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VOLUME TWO
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VOLUME ONE
MICROPROPAGATION
OF
Orchids
THIRD EDITION
Tim Wing Yam and Joseph Arditti
WILEY

Micropropagation of Orchids

Volume I

This, the third and expanded and updated edition of a classic reference work, provides users with comprehensive details of all procedures developed by May 2014 for orchid propagation through the culture of tissues in vitro (micropropagation).

The book opens with an extensive, expanded with new information, and richly illustrated historical chapter which discusses the discoveries, developments, and people that made micropropagation possible. An updated and expanded Chapter 2 discusses in detail the principles and components of culture media used in micropropagation. However, the main component of these volumes is the third chapter, which contains detailed procedures for the culture and micropropagation of hundreds of orchid genera, species, and hybrids. Early, classic, established, and the very latest techniques of mass rapid clonal propagation through the culture of organs, tissues, and cells are presented. Every one of the methods in Chapter 3 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 or involved in orchid micropropagation, growing, and production.

Dr Yam Tim Wing studied Biology (B.Sc.) at the University of Southampton in England. He received his M.Phil. degree in plant breeding and genetics from Cambridge University before returning to Hong Kong where he earned his Ph.D. with a research project on the conservation of the native orchids. From 1988 to 1991, he carried out postdoctoral research at the University of California, Irvine with Professor Joseph Arditti. He is a Principal Researcher at the Singapore Botanic Gardens, National Parks Board, specializing in orchid hybridization and conservation. His hybrids have won awards at local and international shows. He also administers a program designed to conserve the native orchids of Singapore by propagating and introducing these species into urban and natural areas in the country. Dr. Yam is the author of research papers and two books entitled *Orchids of the Singapore Botanic Gardens* and *Native Orchids of Singapore: Identification, Diversity and Conservation*. He also co-authored the book *Biology of* Vanda *Miss Joaquim* with Professors Hew Choy Sin (Western style: Choy sin Hew) and Joseph Arditti. Dr. Yam is married to Pauline Wong and they have two daughters, Grace and Amy.

Professor Emeritus 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 USA, Indonesia, Singapore, and Malaysia. Dr. Arditti retired in 2001 and is now Professor of Biology Emeritus. He continued to write and travel worldwide to lecture at scientific meetings and to orchid groups until 2014 when he retired for a second time. Professor Arditti is acknowledged to be one of the world's leading experts on orchid biology and propagation. He is the proud father of Dr. (forensic psychology) Jonathan O. Arditti who received his bachelor's degree of psychology from the University of Southern California and equally proud father in law to Dr. Alexandria Grabowski who also received a bachelor's degree and a doctorate in dentistry from the same university.

Micropropagation of Orchids

Third Edition

Volume I

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For

My wife Pauline (center) and my daughters Grace (left) and Amy (holding the family dog, Powdy).

The memory of my doctoral studies supervisor,
Dr. Maureen Weatherhead

Tim Wing Yam

My son, Dr. Jonathan Arditti, and my daughter in law, Dr. Alexandria (Alex) Arditti (born Grabowski), both alumni of the University of Southern California, like the old man.

Mike and Anne Grabowski, alumni of the University of Southern California, like their daughter.

Joseph Arditti

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Preface

This book started as a practical appendix (written with a portable manual typewriter which was a gift to Joseph Arditti from his late father, Salomon, 1902–1993) entitled *Clonal Propagation of Orchids by Means of Tissue Culture: A Manual* in the first volume of *Orchid Biology, Reviews and Perspectives* which was intended to generate sales. That it did, so well that it was expanded into the first edition (Wiley, 1993, *MO*1) by Joseph Arditti and the late Robert Ernst (1916–2009). The second edition (Wiley Blackwell, 2008, *MO*2), by Joseph Arditti as sole author followed in 2008. This edition (*MO*3) is the product of a joint effort by Joseph Arditti and his former postdoctoral fellow Tim Wing Yam.

The history chapter in *MO*2 was revised extensively and much new information was added. Revisions of the history chapter in this edition were not extensive and only a small amount of information (kindly provided by the family of the late Dr. Walter Bertsch) was added. Chapters 2, 4 and appendices in this edition were revised to some extent but were not rewritten as extensively as they were for *MO*2.

Several new genera and species have been cultured since the publication of *MO*2. Additional procedures have been developed for orchids for which procedures were included in *MO*2. All of these new procedures are included in this edition.

Like *MO*2, this edition does not contain information on the use of colchicine (as quirkily demanded by a reviewer of *MO*1) because this topic belongs in a book on cytology, cytogenetics, or bioengineering, not one on micropropagation. In view of the large amount of information that had to be included in *MO*2 the 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 second edition grew to be very large. The same policy was followed for this edition. In addition, procedures which use explants from young seedlings or plantlets in vitro are only summarized in this edition due to space limitation.

Orchid nomenclature is fluid, names are changing constantly, and taxonomists frequently disagree about the validity of names. When names of species or even genera change, so do the names of some hybrids. We are not taxonomists and cannot undertake to determine the validity of nomenclatural changes. Therefore we always use the

names of genera, species and hybrids which are used in the original papers. For the latest grex names of hybrids, users of this book should refer to the Royal Horticultural Society International Orchid Register (http://apps.rhs.org.uk/horticulturaldatabase/orchidregister/orchidregister.asp) or, for species, check the International Plant Name Index, IPNI (http://www.ipni.org/ipni/plantnamesearchpage.do).

Information on media, culture conditions, and procedures, which is sufficient for a presentation in *MO* is available in most but not all published papers. Where information was not available, a few logical assumptions had to be made. For example, if a paper did not describe culture conditions in detail an assumption was made that standard culture room temperature and illumination will be suitable. In a few instances, where original papers present few and incomplete details many assumptions had to be made and attention was called to the low quality of these papers.

The second and third editions have 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 we learned from comments about *MO*1 and *MO*2 is that readers appreciated:

- redundancy ["move the tissue to the first medium (Table ABC-1) and after that to the second substrate (Table ABC-2) before returning it to the first solution (Table ABC-1) and then take it to the third (Table ABC-3)" was preferred by users who, when given a choice, chose this type of writing to "move the tissue to the first medium, and then to the second before returning it to the first and then take it to the third"];
- repetitions ("tell me how to mix an alcohol solution every time I need it, don't refer me to 100 pages back");
- details ["sterilize it with 10% Clorox (10 ml Clorox diluted to 100 ml with distilled water)" was preferable to "sterilized it with 10% Clorox"];
- clarity (short declarative sentences rather than long and involved ones);
- unambiguous instructions ("don't give me choices, tell me what to do");
- simple language ("don't use involved chemical names if the substance has a short name, or list both"); and
- self-standing procedures ("list all media and solutions with every procedure, don't send me back and forth across many pages").

