0.57	0.49	0.41	0.53	0.46	0.54	0.45	0.39	0.46	0.28
0.2	0.76	0.78	1.09	1.48	0.88	1.35	0.98	0.90	0.90	0.52	0.58	1.14	0.98	0.83	1.06	0.93	1.07	0.91	0.78	0.91	0.57
0.3	1.14	1.17	1.63	2.22	1.33	2.02	1.48	1.36	1.36	0.78	0.87	1.71	1.48	1.24	1.59	1.39	1.61	1.36	1.17	1.37	0.85
0.4	1.51	1.56	2.17	2.96	1.78	2.70	1.97	1.81	1.81	1.04	1.16	2.28	1.97	1.65	2.12	1.85	2.15	1.81	1.56	1.83	1.13
0.5	1.89	1.95	2.71	3.70	2.22	3.38	2.46	2.26	2.26	1.30	1.45	2.85	2.46	2.06	2.65	2.31	2.69	2.26	1.95	2.29	1.41
1.0	3.78	3.90	5.43	7.40	4.44	6.74	4.92	4.52	4.52	2.60	2.89	5.71	4.90	4.14	5.29	4.65	5.37	4.54	3.91	4.56	2.85
5.0	18.92	19.51	27.14	37.01	22.19	33.74	24.61	22.59	22.62	13.00	15.15	28.54	24.60	20.72	26.43	23.23	26.85	22.71	19.57	22.81	14.23
10.0	37.84	39.02	54.29	74.02	44.38	67.48	49.21	45.19	45.25	26.01	28.89	57.08	49.00	41.44	52.85	46.47	53.71	45.51	39.13	45.62	28.46
Solvent	1Na EtNa	DM EtNa	W EtNa	W EtNa	1Na EtNa	Et 1Na EtNa	1Na EtNa EtHC	Et EtNa EtHC	Et EtNa 1Na	W EtNa	Et EtNa	Et EtNa 1Na	Et EtNa	W EtNa	Et EtNa	1Na EtHC	1Na EtNa	DM EtNa	Et EtNa	1Na EtHC	1Na EtHC
Storage	Fr	Fr	Fr	Fr	Fr	Fr	Fr	Fr	Fr	Fr	Fr	Fr	FR	FR	Fr	Fr	Fr	Fr	Fr	Fr	Fr
Sterilization	A,S	A,S	A,S	A,S	A,S	A,S	A,S	S	A	A,S	A,S	A,S	A,S	A,S	A,S	A,S	A,S	A,S	A,S	A,S	S

[a]Source: Sigma plant cell culture catalog, 1993.

[b]Abbreviations:

Growth regulators: ABA, abscisic acid; Anc, ancymidol; ADS, adenine sulphate; AD, adenine; BA, 6-benzylaminopurine (also known as benzyladenine); *t*CA, *trans*-cinnamic acid; DMSO, 6-(γ, γ-dimethyallylamino)-purine; DZe, dihydro zeatin; 2,4-D, 2,4-dichlorophenoxyacetic acid; KGA, potassium salt of GA_3; GA_3, gibberellic acid; IAA, 3-indolacetic acid; IBA, 3-indolebutyric acid; KIBA, potassium salt of IBA; IPA, indole-3-propionic acid; KIN, kinetin; NAA, α-naphthaleneacetic acid; TDZ, thidiazuron; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; ZT, zeatin; ZTR, zeatin riboside.

Solvents: 1NA, 1N NaOH; DM, dimethylsulfoxide (DMSO); Et, ethyl alcohol, usually 70 or 95%; EtHC, ethyl alcohol (ethanol), 70 or 95% with a few drops of 0.1 N or 1 N HCl; EtNa, ethyl alcohol (ethanol), 70 or 95% with a few drops of 0.1 N or 1 N NaOH or KOH; W, water.

Storage: Fr, freezer.

Sterilization: A, autoclave; S, add in ethanolic solution to still hot and liquid autoclaved medium.

Plant Growth Regulators, Molecular Weights

Name and abbreviation	Molecular weight
Abscisic acid, ABA	264.32
Adenine (anhydrous), AD	135.13
Adenine (trihydrate), ADTH	189.13
Adenine sulfate (anhydrous), ADS	368.34
Adenine sulfate (dihydrate), ADSDH	404.37
Adenosine, ADE	267.25
AMO 1618	354.92
6-Benzylaminopurine, BA or BAP	225.26
Chlorocholine chloride, chlormequat, CCC	158.07
p-Chlorophenoxyacetic acid, CPA	186.16
2,4-Dichlorophenoxyacetic acid, 2,4-D	221.04
3,6-Dichloroanisic acid, Dicamba	221.04
Dimethylaminopurine, DMAP	162.17
Ethephon, ETH	144.50
Gibberellic acid, GA_3	346.38
Dihydrozeatin, 2HZ or DHZ	220.26
3-Indoleacetic acid, IAA	175.19
Indoleacetyl alanine, IAA-ala	246.27
Indoleacetyl glycine, IAA-gly	232.24
Indoleacetyl phenylalanin, IAPhA	322.37
3-Indolebutyric acid, IBA	203.24
Indole-3-ethanol, 3-(2-hydroxyethyl)-indole; tryptophol, IEt	160.19
Indole-3-propionic acid, IPA	189.21
Kinetin, 6-furfurylaminopurine, K or KIN	215.22
6-Methylaminopurine, MAP	148.15
α-Naphthaleneacetic acid, NAA	186.21
1-Naphthylacetic acid, NAA	186.21
2-Naphthyloxyacetic acid, NOA	202.21
Picloram, PIC	241.46
Putrescine, PUT	88.15
2,4,5-Trichlorophenoxyacetic acid, 2,4,5-T	255.49
Thiadiazuron, *N*-phenyl-*N′*-1,2,3-thiadiazol-5-ylurea, TDZ or THI	220.25
2,3,5-Triiodobenzoic acid, TIBA	499.81
Zeatin, 4-hydroxy-3-methyl-*trans*-2-butenylaminopurine, ZT	219.25
Zeatin riboside, ZTR	363.37

Prefixes

- atto = 10^{-18}
- femto = 10^{-15} (one quadrillionth), symbol is f (example: 1 femtomole is written as 1 fmol; 1 femtomolar is written f*M*)
- pico = 10^{-12} (one trillionth), symbol is p (example: 1 picomole is written as 1 pmol; 1 picomolar is written p*M*)
- nano = 10^{-9} (one billionth), symbol is n (example: 1 nanomole is written as 1 nmol; 1 nanomolar is written n*M*)
- micro = 10^{-6} (one millionth), symbol is μ (example: 1 micromole is written as 1 μmol; 1 micromolar is written μ*M*)
- milli = 10^{-3} (one thousandth), symbol is m (example: 1 millimole is written as 1 mmol; 1 millimolar is written m*M*)
- kilo = 10^{3} (one thousand), symbol is K (example: 1000 grams is 1 kilogram or 1 kg)

- mega = 10^6 (million), symbol is M
- giga = 10^9 (billion), symbol is G
- tera = 10^{12}

Pressure

- 1 atmosphere (atm) equals 1.01 bars and 1.03 kg cm^{-2}
- 1 pascal (Pa) is 1 kg s^{-2} m^{-1} and equivalent to 0.00001 atm

Roman numerals

I = 1	XX = 20	CC = 200
II = 2	XXX = 30	CCC = 300
III = 3	XL = 40	CD = 400
IV = 4	L = 50	D = 500
V = 5	LX = 60	DC = 600
VI = 6	LXX = 70	DCC = 700
VII = 7	LXXX = 80	DCCC = 800
VIII = 8	XC = 90	CM = 900
IX = 9	C = 100	M = 1000
X = 10		

Sphere

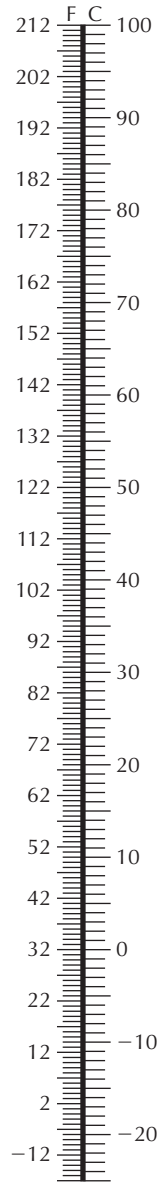
- $\text{Diameter}^2 \times 3.14 = \text{surface}$

Substances used in Media and their Molecular Weights

Name	Molecular weight	Name	Molecular weight
Adenine	135.13		
Adenine·$3H_2O$	189.13	Lysine	146.19
Adenine sulfate	368.34	Malic acid	134.09
Adenine sulfate·$2H_2O$	404.37	Maltose	360.31
Adenosine	267.25	D-Mannitol	182.17
L-Alanine	89.09	Mannose	180.16
p-Amino benzoic acid	137.13	MES	195.24
Arabinose	150.13	Methionine	149.21
L-Arginine	174.21	*myo*-Inositol	180.16
L-Ascorbic acid	176.12	Nicotinamide	122.12
L-Asparagine	132.12	Nicotinic acid	123.11
Aspartic acid	133.10	Ornithine	132.16
Biotin	244.32	Panthotenic acid	219.24
Calcium pantothenate	476.53	Phenylalanine	165.19
Cellobiose	342.30	Proline	115.13
Choline	121.18	Pyridoxine	169.18
Choline chloride	139.63	Pyridoxine·HCl	205.64
trans-Cinnamic acid	148.15	Raffinose	594.52
Citric acid	192.12	Rhamnose	182.17
Cyanocobalamine	1357.64	Riboflavin	376.36
Cysteine	121.15	Ribose	150.13
Cysteine HCl	157.61	Serine	105.09
Cystine	240.29	*mono*-Sodium glutamate·H_2O	187.13
Cystine HCl	275.75	Sodium pyruvate	110.04
Cytidilic acid	323.20	Sorbitol	191.18
Folic acid	441.41	Spermidine	145.25
Fructose	180.16	Spermine	202.35
Fumaric acid	116.07	Succinic acid	118.09
Galactosamine	179.17	Sucrose	342.30
Galactose	180.16	Thiamine·HCl	337.27
Glucosamine	179.17	Threonine	119.12
Glucose	180.16	*alpha*-Tocopherol	430.70
L-Glutamic acid	147.13	Trehalose	342.30
L-Glutamic acid·HCl	183.59	TRIS	121.14
Glutamic acid·H_2O	165.15	Tryptophane	204.22
L-Glutamine	146.15	Tyrosine	181.19
Glutathione	307.33	Urea	60.06
Glycine	75.07	Valine	117.15
Guanylic acid	367.21	Vitamin A	286.44
HEPES	283.31	Vitamin B_{12}	1357.64
Histidine	155.16	Vitamin E	430.70
Histidine·2HCl	228.09	Vitamin E acetate	472.74
Hydroxyproline	131.13	Xylitol	152.15
Isoleucine	131.17	Xylose	150.13
Lactose	360.31		
Leucine	131.17		

Temperature

- To convert °C to °F: $(°C \cdot {}^{9}/_{5}) + 32 = °F$
- Shortcut for °C to °F: $[(°C \cdot 2) - 10\%] + 32 = °F$
- To convert °F to °C: $(°F - 32) \cdot {}^{5}/_{9} = °C$



Volume

- 1 liter (l) is 1 cubic meter (1 m^3). It consists of 10^3 milliliters and is equivalent to 1.06 US quarts (qt)
- 1 microliter (µl) is 1^{-6} liters or 1^{-3} milliliters
- 1 milliliter (ml) is 1 cubic cm (cm^3) and consists of 1000 (10^3) microliters. It is 1^{-3} liters and equivalent to 0.03 fluid ounces (oz) or 0.06 cubic inches (in^3)

Weight

- kg, kilogram (1000 grams or 1000 g)
- g, gram (1000 miligrams or 1000 mg)
- mg, milligram (1000 micrograms or 1000 µg)
- µg, microgram (1000 nanograms or 1000 ng)

APPENDIX SEVEN

Additional Information

This appendix contains information gathered after the manuscript was copyedited. It is divided into categories for convenience.

Contamination

In some cases it may be possible to combat, inhibit or eliminate bacterial contamination through the use of plant preservative mixture, PPM (www.ppm4plant-tc.com) or Kathon (www.sigmaaldrich.com/Brands/Supelco_Home/Biocides/Kathon.html, www.rohmhaas.com/rhcis/markets_and_products/polymeremulKathon.html). A 1% solution of either PPM or Kathon in sterile distilled water can be used to decontaminate tissues. The tissue should be placed in the solution and agitated gently (or not at all if the tissue become bruised) for 24 h. After that the tissue should be cultured in a medium containing 0.2% PPM or Kathon (2 ml l^{-1} medium). The PPM or Kathon can be added before or after autoclaving. However caution is necessary because no information is available on the effects of autoclaved PPM or Kathon on humans. Timentin (www.rxlist.com/cgi/generic2/timentin.htm, www.gsk.com/products/prescription_medicines/us/timentin.htm) at 300 mg l^{-1} can also be used. It is very expensive (Eric Young, eyoung994@hotmail.com provided this information on May 5, 2003 to plant-tc@lists.umn.edu). Users of PPM and Kathon should refer to US Patent No. 5,750,402 and only follow procedures that respect it and do not violate it.

Contaminated explants and cultures can perhaps be saved through the use of a solution which consists of 5 ml PPM of Kathon in 1 l of autoclaved distilled water. The contaminated tissue should be cleaned manually as much as possible and washed with sterile distilled water. After the washing the distilled water should be discarded and replaced with the Kathon or PPM solution leaving very little (just a few milliliters) of air space at the top of the tube. The tissues should be kept in the solution for 24–36 h. Longer exposures of higher Kathon or PPM concentrations may be required in cases of severe contamination, but such treatments may damage the tissues of plants. Lower concentrations of Kathon or PPM should be used for sensitive plants or tissues. Plants or tissues so treated should be cultured a few at a time (Eric Young, eyoung994@hotmail.com provided this information on November 15, 2004 to plant-tc@lists.umn.edu). Users of PPM and Kathon should refer to US Patent No. 5,750,402 and only follow procedures that respect it and do not violate it.

For combating mites and molds see Van Epenhuijsen and Koolaard (2004*a*, 2004*b*) in the list of literature below.