We wrote with these preferences in mind.

A problem with the first edition (1993) was that procedures were not separated. Tables and/or illustrations associated with one procedure extended into another. This was confusing. To eliminate this problem in the second edition (2008) and this one, procedures were completely separated. Desirable as the separation is, it created its own problem: Large blank spaces which created unsightly pages. The problem was eliminated in the second edition and in this one by placing photographs or boxes containing peripheral information in the blank spaces. Both the boxes and photographs in the second edition were received well. We hope that the same will be true for this edition. Those who may find the boxes distracting can simply ignore them.

As are the right and privilege of authors we have expressed opinions in places in the text and a few boxes. We will respectfully consider, but not necessarily accept, other opinions.

Ideally every procedure in this edition and in the previous ones would have been tested. Given the large number of procedures and orchid species and hybrids which are included in this book, testing was impossible. Therefore procedures are presented without having been tested in the hope that they do work.

This edition was completed on June 20, 2014. We edited and revised the manuscript from that date until October 2014. Publication was delayed due to the need to obtain numerous permissions to use photographs, diagrams, quotes, and other items.

Both of us note sadly the passing of an orchid micropropagation pioneer and a good friend, Professor Adisheshappa Nagaraja Rao (b. 1925) of Singapore on Sunday, June 8, 2014. He was professor and head of the Botany Department at the University of Singapore and, later, National University of Singapore from 1967 until 1985. His friendship, wisdom and guidance will be missed. We also note the passing in 2009 of Dr. Robert Ernst, co-author of *MO*1. He and Joseph Arditti collaborated on orchid research from 1967 until 2001.

Many thanks to all those who provided us with PDFs of published papers, copies of illustrations and permissions to use and/or reproduce their work. We thank the following individuals (listed here in alphabetical order), all associated with publication of this book, for their invaluable help, excellent performance and superb professionalism: Ward Cooper, Beth Dufour, Kelvin Matthews, David McDade, Dr. Jolyon Phillips, Jan Ross, Emma Strickland, Kathy Syplywczak, and Bella Talbot. We also thank Dr. Wolfgang Zierau (now retired) for translations from German and Professor (retired) Syoichi Ichihashi for translations from Japanese. Joseph Arditti thanks his brother, Mordechai (Mordi or Mort) Arditti for keeping his computers in good working order.

For those who may be interested in how we managed to collaborate despite the great distance between us: We did it via e-mail and on Skype and WhatsApp at odd hours for one or the other of us due to the 15-hour time difference and during several visits by Joseph Arditti to Singapore. These visits were possible due to the generosity of the Soediono family which provided him with housing as they have done for 30 years.

Tim Wing Yam
Singapore

Joseph Arditti
Irvine, California
April 2017

Preface to the Second Edition

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 & 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*1, *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 procedures in *MO*1 because they are still useful and as many of the methods which 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). 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 next 15 years, but *MO*3 will have to be written by someone else even if I live that long.

Chapter 1 in *MO*1 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 basis 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 subject. 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 colchine 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 which 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 assumption. For example, if a paper did not describe culture conditions in detail I assumed that 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 which were mentioned

in a review of the literature. He/she indicated that a certain amount of expertise is implied in the writing of a book or a review and that readers have a right to expect evaluations, criticisms, praise, opinion, and advice from an expert. *MO*1 is 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 expert.

The author of a second edition has the advantage of hindsight as it were 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 the book appreciated having in *MO*1:

- 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 page xxx first, then the solution in Table MA-3 on page yyy, the substrate in Table VQ-9 on page zzz after that and finish with Table SUN-8 on page 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 which pertain to one procedure are mixed with pages which contain text about another method. I reorganized this edition so that tables and text which pertain 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 which 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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- D. Y. N. Chow, SEGI and Prime College in Kuala Lumpur, Malaysia for many good ideas and having his wife freeze a durian for me;
- my colleague of many years, Dr. Robert Ernst who decided not to coauthor this edition, allowed me to use material from *MO*1 and offered to help with *MO*2;
- Professor Choy Sin Hew, National University of Singapore for advice, permission to use information and illustrations from his publications, helpful comments and being an excellent host when I visit Singapore;
- 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;
- Professor Syoichi Ichihashi, Aichi University of Education in Japan for clarifications and translations of Japanese papers;
- 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);
- Jill and Chin On Mak of Singapore for access to their orchid plants, shopping trips to Johor, and excellent durians;
- Jean Miller who tends the interlibrary loan desk at Science Library at the University of California, Irvine and performs miracles in locating and obtaining rare and/or obscure publications. Writing this book would have been impossible without her and Kathryn Kjaer;
- Professor Helen Nair and her husband James Bonney for hosting me in Kuala Lumpur and providing a place to work while there;
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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.

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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 wordprocessing 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, National Parks Board), and Gu Zhuping (Biology Department, Lanzhou University, Lanzhou, China).

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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

Dr. Robert Ernst, Adjunct Associate Professor of Biology Emeritus at the University of California, Irvine, passed away in Los Angeles on September 1, 2009. He was 93.

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, 2008; 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 in his time.

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;* Hossain et al., 2010; Teixeira da Silva, 2013*b* and literature cited in these reviews) 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 and its commercialization has a more complex history, which is not free of controversy and includes unusual episodes (Arditti, 1977*b*, 1985, 2001, 2008; Arditti and Arditti, 1985; Torrey,

Micropropagation of Orchids, Third Edition. Tim Wing Yam and Joseph Arditti.

1985*b*; Arditti and Krikorian, 1996; Zimmerman, 1996; Easton, 2001; Yam and Arditti, 2004, 2009; Winkelman et al., 2006).

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.

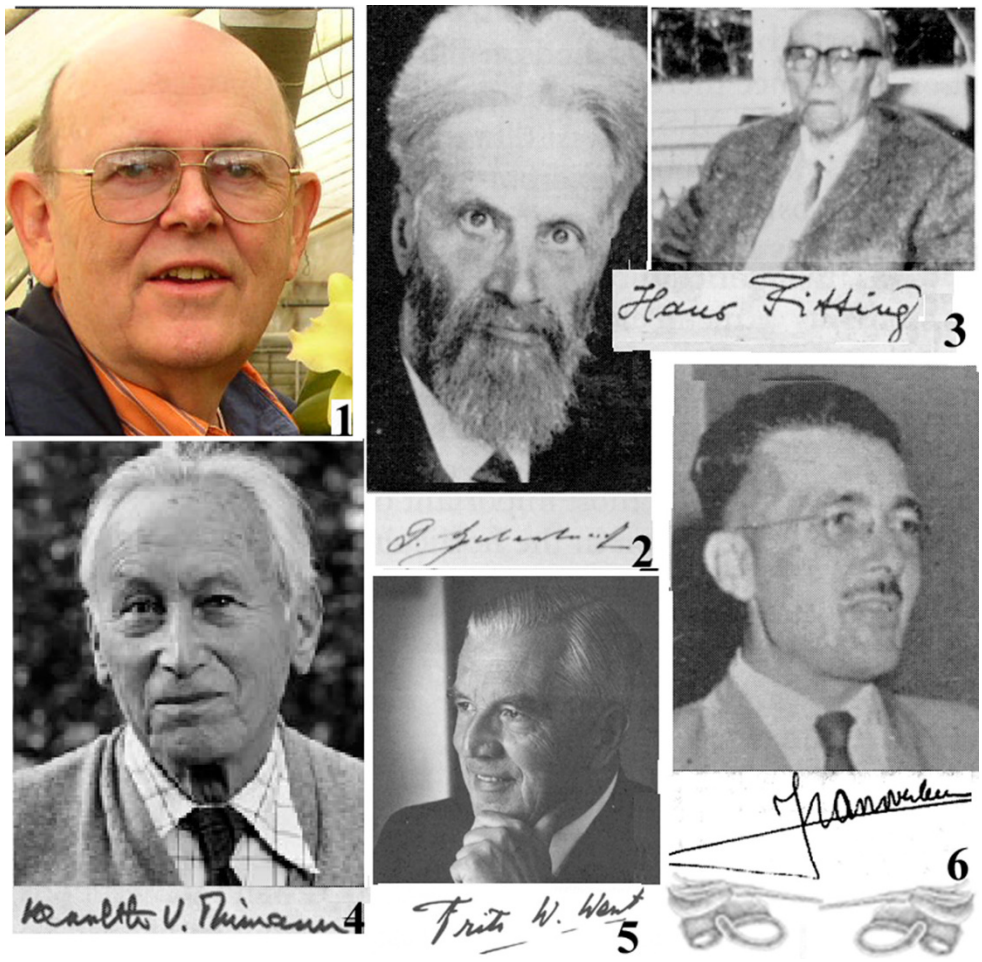
Clone as a term was introduced in 1903 and is based on the Greek word *clon*, which means twig, spray, or slip of the type used for vegetative propagation (Weber, 1903; Krikorian, 1982). Originally referred to plants produced through vegetative propagation methods like cuttings, layering, budding, and 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"



FIGS. 1-1–1-6. **Early plant physiology and tissue culture researchers. 1. Lieutenant and later orchid grower Gene Crocker (courtesy of Keith Davis; and Dr. Harry Gallis by providing a connection with Mr. Davis). 2. Professor Gottlieb Haberlandt (White, 1943). 3. Professor Hans Fitting [photograph by Brigitta H. Flick, signature from a letter to Joseph Arditti (J.A.) which is now in the library of the Singapore Botanic Gardens]. 4. Professor Kenneth V. Thimann (photograph from University of California Santa Cruz web site, signature from autographed book owned by J.A.). 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 J.A. which is now in the library of the Singapore Botanic Gardens). 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).**

(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 we know of 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.

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 erroneously 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. This term must not be used to describe the small, spherical, tuber-like bodies which are produced by germinating seeds.

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 for the second edition with new information, some of it taken verbatim or nearly so, from Arditti and Krikorian, 1996; we thank Professor Emeritus Abraham D. Krikorian for allowing us to use both text and photographs from this paper). 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; Arditti, 2008; Yam and Arditti, 2009). 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, about 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 at the University of 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*, 1909*b*, 1910, 1911, 1921 and a number of letters (now in the library of the Singapore Botanic Gardens) to Joseph Arditti (J.A.) in 1968 and 1969; for reviews see Arditti, 1971*a*, 1979, 1984, 1992; Avadhani et al., 1994; Yam et al., 2009]. 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 came out of retirement to become 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*, 1909*b*) and until his death (in letters to J.A.; for translations into English see Yam et al., 2009) 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; Yam et al., 2009 for reviews). Fitting did not pursue the matter further, probably because he became interested in other phenomena including the sensory physiology of plants (Professor Frits Went in conversation with J.A. in the late 1980s; Arditti and Krikorian, 1996; Yam et al., 2009). Had he continued his work on *Pollenhormon*, Fitting might have discovered auxin.

The first intimation that *Pollenhormon* is or contains auxin was made 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; Yam et al., 2009).

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 at any time. This is coconut water. Coconut milk is a white liquid obtained by extracting, grating, or squeezing the solid white endosperm of 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

FIGS. 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 J.A., 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 (Orchids 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.**

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 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 including orchids.

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).

François Mariat (1921–2003) 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.

FIGS. 1-13–1-18. **Plant scientists. 13. Professor Carlos O. Miller (photograph courtesy C.O. Miller, signature from a note to J.A.). 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.**

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 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 solvents 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). Taro (*Colocasia esculenta*) extract enhanced callus induction and growth in *Doritaenopsis*, *Neofinetia*, and *Phalaenopsis* (Ichihashi and Islam, 1999; for an excellent review on the use and effects of complex organic additives in plant tissue culture and micropropagation see Al-Khayri, 2013).

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; for an excellent review of totipotency, the word, the concept and their history see Krikorian, 2005). 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, Karl Goebel (Fig. 1-18) in 1902, Julius 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), *Ornithogalum 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 today) 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 Austria) 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" or "easy" 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 (or even not 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 (and it most certainly was not!), 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. Hans Karl Albert Winkler (1877–1945) 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 Karl Rechinger (1867–1952) in Vienna tried to culture stem sections and excised buds of *Populus nigra* and *Fraxinus*