Digital Cameras

Thorough reviews of digital cameras can be found at www.dpreview.com.

Freeware

Many freeware computer programs can be downloaded free of charge and completely legally from www.concisefreeware.com.

Kathon

See PPM.

Light Source

Wonderlite (www.orchidlight.com/wonderlight.html, www.wonderlite.co.za, www.thekrib.com/Lights/wonderlite-ad.html) is a wide spectrum light source which may prove suitable for orchid plants or cultures. Lamps are available in two wattages, 160 and 300 W.

Literature, Orchids

Numerous literature references about orchids can be found at http://web.onetel.net.uk/~johntrueman/Orchids.htm.

Literature, Tissue Culture

When available, contact information is provided for authors of the papers listed below. Those of you who may wish to obtain reprints or additional information should contact directly the authors of the relevant papers. Some of the listings below are annotated.

Alam, M. F., P. Sinham, and M. L. Hakim. 2006. Mass clonal propagation of *Vanda teres* (Roxb) Lindle through *in vitro* culture of nodal segments. *Propagation of Ornamental Plants* 6: 140–144. Fax: +880 721 750064; e-mail: falambiotech@lycos.com.

Basker, S., V. N. Bai, L. Jeyakodi, and M. A. Deepa. 2004. *In vitro* propagation of *Dendrobium aqueum* Lindl. An endemic orchid using synthetic seeds. *Seed Research* 32: 174–176. E-mail: narmathabai@yahoo.com.

Bhadra, S. K., M. M. Hossain, and P. Nandy. 2005. Establishment of *in vitro* mass propagation protocol in 'Rasna' *Vanda tessellata* (Roxb.) Hook. ex D. Don.

Journal of the Orchid Society of India 19: 35–40. This paper describes seed germination and the use of seedling-derived explants for micropropagation. Department of Botany, University of Chittagong, 4331 Bangladesh.

Chen, J. T., and W. C. Chang. 2006. Direct somatic regeneration from leaf explants of *Phalaenopsis amabilis*. *Physiologia Plantarum* 50: 169–173. Fax: +886 7 591 9404; e-mail: jentsung@nuk.edu.tw.

Debeljak, N., M. Regvar, K. W. Dixon, and K. Sivasithamparam. 2002. Induction of tuberisation *in vitro* with jasmonic acid in an Australian terrestrial orchid, *Pterostylis sanguinea*. *Plant Growth Regulation* 36: 253–260. Fax: +386 01 2573 390; telephone: 386 01 423 33 88; e-mail: nika.debeljak@k2.net.

Divakaran, M., K. N. Babu, and K. V. Peter. 2006. Conservation of *Vanilla* species *in vitro*. *Scientia Horticulturae* 110: 175–180. Fax: +91 495 2730294; telephone: +91 495 2355038; e-mail: nirmalbabu30@hotmail.com.

Haensch, K.-T. 1999. Somatic embryogenesis in vitro from adult plants of Pelargonium: influence of genotype and basal medium. *Gartenbauwissenschaft* 64: 193–200. Address: Institute for Vegetable and Ornamental Crops Grossbeeren/Erfurt e.V., Grossbeeren, Germany. This paper contains a recipe for a medium called universal medium (U-medium) which was created (according to the author) to be "universally applicable . . . [and] which would meet the average nutritional requirements of a broad spectrum of explants of quite different species . . . [it] was created to meet the average [needs] of a lot of species and explants and therefore it cannot be the best one for orchids and more work might be necessary to adapt . . . I would be very glad if you include [the information] in . . . *Micropropagation of orchids*."

Jelaska, S., N. Bauer, D. Leljak-Levanić, and S. Mihaljević. 2003. Many scopes of plant tissue culture research. *Periodicum Biologorum* 105: 215–220. Fax: +385 1 4826-260; telephone: +385 1 4826-262, 385 1 4877-700; e-mail: molbio@zg.biol.pmf.hr.

Jheng, F.-Y., Y.-Y. Do, Y.-W. Liauh, J.-P. Chung, and P.-L. Huang. 2006. Enhancement of growth and regeneration efficiency from embryogenic callus cultures of *Oncidium* 'Gower Ramsey' by adjusting carbohydrate sources. *Plant Science* 170: 1133–1140. E-mail: pungling@ntu.edu.tw.

Kuo, H.-L., J.-T. Chen, and W.-C. Chang. 2005. Efficient plant regeneration through direct somatic embryogenesis from leaf explants of *Phalaenopsis* 'Little Steve'. *In Vitro Cell and Developmental Biology – Plant* 41: 453–456. E-mail: wcchang2@gate.sinica.edu.tw.

Li, Y., K. Imai, S. Onwona-Agyeman, H. Ohno, and S. Matsui. 2006. Effects of light intensity during acclimatization on antioxidative enzyme activities and growth in mericlone plantlets of a *Cattleya* hybrid. *Journal of the Japanese Society of Horticultural Science* 75: 243–248. E-mail: liyouhong@hotmail.com.

Liu, T.-H. A., J.-J. Lin, and R.-Y. Wu. 2006. The effect of using trehalose as a carbon source on the proliferation of *Phalaenopsis* and *Doritaenopsis* protocorm-like bodies. *Plant Cell Tissue and Organ Culture* 86: 125–129. E-mail: annieliu@mail.dcb.org.tw.

Martin, K. P., and J. Madassery. 2006. Rapid in vitro propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants, and protocorm-like bodies. *Scientia Horticulturae* 108: 95–99. E-mail: marttin@sify.com (if

this address will not work martin@sify.com may be worth trying), hdbiotech@sify.com.
Ogura-Tsujita, Y., and H. Okubo. 2006. Promotion of in vitro shoot formation from protocorm-like bodies of a hybrid between tropical and temperate *Cymbidium* species. *Journal of the Japanese Society of Horticultural Science* 75: 334–336. Email: oguy@kahaku.go.jp.
Shiau, Y.-J., S. M. Nalawade, C.-K. Hsia, V. Mulabagal, and H.-S. Tsay. 2006. In vitro propagation of the Chinese medicinal plant, *Dendrobium candidum* Wall. Ex Lindl., from nodal segments. *In Vitro Cell and Developmental Biology – Plant* 41: 666–670. E-mail: hstsay@mail.cyut.edu.tw.
Shimura, H., and Y. Koda. 2004. Micropropagation of *Cypripedium macranthos* var. *rebunense* through protocorm-like bodies derived from mature seeds. *Plant Cell, Tissue and Organ Culture* 78: 273–276. Fax: +81 11 7062482; e-mail: yasunori@res.agr.hokudai.ac.jp. The terminology used in this paper is wrong and misleading. They state that "protocorm-like bodies (PLB) were formed from germinated seeds." By definition seeds produce protocorms. PLBs are produced by explants. Protocorms produced by proliferating protocorms are protocorms, not PLBs. By the same token PLBs produced from proliferating PLBs are PLBs, not protocorms.
Su, Y. J., J. T. Chen, and W. C. Chang. 2006. Efficient and repetitive production of leaf-derived somatic embryos. *Biologia Plantarum* 50: 107–110. Fax +886 7 591 9404; e-mail: jentsung@nuk.edu.tw.
Van Epenhuijsen, C. W., and J. P. Koolaard. 2004*a*. Monitoring and controlling mould mites in tissue culture facilities. *New Zealand Plant Protection* 57: 196–201. E-mail: vanepenhuijsenk@crop.cri.nz.
Van Epenhuijsen, C. W., and J. P. Koolaard. 2004b. Effective aerosol treatment of mould mites and onion trips in tissue culture. *New Zealand Plant Protection* 57: 202–208. E-mail: vanepenhuijsenk@crop.cri.nz.
Winkelmann, T., T. Geier, and W. Prell. 2006. Commercial in vitro plant production in Germany in 1985–2004. *Plant Cell, Tissue and Organ Culture* 86: 319–327.

Mathematical Equations

A large number of mathematical and algebraic equations and math forums can found at http://eqworld.ipmnet.ru/index.htm.

Photomacrography

Information, articles, and product reviews related to macrophotography can be found at www.photomacrography2.net/index.htm.

Plant Hormones

Information about plant hormones (structure, chemistry, history, functions, and physiology) is available at www.plant-hormones.info/index.htm.

Plant Pathology

Plant diseases are listed on www.ppath.cas.psu.edu/extention.plant_disease.

PPM

"PPM is a mixture of isothiazolone biocides (US Patent No. 5,750,402). Proclin (Supelco) is another mixture of isothiazolones, less expensive. Kathon (product number 500127, 500143, and 500135) from Supelco is a micture of the 5-chloro-2-methyl-4-isothiazolin-3-one with magnesium salts and a little copper nitrate added" (Eric Young, eyoung994@hotmail.com provided this information to plant-tc@lists.umn.edu). Supelco is part of Sigma-Aldrich, whose URL is www.sigmaaldrich.com/Brands/Supelco_Home.html.

When PPM is added to medium and autoclaved at 125°C for 15–20 min there is no substantial difference in its potency (even at concentrations of 0.05%) in comparison to being added after autoclaving if the medium does not contain any protein source. Protein seems to permanently bind the active ingredients during autoclaving. If a medium contains charcoal, the PPM concentration should be increased by 25% (Assaf Guri on the web site plant-tc@lists.umn.edu).

The following is an edited excerpt from a message by Martin Kalin (mkalin@ibek.com to plant-tc@lists.umn.edu):

> I would like you to know that we own US Patent No. 5,750,402 on the use of Kathon (and other isothiazolone equivalents) in plant tissue culture media at concentrations that reduce or prevent microbial contamination, but allow for substantially normal germination of seeds or growth or development of plants, plant organs, plant tissues or plant cells. The text of the patent is accessible on the web site of the U.S. Patent and Trademark Office, or from me upon request. We also have patents and patents pending in other countries of the world.
>
> Plant Preservative Mixture (PPM) is formulated by Plant Cell Technology specifically for plant tissue culture applications. In addition to our method claims, our patent also covers composition claims. The use of Kathon or other isothiazolone equivalents (other than PPM purchased through authorized channels) in countries where it has been patented for the prevention or reduction of microbial contamination of explants prior to placing them in media is covered under our patent. This patent also applies to instances in which remnants of the Kathon (even after washing) are still left on the explants and eventually diffuse into media and reduce or prevent microbial contamination, but allow for the substantially normal germination of seeds or growth or development of plants, plant organs, plant tissues or plant cells.
>
> To avoid patent infringement, I highly recommend you use PPM obtained from us or our distributors (PhytoTechnology Laboratories and others worldwide).
>
> PPM is also an EPA registered product for tissue culture applications and to our knowledge Plant Cell Technology, Inc. is the only EPA registered manufacturer of this chemical for this application and use (i.e., plant tissue culture media).
>
> If you or anyone else would like to discuss this issue, feel free to contact me at mkalin@ibek.com or by telephone at +1 202-778-8522, ext. 1.

Information about PPM, literature references, price lists, online store testimonials, instructions for use, contact information, a materials data safety sheet (MSDS), fax/mail

order form, testimonials, a request for input, and a list of distributors can be found at www.ppm4plant-tc.com.

Research Information

Sharing of information, know-how, and techniques among biology laboratories is available at http://openwetware.org/wiki/Main_Page.

Statistical Calculators

Many statistical calculators can be found at www.graphpad.com/quickcalcs.

Tissue Culture Site

Information about tissue culture, and several media and their components is available at http://aggie-horticulture.tamu.edu/tisscult/database/media/index.html.

Translations

Translation to and from many languages is available at http://babelfish.altavista.com.

APPENDIX EIGHT

Plant Preservative Mixture

Several discussions held while attending 9th Asia Pacific Orchid Conference (APOC 9) in Seoul, Korea convinced me to write this appendix on the use of plant preservative mixture (PPM) with orchids.

The following general instructions regarding the use of PPM (which have been edited only lightly) are presented here courtesy of Plant Cell Technology (PCT).* My additions and recommendations are given in square brackets.

General Instructions

Introduction

PPM™ is a heat-stable preservative/biocide which can be used to effectively prevent or reduce microbial contamination in plant tissue cultures. At optimum doses, PPM™, which stands for Plant Preservative Mixture™, is an extremely effective preservative/biocide which does not impair in vitro seed germination, callus proliferation, and regeneration.

Despite the most stringent use of sterile techniques, contamination of plant cell and plant tissue cultures remains a persistent problem which can result in losses ranging from a small number of cultures to whole batches.

PPM™ can be, and should be, used as a standard ingredient in plant tissue culture media. It is substantially less expensive and less potentially dangerous than commonly used antibiotics.

PPM™ was principally designed to inhibit contamination which airborne, waterborne and human contact induced. However, in many cases it can also be used to reduce endogenous contamination.

The principal PCT scientist involved in the development of the PPM™ application is Dr. Assaf Guri. He holds degrees in genetics, applied genetics, and plant breeding from the Hebrew University in Jerusalem and Michigan State University in the USA. Before joining PCT, he worked at the Volcani Agricultural Research Center in Israel, Michigan State University in East Lansing, Michigan and DNAP in New Jersey.

* I thank Dr. Assaf Guri for allowing me use material from the Plant Cell Technology web site.

Mechanism of Action

PPM™ is a broad-spectrum preservative and biocide, which kills bacterial and fungal cells, prevents germination of spores, and at higher concentrations, can eliminate endogenous contamination from explants. It is heat stable and therefore can be autoclaved.

Previous research has shown that the active ingredients of PPM™ penetrate the cells of the fungi or bacteria and inhibit key enzymes in central metabolic cycles such as the citric acid cycle and the electron transport chain. Our data indicate that PPM™ may also inhibit the transport of monosaccharides and amino acids from the medium into the fungal or bacterial cells.

As in any biocide, a critical ratio of PPM™ molecules per microbial cell is needed to eliminate bacteria and fungi. Keep in mind that a given volume of PPM™ dose has a constant number of PPM™ molecules while the number of spores introduced to tissue culture via endogenous contamination is highly varied. Therefore, explants should not be "squeezed" into a beaker. There should be enough volume for free movement of the solution around the explant material.