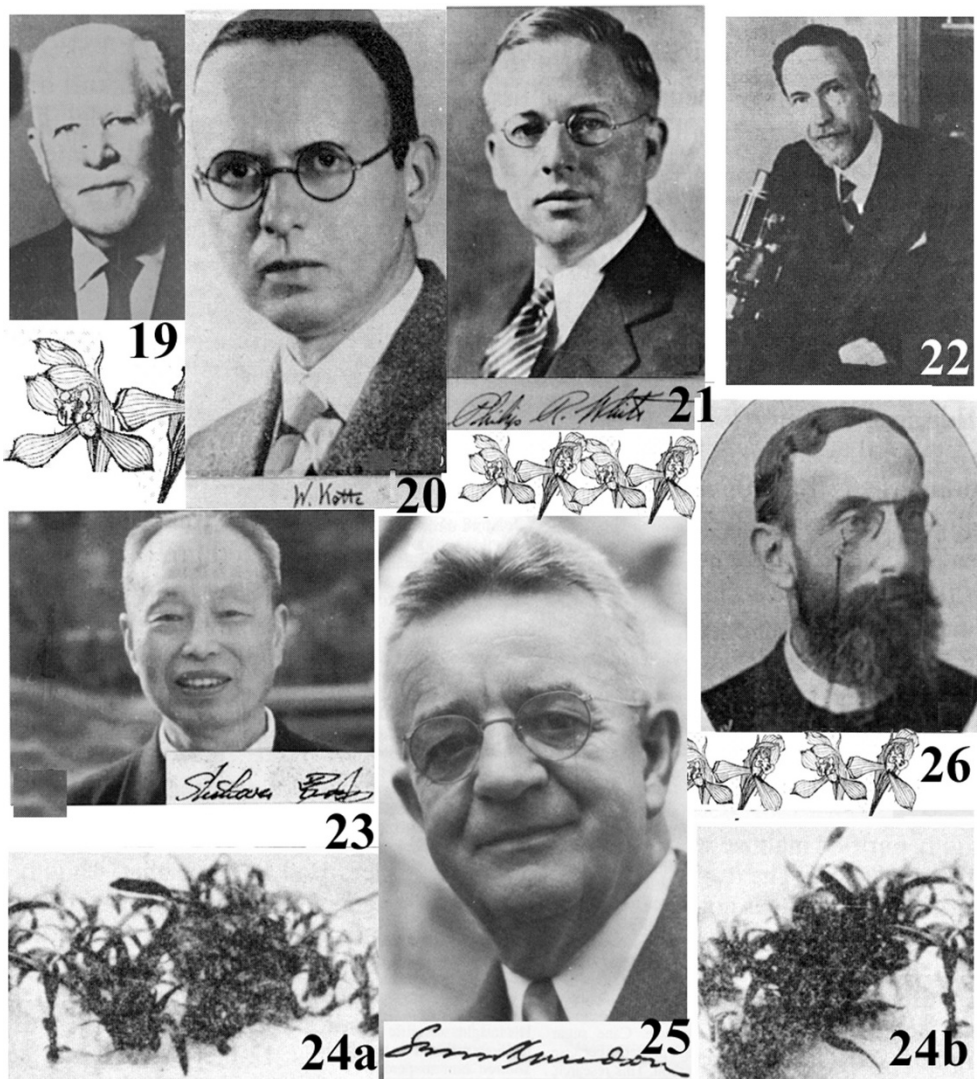
ornus as well as portions of roots on sand moistened with tap water (Rechinger, 1893; Krikorian, 1982; Gautheret, 1983). Rechinger failed in his attempts, but concluded that for proper development sections must be thicker than 1.5 mm. His procedures cannot be called "tissue culture" as the term is understood 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 or medium 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; Appendix 8; 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; Yam and Arditti, 2009; 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 today. 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).



FIGS. 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 Nobécort (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 J.A. which is now in the library of the Singapore Botanic Gardens). 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).**

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 "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. Medium 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.

Although not a vitamin (even if sometimes referred to as one), *myo*-inositol, 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 63 years 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. Still, it must be noted that some media which do not contain *myo*-inositol are effective.

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 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 about 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 around 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*; Morel seems to have had a tendency to claim firsts for himself regardless of whether his claims were justified). 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; he had a tendency to glorify his French compatriots) 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). True, the differences in the time of publication were only 9 and 29 days respectively but December, January and February are not "simultaneous" much as Roger Jean Gautheret (1910–1997) may have wanted them to be. 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*; Yam and Arditti, 2009).

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, 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 due to his indomitable spirit, but returned to his laboratory after the upheaval, resumed research, and trained graduate students until his last days (Arditti, 1999; J.A. was fortunate to meet him about 1986 and become his friend).

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 about 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 and claimants to be first without justification (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 watercress on White's mineral nutrients supplemented with sucrose $20\,g\,l^{-1}$ (w/v) and "hetero-auxin 1 part to 20 millions" (LaRue, 1936). It is interesting to note the level of auxin used by LaRue was much lower than what is added to media today.

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 also clearly stated by "Ernie" Ball in many conversations with J.A. 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 succeeded in culturing shoot tips before Georges Morel did. And, German nursery owner Hans Thomale and Dr. Lucie Mayer, not Georges Morel, were the first to culture an orchid shoot tip (Arditti and Krikorian, 1996). In fact it may be that Morel got the idea to culture orchid shoot tips after reading one of Thomale's writings.

First Micropropagation of Orchids

Nearly 125 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 orchid 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) whereas this report is in French, a language which lost its importance as an international medium of communication long ago; and (4) not many scientists take the time to read the old literature regardless of language and prominence (or lack of it) of journals.

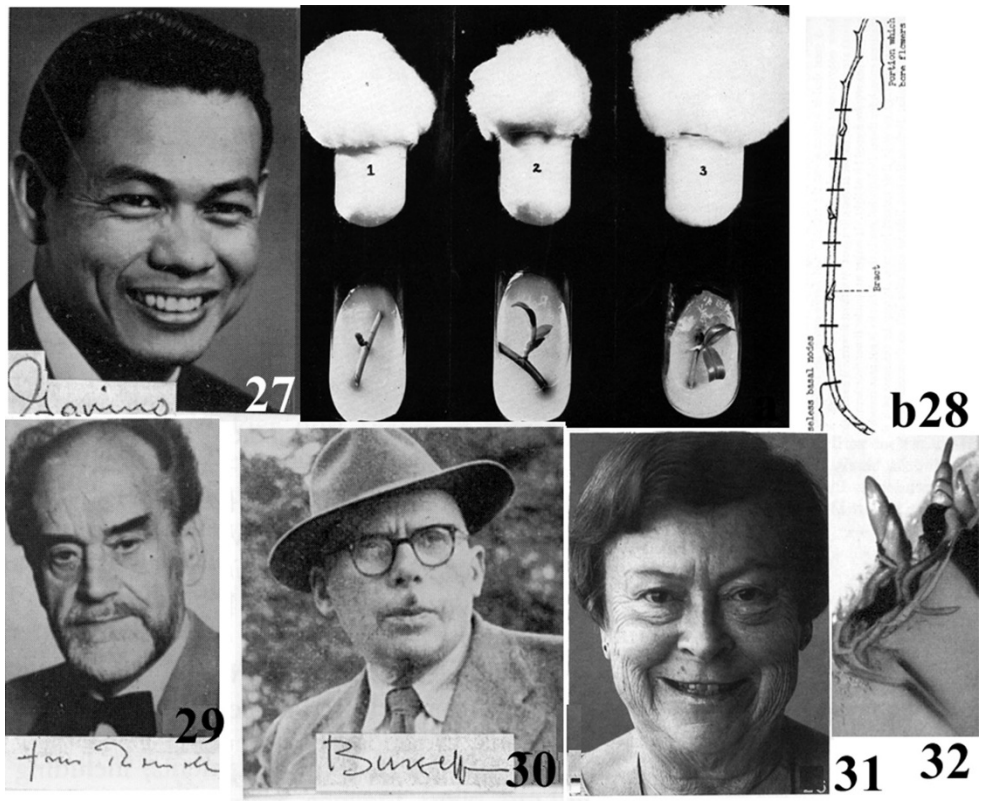
At least one person did notice the articles at the time because according to a short notice a grower named Perrenoud (no first name given) who saw reports in so-called "journaux anglais" placed sections of *Phalaenopsis* roots in humid enclosures and obtained a plant (Anonymous, 1891). This is reminiscent 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 prehistory of orchid micropropagation (Arditti and Krikorian, 1996; Yam and Arditti, 2009).

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 much more (1) relevant to tissue culture, (2) important as a "foreword," and (3) similar to micropropagation than the tree 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). Knudson C (KC), a medium 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) was used as a solution to culture the *Phalaenopsis* nodes.

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 (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 to J.A. by Dr. Rotor) and died Signal Mount, Tennessee on March 8, 2005. His mother, an orchid enthusiast, introduced him to her plants before he was 10 years old. By the time Gavino entered



FIGS. 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 J.A. which is now in the library of the Singapore Botanic Gardens 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 *Orchis maculata* (courtesy Hans Thomale obtained with the help of E. Lucke and Dr. Norbert Haas-von Schmude).**

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 led him to orchid 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 medium 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 (Rotor, 1949) 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 J.A. called his attention to it in response to a direct inquiry by him, perhaps because he was more interested in glorifying his countrymen than writing an unbiased historical account) 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 now and never were 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, not scientific, significance.

During the same period, 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* seedlings (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

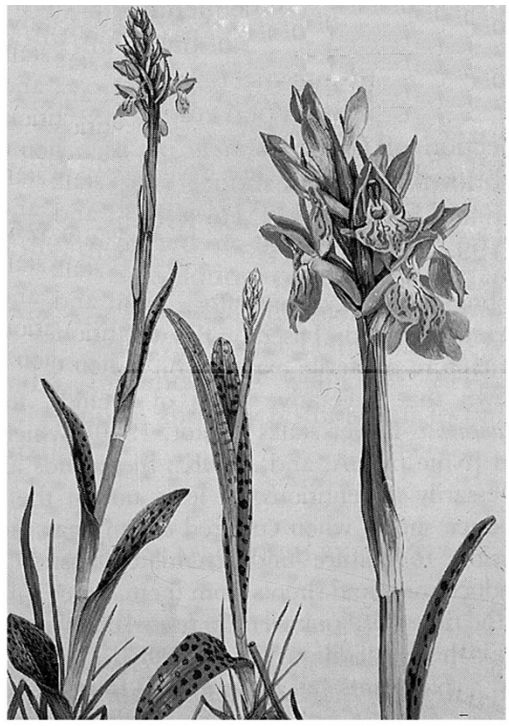
multiplication, and stated: "the practical ability to produce clonal lines of plants of potentially unlimited numbers would be of obvious value in many types of genetic and plant production work." However, there is a major difference between the drawing of attention to potential by Curtis and Nichol 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 first edition (Arditti and Ernst, 1993) withheld a number of facts and sugar coated others (to J.A.'s great and eternal displeasure). A review (Arditti and Krikorian, 1996), published after the first edition (Arditti and Ernst, 1993), more concerned with historical accuracy than with offending individuals, is uncompromisingly accurate. The same is true for a subsequent review (Yam and Arditti, 2009). As already mentioned, these reviews (Arditti and Krikorian, 1996; Yam and Arditti, 2009) served as the basis for a thorough revision of the history chapter of the second edition (Arditti, 2008) and the source of much information. This was done with permission from Professor Emeritus Abraham D. Krikorian (for which we are most grateful). The current chapter is a slightly modified version of the one in the second edition (Arditti, 2008).

Even before the availability of cytokinins (Skoog, 1944; Skoog and Tsui, 1948, 1951; White, 1951; Miller and Skoog, 1955) and the formulation of the MS (Murashige and Skoog, 1962) medium, 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 who later became a pharmacist, 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" (a letter from Thomale to J.A., which is now in the library of the Singapore Botanic Gardens). She became Mrs. Thomale and they parented two sons and a daughter.



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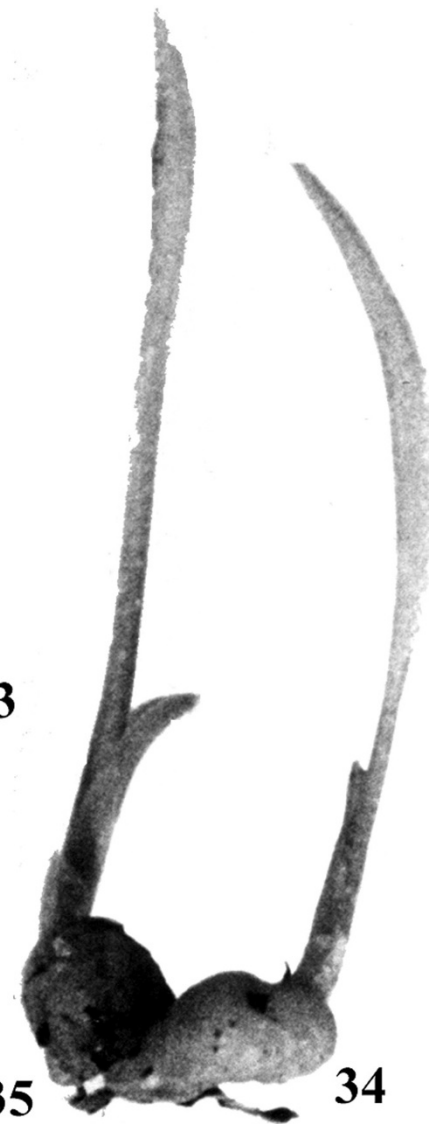
Nur der Vollständigkeit wegen sei darauf aufmerksam gemacht, daß der Wunsch nach einer Vermehrungsart europäischer Erdorchideen dazu führte, dies auf Grund einer Arbeit von Dr. L. Mayer*) durch Kultur von steril gewonnenen Teilstücken auf Agar-Nährböden zu versuchen, was auch gelingt.

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!

35



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FIGS. 1-33–1-35. ***Orchis maculata*** **and the work by Hans Thomale which led 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 the late 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).**

Thomale became interested in orchid seed germination, 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 the above-mentioned 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 and refused the offers. 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 on another plant 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):

> 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 by orchid growers and scientists. 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 on orchids (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). Thomale died on July 25, 2002.

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; Yam and Arditti, 2009), 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. A third reason is the extensive self-publicity by Morel. And, a fourth reason is Morel's many friends and admirers who perpetuated his claims and defended his unearned great reputation.

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 (known as Thomale GD) for this popular genus.

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) without divulging his request.