Advantages of PPM over Antibiotics

1 PPM™ is broad-based and effective against fungi.
2 PPM™ is less expensive than antibiotics, making it affordable for wide and routine use.
3 Since PPM™ targets and inhibits multiple enzymes, the formation of resistant mutants towards PPM™ is very unlikely.
4 PPM™ is heat stable and in general can be autoclaved with media.

Procedures

The procedures described below are generic. Slight modifications may be needed for your specific plant species. For assistance, contact Dr. Assaf Guri at guri1@erols.com or call +1 856 541 1141.

PPM™ significantly simplifies tissue culture working procedures for several reasons:

1 Media containing PPM™ may be dispensed outside laminar flow hoods (LFHs) under ambient conditions [however, the use of hoods is advisable for orchid laboratories]. Culture plates and vessels should be covered shortly after agar solidification. When media are dispensed with pumps, PCT recommends passing autoclaved hot water through the hoses prior to and after media dispensing.
2 Heat-sensitive or heat-stable liquid media containing PPM™ do not need to be sterilized by passing them through millipore filters or autoclaved provided they will be stored in sterile containers and that the stock solutions are not contaminated. In rich media containing 200 mg l^{-1} or more of amino acids or proteins, it is recommended to filter-sterilize the media with the PPM™ [since few orchid media are that rich this should not be necessary].

3 When working in LFHs, tools (forceps or scalpels) do not need to be flamed [for use with orchids, flaming is advisable]. They should be dipped periodically in 70% alcohol. The LFH does not need to be certified [LFHs used with orchids do not require certification anyway]. Work can be carried out outside the LFHs on a clean surface for a period not exceeding 1 h.

4 PPM™ is an acidic liquid solution (pH 3.8) which should be stored at 4°C (see safety information below). At the recommended dose of 0.05–0.2% (v/v), PPM™ is added to the medium before or after autoclavation to prevent airborne and endogenous contamination at low inoculum densities.

5 PPM™ is less effective when exposed to the high densities of bacterial or fungal spores that may be found on seed coats. For in vitro germination, seeds should be surface-sterilized conventionally with an EPA registered bleach [or other methods used for orchid seeds]. Therefore, in the presence of PPM™ (in the germination medium), the seeds can be rinsed under tap water in a non-sterile strainer and left to dry, preferably in the LFH [see below for methods to use with orchid seeds]. If the utensil ends have touched active bacteria or fungi culture or are otherwise suspected of being contaminated, they should be sterilized by autoclaving or through the use of an electric heating element.

6 General dosage levels: With the exception of endogenous contamination, the recommended dose range is 0.05–0.2%. For callus proliferation, organogenesis, and embryogenesis, the recommended range is 0.05–0.075% [different concentrations may have to be used for orchids; see below]. Add PPM to medium pre- or postautoclaving to prevent airborne and endogenous contamination at low inoculum densities or slow growing bacteria. To eliminate higher endogenous contamination densities, higher doses of PPM are needed (see point 7 below).

7 Endogenous contamination:

(a) Stir non-sterilized seeds for 8–12 h in 2–3% PPM™ solution (v/v) supplemented with full-strength basal salts of your routinely used medium. Do not add Tween-20 or pH to this solution. Subsequently, and without rinsing, transfer to germination medium supplemented with 0.05–0.1% PPM™ for herbaceous plants and with 0.2% PPM™ for woody plants. Hard-coated seeds (e.g., *Asparagus*, lupine, ornamental palm, rose, etc.) should be soaked in water for 2–4 h prior to sterilization with PPM™ [different procedures may have to be used for orchids; see below].

(b) Gently and routinely shake/stir 1-cm long explants (or shorter) for 4–12 h in 4–5% v/v PPM™ solution supplemented as above with full-strength MS basal salts [or orchid media salts] without adjusting pH and without Tween-20. Without rinsing, insert explants into a medium supplemented with 0.05–0.1% PPM™ for herbaceous plants and 0.2% PPM™ for woody plants [different procedures may have to be used with orchids; see below].

(c)* For tubers, bulbs, and scales: shake/stir the entire tuber/bulb/scale in bleach. Rinse with water (can be done under non-sterile conditions). Slice the tuber/bulb/scale into thin slices. Shake/stir for 12–24 h in 4–5% PPM™ solution supplemented with full-strength basal salts without

* Paragraphs 7(c) through 10 are intended for ornamental plants and non-North American users only.

adjusting and Tween-20. Without rinsing, insert into a medium supplemented with 0.1–0.2% PPM™ [different procedures may have to be used with orchids; see below].

8* In cases where the protocols above do not yield satisfying results (especially thick explants, highly infested explants, seeds), we recommend the following:
   (a) Shake/stir the explants in water (1 h for soft tissues and 2 h for hard tissues).
   (b) Shake/stir the explants in (50%) PPM™ supplemented with full-strength MS basal salts (without pH-ing and without Tween-20) for 5–10 min.
   (c) Without rinsing, insert the explants into the medium. In cases of fungal contamination, the addition of PPM™ to the medium is optional. However, with bacterial or mixed contamination, the addition of 0.05–0.2% PPM™ to the medium during the first month is essential. Do not discard highly oxidized explants as approximately 50% of the explants will recover within 4–6 weeks [different procedures may have to be used with orchids; see below].

9* To decontaminate contaminated cultures use the following rescue treatment (cultures should not be left visibly contaminated for longer than 1 week):
   (a) Clean the material mechanically using a soft toothbrush under a stream of tap water. Shake/stir in a 50% PPM™ solution (diluted with sterile water) for 5–15 min. For bacterial or mixed contamination we recommend lowering the solution pH to a range of 2.8–3.2 by mixing it 1 : 1 (v/v) full-strength PPM™ (100%) with 0.6 g l^{-1} citric acid solution in sterile water.
   (b) Without rinsing, insert tissues into a medium with 0.05–0.2% PPM™ for at least 1 month. Keep the culture away from high light intensities for the first 10 days. As mentioned above, do not discard oxidized explants. Wait 4–6 weeks as approximately 50% should recover.

10* In some cases the fungal or the bacterial spores are located deep within the explants beyond the reach of PPM. In such cases, and after the water-soaking period, slice the explants and then stir/shake in 50% PPM for 5–15 min [different procedures may have to be used for orchids]. For this decontamination procedure to be effective it is important to ensure that the tissues are completely and thoroughly bathed in the PPM solution.

Elimination of *Agrobacterium*

After co-cultivation, rinse the leaf discs with water. Completely dip the treated leaf discs in a 100% PPM™ solution (supplemented with full-strength basal salts) for approximately 2 min. Blot the discs between two sterile paper towels and place onto a medium supplemented with full-strength, commonly used antibiotics. After 3 weeks, transfer to a medium with PPM™ at 0.05–0.075% and no antibiotics. [This procedure may prove useful for the elimination of other bacteria from orchid cultures.]

General Notes

1 For the first transfer following sterilization with PPM™, we recommend complete insertion of the explants into a semisolid medium.

2 A 50% PPM™ solution can be reused approximately 10 times. The number of uses depends on the volume of explants treated and the inoculum density. Storing the 50% PPM™ solution at 4°C prolongs its activity. If necessary, prepare two PPM™ solutions: one to disinfect endogenous contamination and the second to disinfect in-culture contamination. The second solution should be filtered after each treatment, using 0.2-μm millipore filters. This filtration can be carried out under non-sterile conditions. A single filter can be used for the entire lifespan of the solution.

3 In cases where treatment with 50% PPM™ is still insufficient, full-strength PPM™ (100%) can be used. The treatment with 100% PPM™ is similar to the one described above for 50% PPM™, however, exposure time should not exceed 10 min.

Conclusions

PPM™ will most definitely facilitate work in any plant tissue laboratory and should significantly increase technician and laboratory productivity. However, conditions in each laboratory may vary and this could have a bearing on the effectiveness of PPM™. It is advisable for the staff to follow the guidelines above and thus maximize the effectiveness of PPM™ for a specific laboratory and particular applications. Test results show that:

- PPM™ is effective against airborne and waterborne contamination and contaminants introduced by human contact.
- If used correctly, PPM™ will free seeds or explants from endogenous contamination.
- At recommended dosages (0.5–2 ml l^{-1}), PPM™ does not impair in vitro seed germination, callus proliferation, callus regeneration, or the induction of axillary or adventitious buds.
- The formulation of certain concentrations of PPM and its use in tissue culture to prevent or eliminate microbial contamination is protected by US Patent No. 5,750,402. It is also patent pending in many countries of the world.

Safety Procedures

Safety Issues. PPM™ is non-toxic; however, inhalation and contact with skin and eyes should be avoided since it is an acid. It can be an irritant to skin, eyes, nose, and throat. As a precaution, we recommend that: (1) users wear gloves and splash goggles; (2) users avoid contact with skin and eyes; (3) users avoid inhalation; (4) proper and adequate ventilation is used; and (5) the material should not be applied by spraying it except with directed flow, positive-pressure ventilation, and protective equipment.

First Aid Measures. If swallowed, drink two glasses of water *immediately* and see a physician. Never administer anything by mouth to an unconscious person. In cases of eye and skin contact, *immediately* flush eyes with large amounts of water for at least

15 min. Wash affected skin areas thoroughly with soap and water. Remove and wash contaminated clothing thoroughly. Move subject to fresh air in cases of inhalation.

Important Information

The PPM solution as provided by PCT must/may not be concentrated for any reason.

Disposal Information

Dispose of media containing PPM™ in the same manner as that used to dispose of media without it. In an emergency, contact one of the following numbers: Dr. Assaf Guri, 1 (856) 541 1141 or Martin Kalin, 1 (202) 463-0904 ext. 134 or ext. 0 (for operator). A toxicological assessment has been performed by a qualified toxicologist. This assessment is available upon request. For more information on PPM™, or to request test results, contact: tel: 1 202 778 8522 ext. 1, fax: 1 202 429 9812.

Selected Answers from Frequently Asked Questions (FAQ) for a Previous Version of PPM™

1. PPM™ does not move as easily through plant cell walls as it does through those of fungi and bacteria. That is why explants are more tolerant to it. Plant protoplasts which are not protected by cell walls are very sensitive to PPM™.
2. It is not known if PPM™ affects anaerobic bacteria. However, since tissue cultures are usually well aerated such bacteria are not much of a problem in micropropagation.
3. Since PPM™ targets multiple enzymes it is unlikely that microorganisms can develop resistance to it.
4. In cases of high density contamination, fewer explants should be placed in a specific volume of PPM™.
5. It is possible to use PPM™ to free cultures of both contamination and endogenous contaminants.
6. PPM™ is active at a pH lower than 8.
7. At 0.5–2.0 ml l^{-1} PPM™ can be used in non-sterile media for short periods (1–7 days) without special precautions. For long-term experiments, the culture vessels must be sterile and the stock solution and water must be free of contaminants.
8. PPM™ is a liquid.
9. The effect of PPM™ may be reduced in media that contain proteins. In the presence of 1 g casein hydrolysate the concentration of PPM™ must be increased from 0.5 to 1 ml l^{-1}. Since many orchid micropropagation media contain casein hydrolysate, peptone, and/or tryptone this is an important fact to consider. Should 1 ml PPM™ l^{-1} prove to be insufficient it may be necessary to use a higher concentration, but this must be done carefully. Valuable cultures should not be subjected to high levels of PPM™ without prior testing.

10 PPM™ can be combined with antibiotics and fungicides.
11 Despite the fact that PPM™ is heat stable, autoclaving reduces its effectivity by about 10%. In protein-rich media the reduction may be as high as 90%. Therefore PPM™ must be added after autoclaving in some cases.
12 Once PPM™ has been added to an initial medium it is advisable to incorporate 0.5 ml l^{-1} in subsequent media.
13 PPM™ is a broad-spectrum biocide.

Use of PPM™ with Orchid Seeds

Report 1. Plant Cell Technology provided the following information by Roger Nick of Spring Orchid Laboratory (rn0089@msn.com) in January 2003. The report was edited to conform with style and format of this book.

- Unsterilized orchid seeds harvested at the green capsule stage were sown on half-strength Murashige and Skoog (MS) medium supplemented with 20 g sucrose, 10 mg thiamine, 1 mg pyridoxine, 1 mg niacin, and 100 mg *myo*-inositol l^{-1} and solidified with 8 g agar l^{-1}.
- Seeds from the same capsules were divided and placed on this medium without PPM™ and with 2 ml PPM™ l^{-1}. The medium was dispensed into flasks and autoclaved at 15 psi for 15 min.
- Germination was approximately 90% on both media. After 2 months of growth, no difference could be detected between plants grown on media with or without PPM™.
- The same tests were carried out with hybrid seeds *Cattleya*, *Phalaenopsis*, and *Dendrobium*. After 2 months no contamination was present in flasks containing PPM™, whereas two out of six flasks without PPM™ were contaminated.
- In the Gulf Coast region of the USA, extreme summer temperatures sometimes cause splitting of unripe capsules prior to the anticipated harvest date. Once the capsules have split, the surface can no longer be sterilized with bleach, and most of the embryos are too immature to withstand standard dry seed type sterilization with calcium hypochlorite. I have had great success with obtaining clean seedling flasks from most of the split capsules by utilizing the medium described above with the addition of 2 ml PPM™ l^{-1}, after lightly flaming the outside of the split pod over an alcohol burner. In the past, most capsules which split prematurely were lost completely.
- Occasionally I replace from "mother" flasks and get immediate contamination from what seems to be a "dormant"-type contaminant. I have been able to clean almost all of these contaminated seedlings by placing them in a solution of 20 ml PPM™ l^{-1} for a 24–36-h period on a rotary shaker. The plants are then removed and placed without rinsing on half-strength MS medium as described above with the addition of 2 ml PPM™ l^{-1}. All seedlings remained uncontaminated and grew normally for the remainder of the in-flask period.

Report 2. Marty Kalin of Plant Cell Technology (marty@mk.ibek.com) provided the following information in May 2003.

- PPM™ was incorporated into the final rinse solution used to disperse orchid seed over the surface of nutrient medium at a rate of 4 ml l^{-1}. This solution was used in 255 germination flasks containing 45 varieties of orchid seed. Flasks were sown without the benefit of a laminar flow hood in a non-sterile environment (an open room). Orchid seed was sterilized by normal procedure (10% Clorox for 10 min) then power-filtered and resuspended in PPM™ solution. Flasks were autoclaved in the usual manner and incubated at 23°C after sowing.
- Contamination rates of approximately 10% were observed after a period of 1 month (16 fungal and 5 bacterial). Since these flasks were sown quite heavily with seed and under non-sterile conditions, we consider this to be a significant reduction in the contamination rate that might normally be expected to range at around 45–60% under these conditions.
- Germination of the seed does not appear to have been affected by the presence of PPM™. Seeds of several genera were used including many temperate terrestrial and tropical species.