By the time Thomale was given any recognition (Arditti and Ernst, 1993; Haas-von Schmude et al., 1995; Arditti and Krikorian, 1996; Arditti, 2001; Yam and Arditti, 2009), essentially total credit for priority of discovery had been established for and by

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Tél. : 950 75-22

Références à rappeler :
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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
Kastanienwold 19
LEMGO
Lippe
(Allemagne)

Versailles, le 15 Décembre 1965

Dear Sir ,

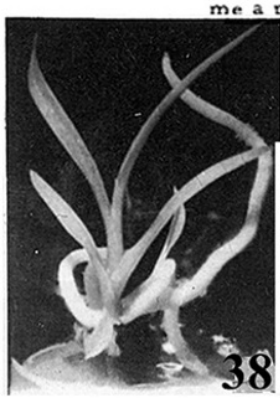
I have been asked by Dr. C. WITHNER to write a chapter on clonal propagation of Orchids for a new edition of his book .

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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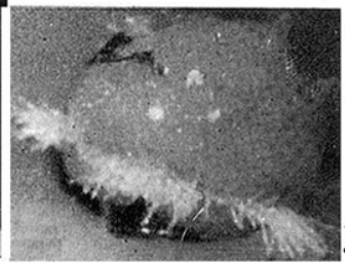

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FIGS. 1-36–1-38. **Correspondence and in vitro cultures by Dr. Georges Morel. 36. Letter from Dr. G. Morel to Mr. Hans Thomale requesting a copy of Fig. 1-32. This letter proves that Dr. Morel knew of Mr. Thomale's work long before he cited it (courtesy 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).**

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; Yam and Arditti, 2009).

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, in a letter to J.A. which is now in the library of the Singapore Botanic Gardens). Chauvinism, 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, but claimed for himself.

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

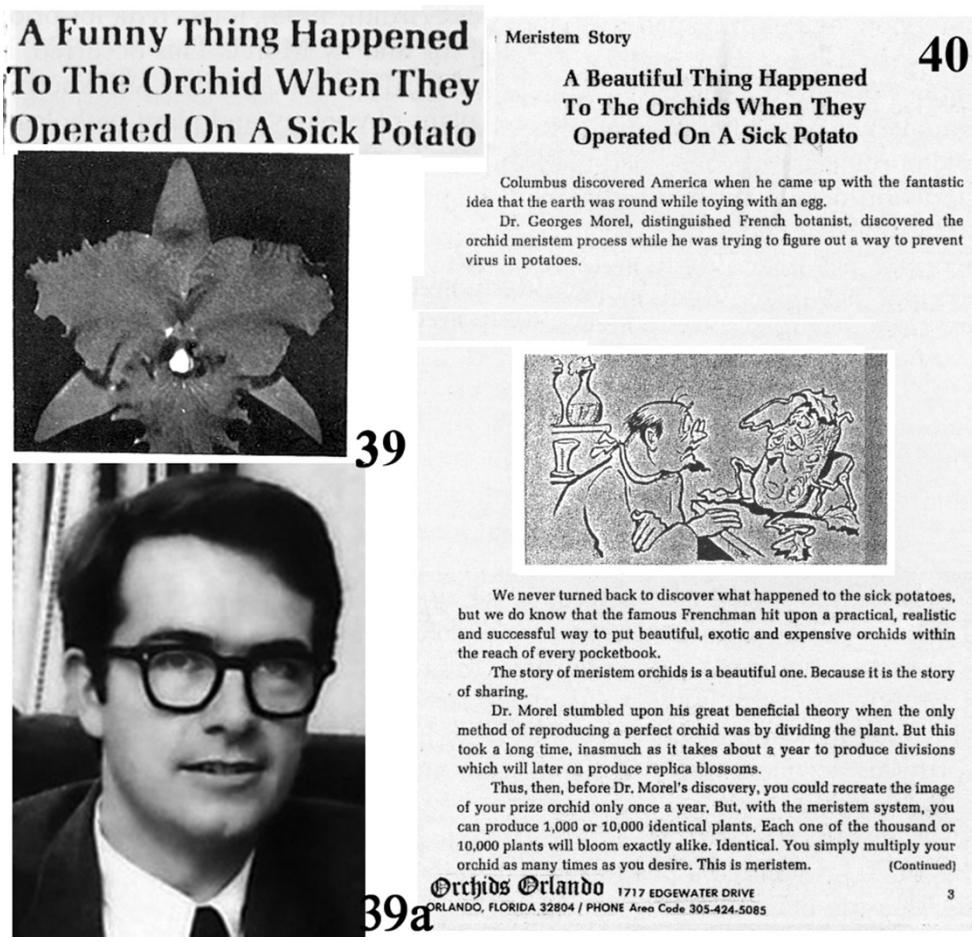
the internal-cork disease in sweet potato, *Ipomea batatas* (Holmes, 1956*a*), as well as aspermy virus (Holmes, 1956*b*) and other viruses (Brierley and Olson, 1956) in *Chrysanthemum*.

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 around 1950 was viral infection of certain potato and *Dahlia* cultivars which would have caused them to be abandoned (Lecoufle, 1974*a*, 1974*b*). Given 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* Alexanderi '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 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).



FIGS. 1-39–1-40. **Vacherot and Lecoufle 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); 39a. Dr. Walter Bertsch. 40. A page from the Orchids Orlando catalog which tells how Georges Morel came to shoot-tip cultures of orchids (Orchids Orlando, no date).**

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; Yam and Arditti, 2009). 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 and the one in the second edition (Arditti, 2008), like two extensive reviews (Arditti and Krikorian, 1996; Yam and Arditti, 2009) dispense 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 is true regarding 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 follow.

- Assertion by the originator of the Murashige and Skoog culture medium: "the potential of propagating orchids through tissue culture was observed first by Morel" (Murashige, 1974).
- Statement by the long-time and excellent chairman of the botany department at the University of Singapore and subsequently the National University of Singapore: "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).
- Pronouncement in an historical account by a "founding father" and self-appointed arbiter of plant tissue culture history (Gautheret, 1983, 1985): "the first application [of micropropagation] concerned the clonal propagation of orchids (Morel, 1960)".
- Sentence in short review by Croation scientists: "G. M. Morel from France began the meristem technology" (Jelaska et al., 2003).
- A review by a prominent expert in the field: "The person who began the meristem culture technology was Dr. Georges M. Morel (Morel, 1960)" (Gamborg, 2002).
- And, since such reviews are often restated, referred to, or quoted in other papers [see for example, "the potentials of tissue culturing for plant propagation … have been … reviewed by Murashige" (Langhans et al., 1977)], an historically incorrect "factoid" has been elevated to truth and dogma.