Report 3. Simon M. Wellinga of SymPhyto in the Netherlands (s.m.wellinga@symphyto.nl) described his findings in May 2003.

- Back in 1998 we ordered a couple of 100-ml bottles and tried PPM™ with our cultures, which at that time consisted mostly of botanical *Cattleya* and *Laelia* species. We did so out of curiosity and to find out whether this mixture would meet its expectations. In an initial trial PPM™ was both used as an ingredient of our standard germination media and as a cure of contaminated seedlings, which after treatment were replated onto media either containing PPM™ or without it. Although PPM™ was used strictly according to the recommendations that came with the product and while following our standard laboratory practices (work done in a laminar flow hood, autoclave cycles as usual, etc.), all our experiments were not planned ahead in what one would call a sound and statistically justifiable setup. Therefore our results cannot be claimed to be scientific evidence, and neither have they been published. The reason that we only tested PPM™ qualitatively, and not quantitatively, was that we would never have been able to utilize this ingredient on a larger scale, both for economical reasons and because of international phytosanitary regulations. Since a considerable proportion of our seedlings are sent in flasks on sterile medium to non-EU destinations (the only way one can get *Paphiopedilum* species and other CITES Appendix I species across international borders), we are not allowed to incorporate any antibiotics in our export flasks or any other compounds that temporarily suppress or mask infections.
- For what it is worth – although we made sure to follow the instructions that came with the product, we never bothered to share our experiences with others – we found that in seed cultures on medium containing PPM™, infections would still occasionally show up. Also, we have never been able to clean contaminated cultures with the help of PPM™, something which with some luck can be achieved in case of hard-leafed material as cattleyas and paphiopedilums with the help of a treatment with sodium hypochlorite. In those instances where treated cultures of infested seedlings seemed to be clean, infection would immediately show up again after replating to PPM™-free medium, and this is why we were led to believe that PPM™ merely suppresses infections but does not eradicate them altogether.

- I understand you are looking for information and references for the forthcoming new edition of *Micropropagation of orchids*. I am sorry that the information above is only anecdotal and that I cannot help you with more valid and statistically relevant data.

Report 4. Esteban McGrath of Loresco Tropical Plants in San Juan, Puerto Rico (lsrjm@prtc.net) wrote of his experiences in May 2003.

- I am a grower and *Hibiscus* breeder and at present no longer deal with orchid seeds. However, in my experience this product (PPM™) should be used for sterilization of not only seed, but utensils as well. I found the losses with PPM™ not to exceed 2%, whereas losses with sodium hypochlorite can be as high as 96%. The "cloro" kill rate effect was worse than its sterilization effectivity, whereas the PPM™ just simply was effective when used as proposed.
- I have used it on in vitro seeds and explants, as well as on extremely difficult to root multiplications of *Hibiscus* with good results. In-flask work was with coconut water.
- No data have been published, but as a field producer, I realize its potential for anything needing extreme pathogenic control.

Report 5. There seems to be only one published report on the use of PPM™ in orchid seed germination: Bautista, N. R., A. B. Quilang, G. B. Taylan, R. F. Madera, and C. C. Pulma. 2001. Effect of Plant Preservative Mixture™ (PPM) on contamination rate and growth of *Vanda sanderiana* Reichb. F. Seedlings (Orchidaceae) *in vitro*. Pages 121–126 *in Proceedings of the World Conference on Science and Technology: harnessing science and technology to meet global challenge* (September 13–15, 2001). Philippine Association for the Advancement of Science, Manila.

- These authors reported using a modified Knudson C medium (without describing the modification) plus 0.125, 0.25, 0.5, 1, 2, and 4 ml of PPM™ for the germination of unsterilized *Vanda sanderiana* seeds in 10 replicas. Culture conditions were 8-h photoperiods provided by fluorescent lights and 21°C.
- Contamination usually appeared 3 days after inoculation. The control and 0.125 and 0.25 ml PPM™ treatments became contaminated after a week. The contaminants were fungi. Approximate contamination percentages were:

Controls:	100%
0.125 ml PPM™:	80%
0.25 ml PPM™:	60%
0.5 ml PPM™:	50%
1 ml PPM™:	30%
2 ml PPM™:	60%
4 ml PPM™:	0%

Use of PPM in Orchid Tissue Culture

Report 1. Roger Nick of Spring Orchid Laboratory (rn0089@msn.com) also reported on the use of PPM™ in orchid micropropagation.

- I also do a considerable number of *Phalaenops*is stem propagations from our most desirable plants. In the past the stems were harvested, washed with mild detergent and water, and placed in 25% Clorox solution with Tween-20 added for a period of 25–30 min. The stems were then rinsed in sterile distilled water three times and placed in quarter-strength MS medium for a period of 24 h. They were then removed and placed in half-strength MS medium as described above with the addition of 0.5 mg naphthaleneacetic acid, 5 mg benzyladenine, and 2 ml PPM l^{-1}.
- When bleach alone was used, I averaged approximately 25–30% contamination. With bleach and PPM™ treatment (20 ml l^{-1}) the contamination rate dropped to approximately 5%. Note has been made that some *Phalaenopsis* hybrids are more sensitive to PPM™ than others. In those cases shorter exposures to 20 ml PPM™ l^{-1} solution were successful.
- To prepare replating medium for the seedlings, I supplemented the medium above with 50 g mashed white potatoes l^{-1}, 100 g mashed green bananas l^{-1}, and 0.2% activated charcoal. The addition of 2 ml PPM l^{-1} to this replating medium was done only after autoclaving.

Report 2. Dr. Victor M. Jiménez of the University of Costa Rica (vjimenez@cariari.ucr.ac.cr) reported that with 2 ml PPM™ l^{-1} contamination sets in within 3 months. If the level of this contamination is low, transfer to fresh medium. However, heavy contamination cannot be cleaned up.

Report 3. Dr. Michael E. Compton, School of Agriculture, University of Wisconsin, Plateville (compton@uwplatt.edu) used 2 ml PPM™ l^{-1} for shoot tip "cultures of *Oncidium* without ill effects. However, PPM™ at this . . . [concentration does not protect shoot tips] from heavy microbial infections."

Report 4. Dr. Asaf Guri of Plant Cell Technology and the inventor of PPM™ (guri1@erols.com) wrote the following when asked how one could use PPM™ with *Paphiopedilum* shoot tips:

> Let me suggest to you to skip sterilization with sodium or calcium hypochlorite and soak the shoot tips in non pHed 4% PPM solution in which full strength MS basal salts are added [if another basal solution is used for culture these salts should be used instead of MS]. I can't tell you the exact exposure time but I'll suggest from 1 to 4 hours exposure in which the tips are agitated very gently. Do not add Tween-20 to the solution. Without rinsing place the tips into the proper medium with 0.1% (v/v) PPM. In case the medium contains any source of peptides (as usually occurs in orchid media) the 0.1% PPM 1 minute before dispensing [some orchid media contain casein hydrolysate, peptone, and/or tryptone which could be sources of peptides]. It [the PPM] will not change the overall pH and the added PPM solution does not need to be sterilized [however the culture medium should be autoclaved regardless of when the PPM is added].

Material Safety Data Sheet

Since this is a relatively new mixture, the material safety data sheet (MSDS) should be consulted by those who will want to use PPM™. It is reproduced here courtesy of Plant Cell Technology (the company which produces and sells PPM™).

PPM™ Identification

- *Chemical name*: PPM™ (Plant Preservative Mixture).
- *Chemical family*: Proprietary.
- *CAS number*: Proprietary (several numbers).
- *Synonyms*: Not applicable.
- *DOT information*: Not corrosive (pH = 3.7).

Hazard	Rating	Scale
Oral toxicity	4	4 = insignificant/none
Eye/skin toxicity/irritation	3	3 = moderate (caution)
Fire	4	2 = high
Reactivity	4	1 = extreme/corrosive

The above rating is Plant Cell Technology's toxicity rating. It incorporates some EPA criteria.

Company identification

Plant Cell Technology, Inc.
1823 Jefferson Place NW
Washington, DC 20036, USA
+1 202 463 0904 ext. 134 (Martin Kalin)
+1 856 541 1141 (Assaf Guri)
PPM™ is a trademark of Plant Cell Technology, Inc.

Data Sheet

1. Physical Data

- *Appearance*: Clear.
- *Color*: Amber to clear.
- *State*: Liquid.
- *Odor characteristic*: Mild, inoffensive odor.
- *pH*: 3.0–4.0.
- *Solubility in water*: Completely soluble.

2. Ingredients

PPM™ is a proprietary chemical product.

3. Fire and Explosion Hazard Data

- *Flash point*:
  Auto-ignition temperature not applicable.
  Lower explosive limit not applicable.
  Upper explosive limit not applicable.
- *Unusual hazards*. Combination generates toxic fumes of the following: hydrogen chloride, nitrogen oxides, sulfur oxides.
- *Extinguishing agents*. Use extinguishing media appropriate for surrounding fire.
- *Personal protective equipment*. Wear self-contained breathing apparatus (pressure-demand MSHA/NIOSH approved or equivalent) and full protective gear.
- *Special procedures*:
  Use water spray to cool containers exposed to fire.
  Minimize exposure.
  Do not breathe fumes.
  Contain run-off.

4. Health Hazard Data

- *Inhalation*. Inhalation of vapor or mist can cause irritation to the nose and throat.
- *Eye contact*. Material can cause corrosion to eyes – permanent, irreversible eye injury.
- *Skin contact*. Skin irritation effects can be delayed for hours. Material can cause irritation to the skin and allergic contact dermatitis.
- *Ingestion*. Material is harmful if swallowed.

5. First Aid Measures

- *Inhalation*. Move subject to fresh air. Give artificial respiration if breathing has stopped.
- *Ingestion*. If swallowed, *immediately* call and see a physician. Never give anything by mouth to an unconscious person.
- *Eye and skin contact*. Flush eyes *immediately* with a large amount of water for at least 15 min. Wash affected skin areas thoroughly with soap and water. Remove and wash contaminated clothing thoroughly. Do not take clothing home to be laundered. Discard contaminated shoes, belts, and other articles made of leather. Get prompt medical attention.
- *Note to physician*. Material has a pH of 3.0–4.0. It may be advised to induce vomiting. Possible mucosa damage may contraindicate the use of gastric lavage. Measures against circulatory shock and convulsions may be necessary.

6. Reactivity Data

- This material is considered stable under specified conditions of storage, shipment, and/or use.
- Thermal decomposition may yield the following: hydrogen chloride, sulfur dioxide, or oxides of nitrogen.
- Hazardous polymerization: production will not undergo polymerization.

- Incompatibility: avoid contact with oxidizing agents, reducing agents, amines, and mercaptans.

7. Spill or Leak Procedures

Dilute with 100× water. Rinse the decontaminated solution to a chemical sewer.

8. Special Protection Information

- *Personal protection*:
  Wear compatible, chemically resistant gloves.
  Material has a pH of 3.0–4.0. If exposed to material during clean-up operations *immediately* remove all contaminated clothing and wash exposed skin areas with soap and water. See section 5 above on first aid for further information.
  Protective clothing made of the following material should be worn to avoid skin contact: butyl, rubber, or nitrite.
- *Engineering controls (ventilation)*. Use local exhaust ventilation with a minimum capture velocity of 0.75 m s^{-1} (150 ft min^{-1}) at the point of dust or mist evaluation. Refer to the current edition of *Industrial Ventilation: A Manual or Recommended Practice* published by the American Conference of Governmental Industrial Hygienists for information on the design, installation, use, and maintenance of exhaust systems.
- *Other protective equipment*. Facilities storing or utilizing this material should be equipped with an eyewash facility and a safety shower.

9. Handling and Storage

- *Storage conditions*. The maximum recommended storage temperature for this material is 55°C (131°F). The minimum recommended storage temperature for this material is 0°C (32°F). Store in a well ventilated area. Do not store this material in containers made of steel.
- *Handling procedures*. See section 8 above prior to handling. As empty containers retain product residues (vapor and/or liquid), follow all MSDS and label warnings even after containers are emptied.

10. Regulation Information

Workplace classification: this product is considered non-toxic.

11. Toxicological Information

- Oral LD_{50} (rat): 30 g kg^{-1}.
- Eye/skin irritation (rabbit): moderate irritation/insult, reversible damage.
- Sensitization data (human): allergic contact dermatitis possible.

12. Ecological Information

Not available.

13. Disposal Conditions

Dispose of PPM™-containing media as you would non-PPM™ media. Items that are contaminated with full-strength PPM™ should be rinsed liberally (100×) in water. See section 7 above.

14. Transportation Information

The US DOT hazard class is 'non-toxic'; it is classified as a skin irritant.

Source of PPM™ and Technical Support

Address: Plant Cell Technology, 1823 Jefferson Place NW, Washington, DC 20036, USA.
Telephone: 202 778 8522 ext. 1, 800 746 8535.
Fax: 202 822 6410, 202 429 9812.
Web site: www.ppmstore.com, www.ppm4plant-tc.com.
E-mail: information@ppm4plant-tc.com, mkalin@ppm4plant-tc.com.
Information: http://ppm4plant-tissueculture.stores.yahoo.net/info.html.
Distributors: http://www.ppm4plant-tc.com/distributors.htm.
Price list: http://www.ppm4plant-tc.com/pricelist.htm.
On-line store: http://ppm4plant-tissueculture.stores.yahoo.net/.
Order form and information: http://www.ppm4plant-tc.com/ppmorderinginformation.pdf
Technical support: http://ppm4plant-tissueculture.stores.yahoo.net/tecsup1.html, guri1@erols.com or call Dr. Asaf Guri on +1 856 541 1141.

Disclaimer

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Users of PPM™ with orchid ovules, immature seeds, unripe fruits (capsules, "pods"), seeds, protocorms, seedlings, explants, callus, protocorm-like bodies, plantlets, mature plants, leaves, roots, tubers, pseudobulbs, canes, soots, young or old growths, stems, inflorescences, flower stalks, flowers, or any other part of the plant or life cycle of orchids do so at their own risk. The author, publisher, and PCT are not responsible for any damage to humans, animals, plants, property, and equipment or any losses which may result from the use of PPM™ even if such use is in line with instructions in this appendix, this book, or the PCT web sites listed above.

Glossary

ABA, *see* Abscisic acid.

Abaxial, facing away from the axis. In a leaf the abaxial surface is the underside because it faces away from the axis of the trunk or branch.

Abscisic acid (ABA), a plant hormone which is associated with seed maturation, desiccation tolerance, suppression of vivipary, some types of seed dormancy, inhibition of seed germination, senescence, and somatic cell embryogenesis in certain plants, but despite its name does not induce abscission (even if it was originally assumed to do so). This hormone was discovered and named by F. T. Addicott and his associates at the University of California, Davis. Dormin, isolated and named by P. F. Wareing and his co-workers at the University College of Wales, Aberystwyth proved to be the same compound.

Absorption, uptake or the movement of water and/or substances across cell membranes as well as the retention of light by a pigment.

Absorption spectrum, a graph depicting the ability of a substance to absorb light of different wavelengths.

Accessory bud, a bud that occurs near an apical or axillary bud and is usually smaller than either.

Acid, a substance: (1) usually of sour taste, (2) which contains hydrogen that can be replaced by a metal or a positively charged radical (and thereby form a salt), (3) that is capable of being a proton donor, and (4) of generally low pH.

Acropetal, sequence of development or blooming, or movement of a substance or water, toward the apex (the opposite of basipetal).

Activated charcoal, charcoal treated in a manner that increases its adsorptive properties.

Active uptake, intake of substances against a concentration gradient. Such uptake requires expenditure of energy.

Adaxial, facing toward the axis. In a leaf the adaxial surface is the upper side because it faces toward the axis of the trunk or branch.

Adenine, a purine base which is a component of DNA and RNA. When added to culture media (usually as the sulfate), adenine acts as a weak cytokinin. *See also* Cytokinins.

Adhesion, attachment of different molecules to each other.

Adsorbent, a substance that adsorbs other compounds or to which other substances adhere.

Adsorption, attachment or concentration of molecules on the surface of another substance at the boundary or interface between two substances or the formation of a solid, liquid, or gas layer on a solid surface.

Adventitious, an organ or a structure arising at an unusual location or a place where it is not normally found, as for example: buds not at a leaf axil or on roots, shoots and roots on a callus, and embryos from a source other than a zygote.

Aerate, provide, supply, or introduce air.

Aerobic conditions, presence of air (opposite of anaerobic).

Agar, a substance prepared from a specific kind of red algae and used to solidify culture media.

Alba, lacking pigmentation and usually white.

Alcohol lamp, a lamp consisting of a wick dipped in alcohol. It produces a clean, smokeless, hot flame which is used to sterilize tools.

Alkali, base.

Amino acid, an acid with the structure, $NH_2–\underset{\displaystyle R}{\underset{|}{C}}–COOH$

Anaerobic, lacking air (opposite of aerobic).

Aneuploid, situation where an exact multiple of the haploid number of chromosomes is not present.

Angiosperms, plants that produce seeds that are borne within a mature ovary (*compare* Gymnosperms).

Anion, negatively charged ion often designated as A^- (*compare* Cation).

Annual, a plant that completes its life cycle (i.e., goes from seed to seed) in a year or less. There are no annual orchids.

Anther, pollen-bearing part of the stamen.

Anthesis, opening of a flower and full bloom.

Anthocyanin, water-soluble flavonoids which can be blue, mauve, pink, purple, or red and are found in vacuoles.

Antiauxin, an auxin antagonist or a substance which reverses, prevents, or inhibits the effects and/or transport of auxins. Examples are *trans*-cinnamic acid (*t*CA), 2,3,5-tri-iodobenzoic acid (TIBA), and 2,4,5-trichlorophenoxyacetic acid (TCPAA).

Antibiotic, a compound which inhibits or kills microorganisms.

Apex, tip, in roots and stems this is the part which contains the apical meristem. This part is usually excised and cultured in orchid "meristem" culture.

Apical dominance, inhibition of lateral bud growth and branching by the apical meristem.

Apical meristem, group of meristematic cells located at the apex.

Ascorbic acid, or vitamin C, one of the water-soluble vitamins, and an antioxidant.

Aseptic techniques, procedures designed and intended to prevent the introduction of unwanted agents (generally bacteria, fungi, mycoplasma, and viruses) into cultures of desirable organisms and explants (cells, tissues, and organs). These techniques may or may not eliminate internal contaminants (i.e., microorganisms which exist within explants).

Asexual embryogenesis, development of embryos from somatic (body) cells without the involvement of gametes. Asexual embryos or embryo-like structures can also develop from callus masses, cells, or explants in vitro.

Asexual propagation, reproduction which does not involve the sexual process (i.e., there is no meiosis and joining of gametes).

Autocatalytic reaction, reaction which is catalyzed by its own product; the effect of ethylene on its own production is an example.

Autoclave, a sealed or closed chamber in which apparatus, liquids, equipment, glassware, media, and other objects are heated (usually by steam) under pressure (103.4×10^3 Pa at 121°C; see Appendix 6 for definitions of units) for the purpose of sterilization. The usual period of sterilization for most media is 15 min.

Autotrophy, ability of an organism (in actuality a green plant) to manufacture its own food.

Auxin, a plant hormone, naturally occurring or synthetic, that regulates cell division, elongation, and enlargement as well as apical dominance, root initiation and growth, flowering in some instances, ethylene production, and other physiological processes in plants. Indoleacetic acid (IAA), which is used widely in orchid tissue culture and micropropagation, is a naturally occurring auxin. Other auxins used in orchid micropropagation are the synthetics naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Auxin was the first plant hormone to be discovered. The discovery was made by F. W. Went (the co-formulator of the widely used Vacin and Went culture medium) while he was a graduate student at Utrecht University in the Netherlands.

Available substance or nutrient, a compound or substance which can be taken up and utilized by a whole plant in soil or an explant in culture.

Axenic culture, a term used to designate a culture of a single organism or one free of both external and internal contaminants.

Axillary bud, a bud located in a leaf axil. Non-adventitious lateral buds are axillary.

BA, *see* Benzyladenine.

Bacteria, plural of bacterium that is unfortunately and erroneously usually used as the singular. Bacteria are prokaryotic (they do not have a well-defined nucleus), single-celled (unicellular) microorganisms which belong to the class Schizomycetes. They can be aerobic or anaerobic, comma shaped (vibrio), corkscrew shaped (spirochaete), filamentous, rod-like (bacillus), spherical (coccus), or spiral (spirillum). Bacteria are common internal or external contaminants.

Bactericide (also bacteriocide), a substance that kills bacteria.

Bacteriostatic substance, a compound that inhibits or slows down bacterial growth and/or reproduction.

Bacterium, *see* Bacteria.

BAP, *see* Benzyladenine.

Bar, a unit used to measure pressure; 1 bar = 1 atmosphere or 10^5 pascals (see Appendix 6 for definitions of units).

Basal, a term with two meanings: (1) Located at the base. (2) Simplest formulation of a culture medium (as in basal versus enriched or supplemented medium).

Base, a term with several meanings: (1) Basal part of a structure. (2) A synonym for basal medium ("base medium"). (3) A nitrogen-containing compound that is a component of RNA and DNA (as in "base pair"). There are five such bases: adenine (A), a purine; cytosine (C), a pyrimidine; guanine (G), a purine; thymine (T), a pyrimidine which should not be confused with thiamin (vitamin B_1); and uracil (U), a pyrimidine. (4) A substance which can accept a proton (H^+), can react with an acid to form a salt, or the hydroxide of a metal.

Basipetal, sequence of development or blooming, or movement of a substance or water, toward the base (the opposite of acropetal).

Benzyladenine (6-benzylaminopurine, N^6-benzyladenine, BA, BAP), a synthetic cytokinin.

Benzylaminopurine, *see* Benzyladenine.

Binomial, the two names (genus and species) of organisms. Example: *Cattleya* (the genus) *intermedia* (the species).

Bioassay, a test (quantitative and/or qualitative) for a substance that utilizes the response(s) of an organism.

Bioreactor, a container, usually a tank, in which organisms, explants, cells, cell extracts, or enzymes carry our life processes or biological/biochemical reactions.

Biotechnology, a term with two meanings: (1) Use of organisms or biological processes in the manufacture of substances. (2) Molecular and/or genetic manipulation of organisms for the purpose of changing them and/or endowing them with new and/or different capabilities.

Biotin, one of the B vitamins. This vitamin is seldom if ever used in orchid tissue culture.

Blackening, darkening of a culture medium due to the release of phenolics by explants. Other terms are bleeding and browning.

Blade, the thin or thick, broad or narrow, flat or terete, extended part of a leaf above the petiole. Lamina is another term.

Bleach, an agent (liquid or powder) which contains free chlorine ions capable of whitening. The most common bleaches used in laboratories are calcium hypochlorite solutions (which must be prepared fresh since they lose their activity within 12 h) and commercial sodium hypochlorite products that are commonly known as domestic bleaches. Clorox, Domestos, Milton's, Snow White, and Purex are some brand names of such solutions, but there are many more. Domestic bleaches usually contain 4.75–6.0% sodium hypochlorite. They are more stable than calcium hypochlorite solutions, especially in unopened bottles, but if a bottle has been opened for a prolonged period it may be wise not to use the bleach for critical work.

Bleeding, *see* Blackening.

Bract, a modified leaf that may occur below the flowers or inflorescences.

Breeding, using sexual reproduction to produce new forms or offspring.

Browning, *see* Blackening.

Buchner flask, thick-walled, flat-bottomed, conical flask with a tube extending from its neck. The tube can be connected to a vacuum source to create suction and to accelerate filtering.

Bud, an undeveloped vegetative shoot and/or flower consisting of undifferentiated meristematic tissue as well as organs in various stages of differentiation. Buds are usually covered and protected by scales (which are modified leaves).

Buffer, a solution which can stabilize pH and resist acidity or alkalinity changes. Hans Burgeff, the German orchid expert, proposed the use of a K_2HPO_4/KH_2PO_4 buffer for the Knudson C medium to reduce acidification caused by seedlings (see Appendix 5 for the recipe). More recent work with orchid seedlings has shown that this acidification may not cause any harm.

Bulb, a modified bud consisting of a flattened, disk-shaped stem and fleshy scale leaves.

Bundle sheath, a sheath of parenchyma cells that surrounds vascular bundles.

Bunsen burner, a commonly used burner in laboratories that mixes air and gas to produce a hot, clean, smokeless flame. This burner was invented by the chemist Robert Bunsen ca. 1855 when he moved to the University of Heidelberg. As a condition for the move, Bunsen wanted a new laboratory building with gas piping. His innovation was to mix air and gas, not at the point of combustion, but before that. He (with co-author Henry Roscoe) wrote: "The principle of this burner is simply that city gas is allowed to issue under such conditions that by its own movement it carries along and mixes with itself precisely enough air so that the resulting air-bearing gas mixture is just at the limit where it has not yet acquired the ability to propagate the flame through itself." *See also* Erlenmeyer flask (which was invented at the same university).

C_3 or C3 cycle, the Calvin–Benson–Bassham pathway of carbon fixation in photosynthesis in which the three-carbon molecule, 3-phosphoglyceric acid, is the first stable product.

C_4 or C4 cycle, the Hatch and Slack pathway of carbon fixation in photosynthesis in which four-carbon molecules are the first stable products.

Callus, a term with three meanings: (1) A mass of (usually) large, thin-walled, and undifferentiated cells, which develops on a plant following wounding or infection by certain bacteria. (2) A group of cells as in meaning 1 that develops on a plant following hormone applications. (3) An undifferentiated or partially differentiated, unorganized, actively dividing mass of cells developing from an explant or individual cells in vitro.

Callus culture, the culture of a callus mass on solid medium or in liquid medium for the purpose of increasing its size or inducing organogenesis.

Calory, heat required to raise the temperature of 1 g of water by 1° from 14.5 to 15.5°C. This is also known as a gram-calory; 1 calory = 4.19 joules, 1000 calories = 1 kilocalory.

CAM, *see* Crassulacean acid metabolism.

Cambium, a one- or two-cell-thick layer of meristematic cells which gives rise to secondary tissues. Most monocotyledonous plants, orchids included, do not have cambium.

Capsule, a simple, dry, dehiscent fruit consisting of two or more carpels. Orchid fruits are capsules, not pods.

Carbohydrate, a substance composed of carbon, hydrogen, and oxygen in the ratio of 1C : 2H : 1O (CH_2O). Sugars are carbohydrates.

Carbon fixation, a reaction in which carbon from carbon dioxide is attached to a receptor and made part of an organism (essentially what occurs in photosynthesis).

Carcinogen, a substance that can cause cancer.

Carotene, a lipid-soluble, orange or orange-reddish, plastid pigment.

Carotenoid, lipid-soluble, orange, red, or orange-red pigments located in chromoplasts in fruits and flowers.

Carpel, a modified leaf (floral leaf) that bears and can enclose ovules.

Casein hydrolysate, a complex organic mixture, sometimes added to culture media, consisting of peptides and amino acids. It is produced through hydrolysis (acid or enzymatic) of casein (the proteins found in milk).

Cation, positively charged ion often designated as M^+ (*compare* Anion).

Cation exchange, replacement of one cation by another on negatively charged particles.

Cell, structural and physiological component of organisms, the smallest unit capable of carrying out life processes.

Cell culture, in vitro culture of cells isolated from multicellular organisms.

Cell cycle, stages in the life of a cell from one division to the next.

Cell differentiation, process in which cells become specialized.

Cell division, production or formation of two or more cells from an existing cell.

Cell fusion, formation of one hybrid cell through the joining of two cells.

Cell hybridization, fusion of two different cells to form a new hybrid cell.

Cell membrane, a membrane that surrounds the cytoplasm and is found just inside the cell wall.

Cell sap, aqueous solution of sugars, amino acids, organic acids, pigments, wastes, and other materials found in vacuoles.