Once such a transformation happens (i.e., a factoid becomes a fact), the forces which usually resist knowledge tend to maintain the status quo and thus strive to support dogma (Gaffron, 1969). Such is the persistence, perseverance, pertinacity, doggedness, and tenacity (and unfortunately sometimes incomplete knowledge of the history of the subject) of those who believe in and perpetuate the "Morel discovered it" urban legend that it is being repeated (Gamborg, 2002) even after the publication of several correct versions of the history of orchid micropropagation (Arditti, 1984, 1985, 1992, 1999, 2001, 2008; Yam and Arditti, 1990, 2009; Arditti and Krikorian, 1996; Easton, 2001). That is why it has been necessary to publish the correct history several times in a number of forms in different journals, proceedings, and hobby magazines (Arditti, 1984, 1985, 1992, 1999, 2001, 2002, 2004, 2008; Yam and

Arditti, 1990, 2004, 2008, 2009; Arditti and Krikorian, 1996; Easton 2001). These efforts have had an effect. A recent review properly credited "orchid tissue culture starting from the pioneering work of Rotor" and "Wimber (1963) [who] published the first detailed protocol for in vitro production of *Cymbidium* starting with meristem culture" (Chugh et al., 2009).

The belief that Morel made the discovery has come to bear on and alter the history of orchid micropropagation. Attempts to question the accepted view led to sharp exchanges in the literature some years ago (Arditti, 1985 vs. Torrey, 1985*a*, 1985*b*). Editorial demands for changes in manuscripts (Arditti and Arditti, 1985; Lucke, 1994) had to be agreed to in the not so distant past. Even the history chapter in the first edition of *Micropropagation of Orchids* (Arditti and Ernst, 1993) had to be revised because of insistence and strong pressure by one of the authors because of his personal friendship with the owners of the Vacherot and Lecoufle orchid establishment. Only recently has it become possible to freely publish the correct history (Arditti, 1984, 1985, 1992, 1999, 2001, 2002, 2004, 2008; Yam and Arditti, 1990, 2004, 2008, 2009; Arditti and Krikorian, 1996; Easton 2001) without interference by adherents to the fiction that "The person who began the meristem culture technology was Dr. Georges M. Morel" (Gamborg, 2002).

Immediately after publication of the first edition of *Micropropagation of Orchids* (Arditti and Ernst, 1993) the accepted history was examined carefully (Arditti and Krikorian, 1996) for the sole purpose of placing historical facts in the most accurate perspective possible. This careful reexamination resulted in a review which combined parts that were removed from the first edition of *Micropropagation of Orchids* (Arditti and Ernst, 1993), previously published information by Professor Abraham D. Krikorian (Krikorian, 1982, 1988, 1989*a*, 1989*b*, 1994*a*, 1994*b*, 1995; Krikorian and Berquam, 1969) and facts newly discovered at the time (Arditti and Krikorian, 1996). This review served (word for word in places) as a basis of what was presented in the second edition of *Micropropagation of Orchids* (Arditti, 2008) and is outlined here (we thank Professor Emeritus A.D. Krikorian for permission to do so). 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 famed physicist Ernst Mach (1838–1916) as 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 and the one in the second edition (Arditti, 2008) may appear, to some at least, to be "unjust" only because many previous accounts have been imprecise enough to have done considerable violence 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. In fact, what is unjust is the attribution of the discovery to a person who did not make it and the lack of recognition of those who did.

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 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 (Jacquet, 2007). On returning to 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 at the time (Arditti, 1985; Torrey, 1985*a*, 1985*b*), and the late Howard A. Schneiderman (1927–1990), an entomologist and developmental biologist who held Morel in high regard (and told J.A. about it). Schneiderman became Dean of Biological Sciences at the University of California, Irvine (UCI) during the late 1960s and 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. In fact he was highly antagonistic.

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 without listing the full composition of the solution which was used. 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 of the Royal Botanic Gardens, Kew 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; Yam and Arditti, 2009 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, 1955*b*). It would have been impossible for anyone to repeat Morel's work because this article, such as it is, does 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 PLB 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.

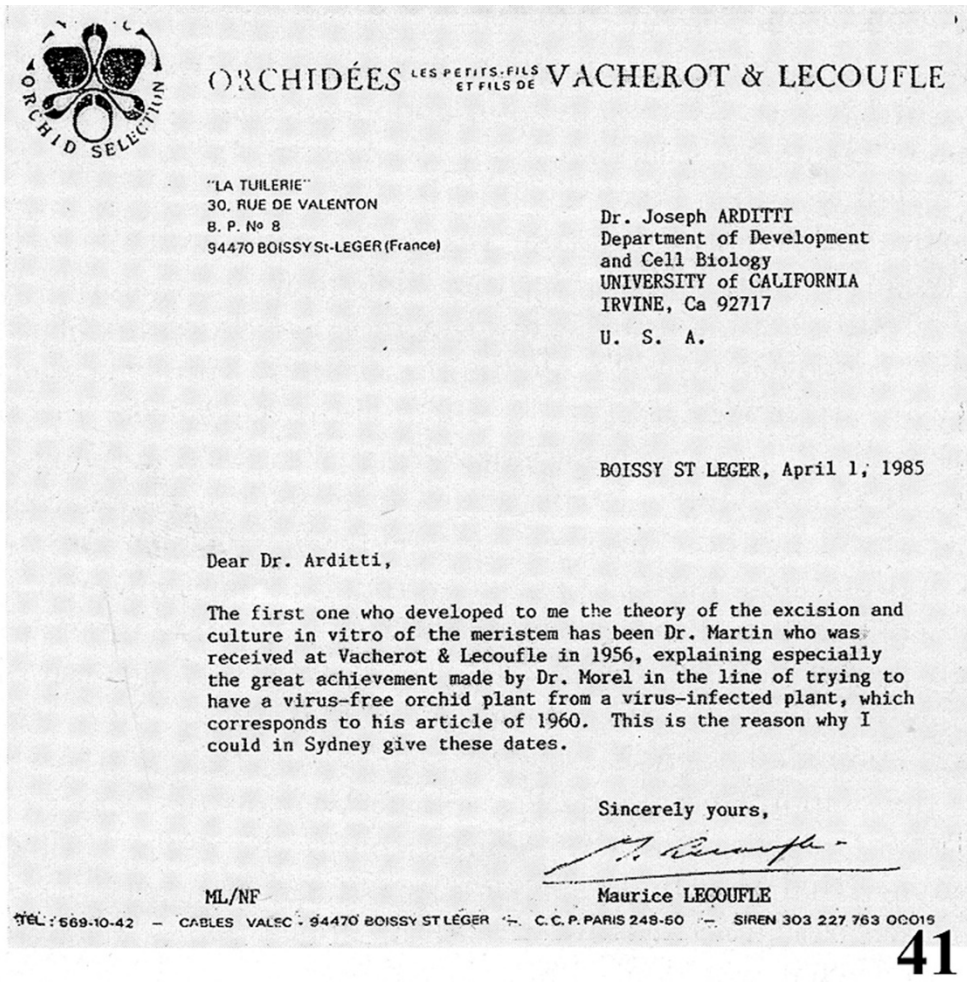
There is also a suggestion that some "meristem-cultured plants may mature more quickly than plants raised from seeds" (Lecoufle, 1967). One example is plantlets of *Odontonia* Boussole 'Blanche' and *Odontonia* Moliere 'Lanni' which were removed from their flasks on April 30, 1965 and "flowered ten to 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 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; this letter is now at the library of the Singapore Botanic Gardens).
3. A short conversation J.A. 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.