Cell suspension, cells in culture in a liquid medium.

Cell wall, a rigid external structure that surrounds plant cells.

Cellulase, enzyme which breaks down cellulose.

Cellulose, carbohydrate found in plant cell walls. It is an unbranched chain of β-D-glucose in 1,4 linkages.

Centrifuge, laboratory apparatus that separates particles of various sizes and weights by generating gravity forces through rotation.

Chemically defined medium, a culture medium containing components which are of known and defined chemical nature.

Chlorenchyma, parenchyma tissue containing chloroplasts (and chlorophyll).

Chlorophyll, green pigment found in chloroplasts. It absorbs light energy which is used in photosynthesis.

Chloroplast, a chlorophyll-containing plastid.

Chlorosis, reduction in or loss of chlorophyll content.

Chromoplast, a plastid that contains yellow, orange, or red lipid-soluble pigments.

Cladophyll, a shoot which resembles a leaf.

Clonal propagation, multiplication of genetically identical plants.

Clone, cells or organisms (plant or animal) which are derived from a single cell or organism (plant or animal) and are genetically identical (except for possible genetic changes which can occur or may be induced by the propagation procedure).

Cloning, production of clones.

Coalescence, fusion of separate segments in a floral whorl.

Coconut milk, a white liquid obtained by grating or squeezing the solid endosperm or copra. *See* Coconut water.

Coconut water, colorless, liquid endosperm of coconuts, sometimes erroneously called coconut milk, which is added to some culture media. *See* Coconut milk.

Co-enzyme, a non-protein, low molecular weight substance which is necessary for or promotes enzyme activity.
Cohesion, union of floral parts of the same floral whorl.
Colchicine, alkaloid derived from *Colcichum autumnale*, which inhibits spindle formation during cell division, thereby preventing the separation of chromosomes and leading to cells which have multiple chromosome sets. It is used to increase (usually double) chromosome numbers in orchids and other plants.
Collenchyma, strengthening tissue consisting of thick-walled cells.
Colony, closely associated, poorly differentiated cells in culture.
Compensation point, conditions under which photosynthesis equals respiration.
Complete flower, a flower that has sepals, petals, stamens, and carpels.
Complex additive, an additive to a medium whose composition is either not fully known or very elaborate. Examples are coconut water and banana homogenate.
Conduction, movement of water and other substances through the plant.
Corm, a modified, enlarged, food-storing stem usually found underground.
Cortex, parenchymatous tissue in roots and stems located between the epidermis and pericycle or phloem, respectively.
Crassulacean acid metabolism (CAM), fixation of carbon to malic acid in the dark by succulent plants.
Cryopreservation, preservation of cells, tissue, seeds, and pollen under very low temperatures.
Cryoprotectant, a substance which prevents cell damage during freezing and thawing in cryopreservation. Examples include dextrans, dimethylsulfoxide, ethylene glycol, glycerol, hydroxyethyl starch, polyvinylpyrrolidone, and sugars.
Cultivar, plants found only in cultivation and possessing specific characteristics which distinguish them from other plants of the same kind.
Cuticle, cutin layer on the outer walls of epidermis cells. *See also* Cutin.
Cutin, a waxy substance, slightly permeable or impermeable to water vapor, liquid water, and gasses.
Cytogenetics, study of chromosomes and their role in taxonomy, genetics, variability, and other plant characteristics.
Cytokinesis, cytoplasm division in cells; it follows nuclear division (karyokinesis). *See* Meiosis, Mitosis.
Cytokinins, plant hormones, many (but not all) of which contain adenine. They control cell division and differentiation. In vitro they induce bud and shoot formation. Examples are the naturally occurring zeatin and isopentenyl adenosine as well as the synthetic benzyladenine, dimethylaminopurine, kinetin, and thidiazurone. Kinetin, the first known cytokinin was discovered and isolated ca. 1955 by Carlos Miller, Folke Skoog, and their associates in the botany department at the University of Wisconsin.
Cytology, science of cell structure and function.
Cytoplast, intact cytoplasm that remains following the removal of the nucleus.
Cytotoxic, toxic to cells.
2,4-D, *see* 2,4-Dichlorophenoxyacetic acid.

Day-neutral plant, a plant that flowers regardless of day length (*compare* Long day plant, Short day plant).

Deoxyribonucleic acid (DNA), a large helical molecule that carries hereditary information.

Development, changes in organisms, tissues, and cells, which result in the formation and appearance of features and characteristics which make possible the performance of specific and characteristic functions.

Dextrose, glucose.

2,4-Dichlorophenoxyacetic acid (2,4-D), a synthetic auxin and selective herbicide (it kills dicotyledonous plants) which is often added to tissue culture media at very low concentrations.

Dicotyledonous plant, a plant with two seed leaves (cotyledons). Also referred to as a broad leaf plant. Orchids are monocotyledonous plants, but the seeds of most species do not have a cotyledon.

Differentially permeable membrane, a membrane through which different substances move at different rates and varying ease. Some substances cannot diffuse through such membranes. Others may move freely.

Differentiation, formation of new functions, structures, organs, and specializations.

Diffusion, movement of molecules of a substance from an area of its own higher concentration to an area of it own lower concentration.

Dimethylsulfoxide (DMSO), a versatile solvent.

Diploid, presence of two sets of chromosomes, $2n$, as in body (somatic) cells. Contrast with haploid, $1n$, as in reproductive cells.

Disaccharide, a sugar consisting of two linked, simple, sugar molecules. Sucrose, which consists of one glucose molecule and one fructose molecule, is an example.

Disinfection, removal or destruction of undesirable microorganisms.

Diurnal, an event which occurs every 24 h.

DMSO, *see* Dimethylsulfoxide.

DNA, *see* Deoxyribonucleic acid.

Dormancy, state of arrested growth during which buds, seed, tubers, corms, bulbs, and other structures do not grow. Environmental cues such as cold temperature and photoperiod can break dormancy and bring about the resumption of growth.

Dorsiventral, a structure with two distinctly different upper and lower surfaces, as for example a leaf.

Dry weight (DW or D. W.), water-free weight of tissue.

EDTA, see Ethylene diamine tetraacetic acid.

Egg (ovum), female gamete.

Embryo, young sporophytic plant or immature organism before the start of rapid growth and development.

Embryo culture, the culture of embryos on a medium in vitro.

Embryo sac, female gametophyte of seed-producing plants (angiosperms).

Embryogenesis (embryogeny), development or formation of an embryo.

Embryoid, embryo-like structure, which may develop in vitro.

Emulsion, suspension in liquid of fine particles.

Endemic, an organism which is found or restricted to a specific region.

Endodermis, a single layer of thick-walled cells which forms a sheath around the vascular tissue, mostly in roots, but sometimes in stems. Cells of this layer have thickenings called Casparian strips. It is the innermost layer of the cortex.

Endogenous, from within. Opposite of exogenous.

Endosperm, nutritive tissue found in seeds. Most orchid seeds lack an endosperm.
Epidermis, the outermost layer of cells on all plant parts.
Epiphyte, an organism, often a plant, which grows on another plant. Many orchids are epiphytes.
Erlenmeyer flask, flat-bottomed conical flask with a wide or narrow neck, often used as a culture vessel, especially in research laboratories. Named after Emil Erlenmeyer, Professor of Chemistry in Heidelberg between 1863 and 1867, who designed it. *See also* Bunsen burner (which was invented at the same university).
Essential element, mineral elements required for normal plant growth and/or completion of the life cycle; also referred to as essential mineral or essential inorganic nutrient.
Ethanol, a disinfectant and solvent (ethyl alcohol, drinking alcohol).
Ethephon, a substance which breaks down to produce ethylene. It can be added to cultures, but it is almost never used in orchid micropropagation.
Ethyl alcohol, *see* Ethanol.
Ethylene, a gaseous plant hormone involved in senescence, ripening, and abscission.
Ethylene diamine tetraacetic acid (EDTA), a chelating agent used to chelate iron in culture media.
Etiolation, excessive stem elongation, abnormal development, and reduced or no chlorophyll content associated with insufficient illumination.
Eukaryote, organism with a well-defined nucleus surrounded by a membrane. Higher plants including orchids are eukaryotes.
Euploid, an organism with a chromosome number which is an exact multiple of the 1*n* number.
Ex situ conservation, conservation through the removal of a plant (or another organism) from its native habitat and maintaining it in a different location.
Exogenous, from an external source. Opposite of endogenous.
Explant, a section of tissue (usually small) or part of an organ taken from a plant and placed in a culture medium.
Exudate, produced through exudation.
Exudation, slow release or leakage of a substance.

F_1, first filial generation of a cross or mating ($P♀_1 \times P♂_1 = F_1$).
F_2, second filial generation produced by crossing or self-pollinating individuals of the F_1 generation. The offspring of the F_1 generation and "grand offspring" of the parents of the P_1 generation ($F♀_1 \times F♂_1 = F_2$). These terms are often misused by practical breeders.
Family, a group of genera. The Orchidaceae is a family.
Fertilization, a term with two meanings: (1) Addition of nutrients. (2) Fusion of two gametes.
Filter sterilization, sterilizing a liquid by passing it through a filter with holes which are small enough to retain contaminating microorganisms, but large enough to allow the passage of solute(s) and solvent(s).
Filtration, a term with two meanings: (1) Separation of particles from liquid or each other by passing a mixture through a paper or porous membrane with appropriate size pores. (2) Separation of cell clumps or aggregates from single cells in a culture.
Flavonoids, water-soluble pigments of colors ranging from yellow to blue.

Flaming, sterilizing an instrument by dipping it in alcohol, which is ignited by passing it through a flame.

Foot candle, a unit of light intensity mainly designed to measure brightness in terms of the human eye. The unit is not an appropriate measure of light energy as it applies to plants. 1 ft-c = 10.76 lux or meter candles (see section on light in Chapter 2).

Formula weight (FW), usually used to describe the molecular weight of a substance plus waters of hydration.

Freeze drying, drying cells, tissues, and organs under vacuum while they are frozen (lyophilization, cryopreservation).

Fresh weight (FW or F. W.), weight of tissues or organs including their water content.

Friable, callus which crumbles or falls apart easily.

Fungicide, a substance which can kill fungi.

FW, *see* Formula weight, Fresh weight.

Gamete, reproductive cell which can fuse with another cell of the same nature, but of the opposite sex. The result is a zygote.

Gametophyte, stage in the flowering plant life cycle that can produce gametes. This stage has 1*n* number of chromosomes. In flowering plants the pollen grain is the male gametophyte and the embryo sac is its female counterpart.

Gel, a jelly-like colloidal state.

Gellan gum, polysaccharide derived from *Pseudomonas* and used as a gelling agent. *See* Gelrite, Phytagel.

Gelrite, a proprietary trademark and brand name of a refined polysaccharide derived from *Pseudomonas* and used as a gelling agent.

Gene, unit of heredity consisting of DNA base pair sequences, which codes for RNA and protein.

Genetic engineering, manipulation of heredity and hereditary material for industrial, agricultural, medical, military, or other purposes.

Genetics, study and science of heredity.

Genome, entire genetic information in an organism or cell.

Genus, unit of classification consisting of one or more species. Several genera make up a family.

Geotropism, a term for gravitropism which is no longer used.

Germination, start of growth of a seed, spore, or pollen grain.

Gibberellins, a group of plant hormones that control or regulate elongation, flowering, fruit development, bolting, germination, vernalization, enzyme production, and other processes. These hormones were discovered by E. Kurosawa in 1926 while he was studying a rice disease in Taiwan (then called Formosa and part of the Japanese Empire).

Glucose, a six-carbon sugar. It is the most common sugar in plants, and is found in blood (hence called blood sugar). Also known as dextrose.

Glycine, an amino acid which is added to some culture media based on the Murashige–Skoog medium.

Gram molecular weight, number of grams equivalent to the molecular weight of a substance, a mole.

Gravitropism, response of plant organs to gravity.

Gro-Lux lamps, a trademark and brand name for wide spectrum fluorescent lamps which are used to illuminate plant cultures. Similar lamps are also marketed under other brand names (see section on light in Chapter 2).
Ground meristem, a meristem which produces ground tissues such as cortex and pith.
Growth, increase in size, weight, volume, and/or number of cells.
Growth factor, substance that affects growth.
Growth inhibitor, substance that inhibits growth.
Growth regulator, substance that regulates growth.
Growth retardant, substance that inhibits or retards growth.
Guard cells, two epidermal cells which surround and regulate the size of a stoma.
Gymnosperms, plants that produce seeds not in an ovary. Cone-bearing plants (conifers) are an example (*compare* Angiosperms).
Gynoecium, all of the carpels in a flower.

Hair, an outgrowth consisting of one or more cells which looks like a human hair (hence its name). Plant hairs can be adsorptive (like root hairs and rhizoids) or secretory (glandular). Despite its frequent use, the term "hair" is not favored by plant scientists who prefer the terms trichome or rhizoid except in the case of "root hairs."
Haploid, organism or life cycle phase with only a single set of chromosomes ($1n$). Contrast with diploid $2n$, as in somatic cells.
Herb, a plant lacking woody tissues; a soft, grass-like plant.
Herbaceous, adjective pertaining to herb.
Herbicide, substance which is toxic to or can kill plants. Some herbicides are hormonal in nature and if used in very low concentrations can be beneficial in tissue cultures. An example is 2,4-D.
Heterotrophic, deriving nutrients from another source.
Heterozygous, containing different alleles of the same trait, as for example, T (tall) and t (short) in the same plant (Tt). Appearance is not always indicative of the alleles that may be present in a plant.
Hormone, a substance with a very specific function which may be produced in one part of a plant and transported to another where it exerts its effects. Hormones usually are effective at very low concentrations. Synthetic analogs are available for some hormones. The first person to suggest that plants may have hormones was the German plant physiologist, Hans Fitting in 1909. He made the suggestion as a result of his work on the effects of pollen on *Phalaenopsis* flowers at the Bogor Botanical Gardens in Indonesia.
Hydrolysis, breaking down or splitting of a substance through the addition of water.
Hydroxy group, OH^-.
Hypertonic, solution of a concentration or osmotic potential relatively higher than that of another (sometimes adjacent) solution or cell. It is important to keep in mind that tonicity is determined by the molarity of a solution, not by its weight, percent, or parts per million of a solute.
Hypha, fungal filament.
Hypotonic, solution of a concentration or osmotic potential relatively lower than that of another (sometimes adjacent) solution or cell. It is important to keep in mind that tonicity is determined by the molarity of a solution, not by its weight, percent, or parts per million of a solute.

IAA, see Indoleacetic acid.
IBA, *see* Indolebutyric acid.
Illumination, providing light.
In vitro, literally "in glass," but meaning "in a culture vessel." Sometimes used (not entirely correctly) as the opposite of ex situ.
In vitro propagation, plant propagation in culture media in vessels. *See also* Micropropagation.
Indoleacetic acid (IAA), a naturally occurring auxin which is used in many culture media.
Indolebutyric acid (IBA), an auxin which is used in many culture media.
Inoculate, placing explant or cells into or on culture medium.
Inositol, hexahydroxycyclohexane, a polyol (sugar alcohol) which is added to many culture media.
Internode, area of a stem or inflorescence between two nodes.
Isodiametric, of the same diameter in all directions as in "isodiametric cell."
Isotonic, a solution of a concentration or osmotic potential equal to that of another (sometimes adjacent) solution or cell. It is important to keep in mind that tonicity is determined not by weight, percent, or parts per million of a solute, but by its molarity.

J m^{-2}, joule per square meter, a unit of light measurement (but *see* PAR). Also written as J/m^2, which is not the preferred form. *See also* Joule.
Joule (J), energy required to apply a force of 1 newton (N) over a 1-m distance.
Juvenility, early stages of development. During these stages, plants usually do not flower although in some cases very young orchid plantlets or seedlings have flowered in vitro.

Karyokinesis, division of the nucleus; it precedes cytokinesis. *See* Meiosis, Mitosis.
Karyotype, all of the chromosomes of an individual plant usually during metaphase arranged according to length, in a manner which shows their morphology and position of the centromere.
Kinetin, a synthetic cytokinin (6-furfuryl aminopurine, furfuryl adenine) which is often added to culture media. This was the first cytokinin to be isolated and identified. The work was carried out by Carlos Miller (then a graduate student) and his mentor, Folke Skoog, at the department of botany, University of Wisconsin.
Kranz anatomy, wreath-like (from the German *Kranz* meaning wreath) layers of chloroplast-rich cells which surround vascular bundles. Typical of C_4 plants.

Lactose, milk sugar; a disaccharide consisting of one galactose and one glucose molecule, and never used in orchid tissue culture media.
Lamina, *see* Blade.
Laminar flow hood, a cabinet for the preparation of cultures and other work which requires sterile conditions. Sterility of the air inside the hood is maintained by passing it through a filter. It is important to keep in mind that the work surface inside the hood as well as any equipment and apparatus inside it will not be sterile unless they are sterilized properly.

Lateral bud, a bud found on the side of a stem in the axil of a leaf (axillary bud).
Lateral meristem, a meristem that produces secondary tissues like the vascular cambium (itself a meristematic tissue).
Lateral root, a root that branches out of an older or primary root.
Leaf primordium, a lateral outgrowth on the apical meristem which develops into a leaf.
Levulose, fructose, a six-carbon sugar.
Liquid medium, a medium that does not contain a solidifier.
Log phase (log growth phase), phase during which growth or increase in number of cells is most intense or vigorous.
Long day plant, a plant that requires a light period longer than a specific critical minimum to flower.
Lux, a measure of illuminance. This is a metric unit designed to replace the foot candle (1 lx = 0.09 ft-c). Both of these units measure light intensity in terms of human vision rather than the wavelengths which support plant growth. *See also* PAR and section on light in Chapter 2.
Lyophilize, dehydration that follows rapid freezing.
Lysosome, a vacuole that contains digestive enzymes.

Macerate, breaking down tissue through cutting or digesting with enzymes to cause cell dissociation or protoplast formation.
Macroelement, an essential element which is required in relatively large amounts. A recent suggestion is that to be a macronutrient, an element must be present at a concentration higher that 0.5 mmol l^{-1}.
Mannitol, a sugar alcohol commonly used as an osmoticum in suspension solutions or media for plant protoplasts.
Media, plural of medium, which is routinely used incorrectly as the singular. *See* Medium.
Medium, a solution, formulation, or combination of nutrients and growth factors used for the culture of cells, explants, tissues, or organs.
Megaspore, the haploid ($1n$) spore in higher plants which gives rise to the female gametophyte.
Meiosis, two successive divisions of the nucleus which reduce the chromosome number from $2n$ to $1n$. Segregation of genes results from the reduction in chromosome number.
Mericloning, a practically and commercially very clever, but scientifically incorrect and linguistically abominable (especially when made into a verb), term derived by compressing "meristem" and "cloning." It was proposed by Gene Crocker, then a lieutenant and subsequently an orchid grower for Carter & Holmes Orchids (Newberry South Carolina). Gordon W. Dillon, long time editor of the *American Orchid Society Bulletin* called attention to the term in 1964 and it became popular instantaneously.
Meristem, undifferentiated and constantly young cells capable of continued division, found in tissues located at stem and root apices.
Meristem culture, culture in vitro of excised apices which contain a meristem. The most commonly cultured apices in orchids are those of shoots, but root tips are also cultured. This term is actually a misnomer because what is actually being

cultured is a shoot tip. In common usage the word meristem has been adulterated into a verb ("to meristem") and a professional description ("a meristemmer"). Both are linguistic abominations. A commonly accepted misconception is that Georges Morel of France was the first to culture a meristem. Actually, Ernest Ball (in the USA) and Loo Shih Wei (in the USA, but he returned to his native China soon after reporting his pioneering work) were the first to culture meristems (independently of each other) in 1946 and 1945–1946, respectively. The first to culture an orchid meristem was Hans Thomale in Germany in 1954. Morel reported his work in 1960 in an article which does not meet scientific standards.

MES, *see* Morpholineethane sulfonic acid.

Metabolism, chemical processes that occur in living cells and organisms.

Microelement, an essential element which is required by plants in very small amounts.

Micrometer, one millionth (10^{-6}) of a meter, or 0.001 mm; abbreviated as μm.

Micromolar, a concentration of one micromole per liter; abbreviated as μ*M*.

Micromole, one millionth of a mole; abbreviated as μmole, not μ*M*, which means micromolar.

Micron, an older, no longer used name for a micrometer; abbreviated as μ.

Micronutrient, *see* Microelement.

Micropropagation, multiplication by means of explants cultured in vitro.

Middle lamella, layer of pectic substances which cements two adjacent cells.

Milligram, one thousandth (0.001 or 10^{-3}) of a gram; abbreviated as mg.

Millimeter, one thousandth (0.001 or 10^{-3}) of a meter; abbreviated as mm.

Millimole, one thousandth (0.001 or 10^{-3}) of a mole; abbreviated as mmole, not m*M*, which means millimolar.

Mitochondrion, a small cell organelle where aerobic respiration, oxidative phosphorylation, and adenosine triphosphate (ATP) production take place.

Mitosis, division and separation of chromosomes that leads to the production of two nuclei. It is followed by cytokinesis, which results in two new cells.

Molality, number of moles added to a liter of solvent (i.e., solute in 1 l of solvent).

Molar, usually referring to concentration in terms of moles; usually abbreviated as M or *M*.

Molarity, number of moles in a solution adjusted to the total volume of one liter (i.e., the volume of the solute plus the volume of the solvent together are 1 l). The difference between molarity and molality is that in the latter, the volume of solvent is always exactly 1 l, whereas in the former the volume of solvent is 1 l minus the volume of the solute. A molal solution is prepared by mixing the solute with 1 l of solvent. Molar solutions are prepared in volumetric flasks by adding solvent to the solute until the total volume reaches 1 l. Equimolar, not equal weight, solutions must be used in comparing the effects of substance on explants.

Mole, weight of a substance in which the number of grams is equal to the molecular weight of the compound. A mole contains Avogadro's number of atoms, 6.023×10^{23}.

Molecular biology, combination of genetics and biochemistry.

Molecular weight, weight of a molecule of a substance.

Molecule, smallest unit of matter or substance which retains its chemical identity; it may consist of one or more atoms.

Monocotyledonous plant (monocotyledon, monocot), a plant whose embryo has one cotyledon (seed leaf). Orchids are considered to be monocotyledonous plants despite the fact that the seeds of most species do not have cotyledons. They have other monocot characteristics.

Monoploid, *see* Haploid.

Monosaccharide, a simple sugar, as for example the six-carbon sugar glucose, which cannot be broken into a smaller sugar molecule.

Morphogenesis, development of form and shape.

Morpholineethane sulfonic acid (MES), a compound used in the formulation of buffers.

Morphology, a term with two meanings: (1) The science and study of form and its development. (2) Generally, shape and form.

MS medium, *see* Murashige–Skoog medium.

Murashige–Skoog (MS) medium, a medium used in tissue culture.

Mutant, variant caused by sudden genetic change(s).

Mutation, process of causing a mutant.

Mutualism, the living together of two or more organisms in a mutually beneficial manner.

MW, molecular weight.

Mycelium, hyphae that make the body of a fungus.

Mycorrhiza, a mutualistic association between a fungus and the roots of a plant. The term was coined by the German botanist Albert Bernhard Frank (1839–1900). Orchids have mycorrhiza.

NAA, *see* α-Naphthaleneacetic acid.

Nanogram, one millionth (10^{-6}) of a milligram or one billionth (10^{-9}) of a gram; abbreviated as ng.

Nanometer, one millionth (10^{-6}) of a millimeter or one billionth (10^{-9}) of a meter; abbreviated as nm. Older terms are millimicron (mμ) or 10 angstrom (10 Å).

Nanomole, one millionth (10^{-6}) of a millimole or one billionth (10^{-9}) of a mole; abbreviated as nmole, not n*M*, which means nanomolar and refers to concentration.

α-Naphthaleneacetic acid (NAA), a synthetic auxin used in many culture media.

Niacin (nicotinic acid), a water-soluble vitamin often added to tissue culture media.

Nitrate, NO_3^-, a form of nitrogen which can be taken up and used directly by plants.

Nodal culture, culture of lateral buds usually with some adjacent stem tissue.

Node, location on a stem where leaves and buds arise and are located.

Nucleus, a body in a eukaryotic cell enveloped by a membrane and containing chromosomes and DNA. All plant cells except sieve tube elements have nuclei.

Nurse culture, culturing plant cells or tissue of one kind of plant on a culture of another kind of plant. The two cultures may be in direct contact or separated from each other by a membrane or filter paper.

Nutrient, a substance that provides sustenance.

Nutrient solution, a combination of macro- and microelements, a carbon (energy) source (most commonly sucrose), hormones, vitamins, sometimes amino acid(s), and a polyol, as well as complex additives (coconut water, casein, banana homogenate, etc.) used for the culture of cells, organs, tissues, and other explants.

Offshoot, a side growth from a stem often produced from the crown near soil level.
Offspring, new progeny produced by an existing organism. Both the plural and singular forms of this word are the same.
Oligosaccharide, a larger sugar molecule consisting of several linked sugar molecules.
Organ, several tissues that function together morphologically and physiologically and form a specific part of an organism.
Organ culture, culture of an entire organ in vitro.
Organelle, a specialized structure within a cell which performs a specific function, as for example chloroplast or mitochondrion.
Organic, a compound containing carbon.
Organogenesis, formation of organs.
Ortet, a term coined in 1929 to designate the "original plant of seedling origin from which members of a clone" have originated (for more extensive discussions of the term see Stout, 1929; Krikorian, 1982).
Osmosis, diffusion through a semipermeable membrane.
Ovary, enlarged basal part of the pistil; it becomes the fruit.
Ovule, a reproductive structure consisting of the embryo sac, egg cells, and integuments surrounded by the nucellus.

Palisade parenchyma, a tissue layer consisting of elongated chloroplast-rich cells positioned at a right angle to the surface and found just below the upper epidermis of a leaf.
PAR, *see* Photosynthetically active radiation.
Paradermal section, a section cut parallel to a flat surface.
Parenchyma, tissue made up of parenchyma cells. *See* Parenchyma cell.
Parenchyma cell, living, thin-walled, undifferentiated cells; the most common cell type in plants.
Parthenocarpy, development of a fruit without fertilization.
Parthenogenesis, formation of a seed without fertilization.
Parts per million (ppm), 1 mg per liter.
Pectin, a jelly-like substance which forms the middle lamella and cements cells to each other.
Pedicel, stalk of an individual flower on an inflorescence; it is located just below the ovary and is contiguous with it.
Peduncle, stalk of an inflorescence or a flower born singly.
Pentose, a five-carbon sugar.
Permeable, a membrane through which substances can diffuse.
Petal, part of the corolla (the usually colored floral segments).
Petiole, stalk of a leaf.
Petri dish, flat, round culture dish. Invented by L. Petri (1875–1946).
pH, a term or symbol which indicates the concentration of hydrogen ions (H^+) in a solution. pH 7 (the reciprocal of 1×10^{-7} which is the number of dissociated water molecules) is neutral. Values from 0 to 7 are indicative of acidity (the lower the number, the higher the H^+ level and the more acid the solution). Numbers between 7 and 14 are alkaline (the higher the number, the lower the H^+ levels and the more alkaline the solution). pH levels can be measured electronically or with indicator papers.

Phenolics, substances which have hydroxyl group(s) on benzene rings. They are produced from newly excised tissues, diffuse into the culture medium, become oxidized, and form dark colored compounds, which in addition to being unsightly may also be deleterious.

Phloem, conductive tissue in plants made of living cells called sieve elements, which transports organic substances. The sieve elements have no nuclei. They share the nuclei of adjacent companion cells.

Photon, elementary light particle; a quantum of light whose energy is proportional to its frequency and is expressed by the formula $E - h\nu$ where E is energy, h is Plank's constant or 6.62×10^{-27} erg-second, and ν is frequency.

Photoperiod, period of illumination, relative period of light, or length of day.

Photoperiodism, response of organisms to relative periods of light and dark.

Photosynthesis, synthesis of carbohydrates from carbon dioxide and water in the presence of chlorophyll, using light energy which was converted to chemical energy.

Photosynthetically active radiation (**PAR**), the wavelengths of light that are active in photosynthesis.

Phototropism, growth or bending toward light.

Phyllotaxy, phyllotaxis, arrangement of leaves on a stem.

Physiology, physical and chemical activities and functions of living organisms.

Phytagel, a proprietary trademark and brand name of a refined polysaccharide derived from *Pseudomonas* and used as a gelling agent.

Phytoalexin, a substance produced by plants to ward off attacks by fungi and bacteria. The French botanist Noël Bernard, discoverer of the role of mycorrhiza in orchid seed germination, also discovered phytoalexins while working on the interactions between orchids and their mycorrhizal fungi, just before his death. His findings were published in 1911 in a posthumous paper written by his mentor J. Costantin and his cousin J. Magrou.

Phytohormone, plant hormone, chemical regulators of plant growth and development which include abscisic acid, auxins, cytokinins, ethylene, and gibberellins. The first person to suggest the existence of plant hormones was the German plant physiologist Hans Fitting who formulated the concept as a result of his work (in the Bogor Botanical Gardens in Indonesia) on the effects of pollination on *Phalaenopsis* flowers, which was published in 1909. Auxin, the first plant hormone and the one intimated by Fitting who called it *Pollenhormon*, was discovered by Frits W. Went in 1926.

Phytokinin, an older and no longer used name for cytokinin.

Pigment, a substance which absorbs light, usually selectively.

Plantlet, a small complete (roots and shoots) plant derived from cultured cells, an explant, or a callus. It is incorrect to use "seedling" to describe such a plant.

Plasma membrane, *see* Plasmalemma.

Plasmalemma, a membrane that surrounds the cytoplasm.

Plasmolysis, shrinking of the cytoplasm and separation from the cell wall due to loss of water.

Plastid, a cytoplasmic organelle which contains lipid-soluble pigments.

PLB, *see* Protocorm-like body.

Ploidy, number of chromosome complements (sets), as for example haploid ($1n$), diploid ($2n$), triploid ($3n$), tetraploid ($4n$) to N-ploid (Nn).

Polyploid, cell, tissue, or organism with more than one complement (complete set) of chromosomes.

Polysaccharide, long chain molecule like cellulose and starch composed from monosaccharides.

Polyvinylpyrrolidone (PVP), a substance used in micropropagation media as an adsorbent, antioxidant, or osmoticum.

ppm, parts per million.

PPM, Plant Preservative Mixture, a proprietary preparation that combats contamination in cultures.

Primary growth, a growth that originates from primary meristems.

Primordium, a mass of cells which produces a specific structure like an organ.

Progeny, offspring.

Proliferation, relatively rapid increase (but not always so) brought about by constant, frequent, and repeated cell division.

Protein, a large molecule consisting of polypeptides which are made of amino acid chains. The term was coined in 1838 by the Swedish chemist Jons Jacob Berzelius (1779–1848) who also named methyl alcohol and invented modern filter paper. Despite his many achievements, Berzelius repressed the French chemist Auguste Laurent who proposed valid ideas regarding organic structures.

Protocorm, a structure produced by germinating orchid seeds. The term was coined by Mechior Treub (1851–1910), long time director of the Bogor Botanical Gardens in Indonesia, to describe the early stages of lycopod seedlings. It was not coined by Noël Bernard, as is often stated erroneously [most recently by P. J. Cribb (1999) in the first volume of the otherwise excellent *Genera Orchidacearum*]. This term should be used only for structures produced by seeds. *See* Protocorm-like body.

Protocorm-like body (PLB), a structure which resembles a protocorm but is produced by an explant or a tissue derived from an explant.

Protoplasm, living substance of cells.

Protoplast, cytoplasm surrounded by plasmalemma and freed of the cell wall.

Purine, one type of nucleotide bases (adenine and guanine) found in DNA and RNA. Adenine is a component of cytokinins.

Pyridoxin, vitamin B_6, which is often added to culture media.

Pyrimidine, a single ring nitrogenous base found in DNA and RNA such as cytosine or thymine (which should not be confused with vitamin B_1, thiamin, or thiamine).

Quantum, elemental unit of light energy whose value is hv, where h is Plank's constant, 6.62×10^{-27} erg-second and v is the vibration frequency of the light wave. *See also* Photon.

Quarantine, a term with two meanings: (1) Keeping an organism or human in isolation if there is a danger of a disease. (2) Restrictions in the shipping of organisms to prevent the spread of diseases.

Raceme, an inflorescence with an elongated main axis (rachis) which bears flowers that have pedicels (floral stalks). Most orchid inflorescences are racemes, not spikes.

Rachis, axis of an inflorescence. Traditionally the term was applied to the axis of a spike. However, at present usage has extended to the axis of most inflorescences.

Raphide, a needle-shaped crystal made of calcium oxalate and found in many cells.
Reproduction, giving rise to offspring sexually or asexually.
Respiration, biological oxidation of molecules with the release of energy.
Rhizoid, a hair-like structure.
Rhizome, a modified stem usually located underground or at ground level.
Ribose, a five-carbon sugar.
rpm, revolutions per minute.

Sap, the liquid content of phloem, vacuoles, and xylem.
Seed, a mature ovule.
Seedling, a young plant formed by a germinating seed.
Semiliquid, *see* Semisolid.
Semipermeable membrane, a partially permeable membrane; some substances can pass through it, but others cannot.
Semisolid, a medium solidified with a small amount of solidifier; semiliquid.
Senescence, an aging process in which cells and tissue lose functions, degrade, deteriorate, and eventually die.
Sheath, a term with three meanings: (1) Part of a leaf which envelops and wraps around a stem. (2) A layer of tissue which envelops or surrounds another tissue. (3) In some orchids (*Cattleya*, for example) a structure which envelops young flower buds.
Shoot, a young branch growing out of an existing stem or a young plant emerging from the soil, an explant, or a callus mass.
Shoot apex, *see* Shoot tip.
Shoot tip, terminal portion or bud of a plant (the top 0.1–1.0 mm) consisting of the apical meristem (upper 0.05–0.1 mm) and adjacent leaf primordia and stem tissues.
Short day plant, a plant that requires a dark period longer than a specific critical minimum to flower.
Sieve cell or element, a long, slender, tapering cell which is a component of the xylem.
Sieve tube, a series of sieve cells attached end to end.
Single node culture, culture of a single lateral bud with a stem section attached to it. *Phalaenopsis* flower-stalk node cultures are an example. The culture of these nodes was first reported by Dr. Gavino Rotor in 1949. This is the first instance of orchid micropropagation. It preceded Georges Morel's supposed first culture of an orchid explant by 11 years. Thus Rotor, not Morel, is the discoverer of orchid micropropagation.
Solid medium, a nutrient solution that has been solidified by a solidifier such as agar.
Solute, *see* Solution.
Solution, a mixture in which molecules of one substance (the solute) are homogeneously dispersed among the molecules of the other (the solvent).
Solvent, *see* Solution.
Species, a group of similar organisms which share many common characteristics and interbreed freely.
Spike, an inflorescence with an elongated main axis (rachis) which bears sessile flowers [i.e., flowers that do not have pedicels (floral stalks)]. Most orchid inflorescences are not spikes, they are racemes.

Starch, an insoluble polysaccharide consisting of branched or unbranched chains of α-D-glucose, which is the main food storage substance in plants.
Stele, the central vascular cylinder, located inside the cortex, in roots and stems of vascular plants.
Sterile, a term with two meanings: (1) Apparatus, condition, container, medium, tool, and other objects which do not contain microorganisms. (2) Incapable of fertilizing or being fertilized.
Sterilization, process of making sterile.
Stock solution, a concentrated solution of a medium component prepared in advance for convenient and repeated use.
Subculture, transfer of part of an existing culture to a new medium and vessel.
Substrate, a term with three meanings: (1) Substance that is changed by an enzyme. (2) Medium that supports explants or organisms in vitro. (3) Medium that supports a static organism.
Succulent, plant with fleshy stems and/or leaves which store water.
Sucrose, a disaccharide consisting of α-D-glucose and fructose.
Surfactant, surface active or wetting agent like Tween, and the components of domestic detergents and soaps.
Suspension culture, a culture of cells, explants, callus sections, protocorms, protocorm-like bodies, and other tissues growing while suspended in liquid and usually shaken.
Symbiosis, close association of two organisms. When this association is of mutual benefit it is said to be mutualistic (the phenomenon of mutualism).

TDZ, *see* Thidiazuron.
Terminal bud, bud at the tip of a stem.
Tetraploid, four complements (sets) of chromosomes ($4n$).
Thiamine, vitamin B_1, which is added to many media. Sometimes it is spelled thiamin.
Thidiazuron, *N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea (TDZ), a compound with high cytokinin-like activity. Its cytokinin-like activity was first discovered by M. C. Mok, D. W. S. Mok, and D. J. Armstrong at the Department of Horticulture, Oregon State University, Corvalis.
Tissue, a group of similar cells.
Tissue culture, maintenance of tissue(s) and other explants on a medium in vitro, under conditions which promote growth, proliferation, and differentiation. Often used synonymously with micropropagation.
Trichome, a filament or hair.
Triose, a three-carbon sugar.
Tropism, curvature, growth, or movement in a specific direction due to an external stimulus (gravitropism, tropism induced by gravity; phototropism, tropism induced by light).
Tuber, a modified food-storing root (as in *Dahlia*) or stem (as in potato).
Turgid, swollen, crisp, or distended due to an influx of water.
Turgor pressure, internal pressure resulting from an influx of water.

Undefined component, a medium component whose composition is not known (partially or fully).

Undefined medium, medium whose composition is not known (partially or fully).
Undifferentiated, tissue or cells that lack specialization.
Unorganized growth, a tissue with few or no differentiated cells or regions and no clear, defined, or recognizable structures.

Vacuole, a cytoplasmic component consisting of a solution of various substances in water (sap) bounded by a membrane.
Vacuum, free of air or under greatly reduced atmospheric pressure.
Variegation, presence of colored and non-colored tissues.
Variety, subdivision of a species.
Vascular, pertaining to conducting tissue.
Vegetative propagation, a method of asexual propagation using parts or organs.
Venation, arrangement of veins. In most orchids, leaf venation is parallel.
Vernalization, temperature (usually low) treatment for the purpose of inducing flowering or growth.
Vessel, a term with two meanings: (1) A short cell with a lignified wall, which is a xylem component. (2) A container (bottle, Erlenmeyer flask, jar, Petri dish, or test tube) used for tissue culture.
Virus, a pathogen consisting of a protein coat or capsule that contains DNA or RNA.
Vitamin, a substance required by living organisms to maintain normal growth, development, metabolism, and physiology. The commonly used vitamins in orchid tissue culture are B_1 (thiamin or thiamine), B_6 (pyridoxin), niacin (nicotinic acid), and sometimes (rarely) biotin and C (ascorbic acid).
v/v, adding or mixing on a volume to volume basis, as for example ml/ml.
v/w, adding or mixing on a volume to weight basis, as for example ml/mg.

Water stress, lack of sufficient water.
Wet weight, weight of completely hydrated tissue. *See also* Dry weight, Fresh weight.
Wetting agent, *see* Surfactant.
w/v, adding or mixing on a weight to volume basis, as for example mg/ml.
w/w, adding or mixing on a weight to weight basis, as for example mg/mg.

Xanthophyll, a yellowish plastid pigment.
Xerophyte, resistant to drought.
Xylem, water conduction tissue in plants.

Yeast extract, an undefined or complex additive to some media consisting of substances from yeast.

Zone of elongation, part of a young root, just above the meristem, where cells are elongating.
Zygote, fusion product of two gametes.

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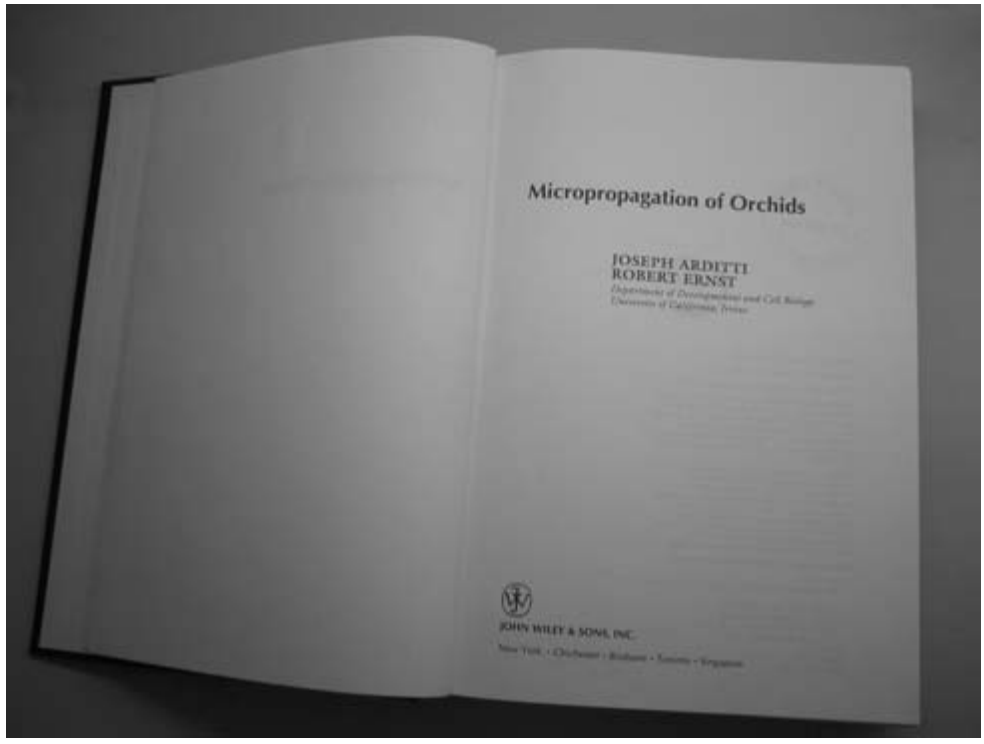
Page numbers in *italic* refer to tables; those in **bold** refer to figures and text or illustrations in boxes. Page numbers followed by 'n' refer to footnotes. Subentries are arranged in the order: general entries, species names, variety names.



Micropropagation of Orchids

JOSEPH ARDITTI
ROBERT ERNST
Department of Developmental and Cell Biology
University of California, Irvine

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