ORCHIDÉES LES PETITS-FILS ET FILS DE VACHEROT & LECOUFLE

"LA TUILERIE"
30, RUE DE VALENTON
B. P. Nº 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 VALEC - 94470 BOISSY ST LEGER — C.C.P. PARIS 248-50 — SIREN 303 227 763 00015

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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.**

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 PLB mentioned earlier and revealed attempts to extend the *Cymbidium* method to *Odontoglossum*, *Miltonia*, and *Phaius* (Morel, 1963). However he did not provide additional information about excision and culture medium or media and 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) and the reference to "Knop's" vs. "Knudson III" in the first orchid paper by Morel were intentional or inadvertent. What is very clear is a consistent practice of omitting critical information or presenting it incompletely and/or in a confusing manner.

Altogether the content (or lack of it) of the first and second papers (Morel, 1960, 1963) tend 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 KC 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) by a scientist of Morel's stature 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 (1923–2009) 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; Yam and Arditti, 2009).

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; Yam and Arditti, 2009).

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., 1974/1975), 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 ½ concentration of Knop solution, 10 drops of Berthelot solution (Morel, 1948)."

Basil Kassanis (1911–1985) 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 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 book 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 or Arditti, 2008). 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 KC 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*, 1965*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*, 2006; Arditti and Ernst, 1993; and the present book 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) 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 there was 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 in this century by statements that "It was here [the firm of Vacherot and Lecoufle], around the end of 1959 … an American student in botany … Walter Bertsch (Fig. 1-39a)… 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 led 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 most of the participants (Georges Morel, Walter Bertsch, Maurice Lecoufle and others) dead, and the current generation of V&L owners 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).

A recent exchange of e-mail messages involving Professor Joseph Arditti sheds additional light on the role of Dr. Walter Bertsch (Fig. 1-39a). It started with a message dated February 23 from Yanlo Yue: "My name is Yanlo and my partner is Tim Bertsch … [who] lived with his father [Walter Bertsch] and mother (Susan) when Walter worked for Vacherot and Lecoufle … If you're interested, Tim and Susan will be happy to discuss with you their knowledge of what happened during that time." The reply to Yanlo (an enthusiastic "yes") elicited two messages. One was from Dr. Tim Bertsch: "I can't personally shed much light on this, although as a young child I do remember spending quite some time at V&L's place outside Paris with my father. This was after the events anyway – I was born in 1963 in Tennessee. However I have forwarded these e-mails to my mother, who was working for V&L at the time … If anyone remembers the gossip and events from then it will be her – she is 75 or so now."

Susan (Bertsch at the time and now Moody) replied quickly and provided interesting information:

> Your e-mail to Yanlo Yue was passed on to me. Murky and unsubstantiated claims notwithstanding, here is what happened, to the very best of my remembrance. I was working for French orchid growers Vacherot & Lecoufle, who were based in Boissy-St. Leger. In 1960 [probably mid to late 1950s] they were visited by Dr. Walter Bertsch, a young post graduate biologist working at the Cyclotron in Gif-sur Yvette … He had recently read about Professor Morel's experiments in cloning potatoes and was convinced the same could be done with orchids. With the help of Maurice Lecoufle and Michel Vacherot, and the laboratory facilities at V&L, he eventually succeeded in propagating tiny sections of orchid roots … Though by this time, other orchid growers were interested, Dr. Bertsch, as a trained biologist was able to propel V&L in the very front of cloning, and set them some years ahead of rival commercial orchid growers. Claiming the contrary was spurious. I was there and they were exciting times.

In a second e-mail Ms. Moody (formerly Bertsch) wrote: "With regards to cloning potatoes being adapted to orchids, I have no recollection at all of Prof. Morel coming to V&L to work with Lecoufle, Vacherot or Bertsch. I am sure that it was Walter who got the idea: I can remember his enthusiasm for the possibility and the new world of

meristemming it would open up." As to the source of explants, Ms. Moody wrote: "I am not sure of the difference between roots and shoots. It was the white bits hanging down that they cut the tip of, and then cut the tip into tiny segments."

And so, while shedding some light on the mystery of who developed shoot-tip cultures for V&L, Ms. Moody left one question not fully answered and created a new one. There is some evidence that Professor Morel and at least one of his associates were involved with V&L (Figs 1-30, 1-40 and 1-45, and texts associated with them), but Ms. Moody's message implies that they were not. All evidence to date suggests with considerable certainty that shoot tips were the explants which were cultured at the time. Ms. Moody's message raises new questions for which there are no answers: Were root tips cultured? If so, were they the only explant or were both shoot and root tips cultured? What was/were the medium/media used?

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 by 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 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 questions: "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 in a letter which is now in the library of the Singapore Botanic Gardens), never did write. Thus, questions still remain. The best service Morel's admirers can provide to his memory is to set the record straight openly and clearly.

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

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 question 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 and perhaps shy man.

The only point left to consider at this stage 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) had independently discovered shoot-tip cultures (Figs 1-48 and 1-49), published a detailed scientific paper, 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 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;

FIGS. 1-46–1-51. **Orchid micropropagation, people, and medium components. 46. Professor Donald E. Wimber (photographed in 1988 in Hiroshima by J.A., signature from a letter to J.A. which is now in the library of the Singapore Botanic Gardens). 47. Samuel Mosher, founder-owner of the Dos 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 J.A.). 51. Electron microscope photographs of charcoal (courtesy the late Dr. Maureen Weatherhead).**

Thomale, 1956, 1957) before Morel formulated his own by modifying existing ones. Several explant types (shoot tips, buds and nodes) from monocotyledonous plants in general (Robbins, 1922*a*; 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 PLBs 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 some 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 *Nasturtium 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). It should be noted here that Professor Arditti knew Morel and had short conversations with him in Los Angeles, California, Sydney, Australia and Hong Kong.

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 manage it and study orchid chromosomes.

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. Dr. Wimber 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 J.A. on December 13, 1976 (this letter and additional correspondence between J.A. and other orchid scientists are now part of the Joseph and Jonathan Arditti orchid reprint and documents collection at the Singapore Botanic Gardens).

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. After that 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. PLBs developed at the bases of the embryonic leaves and along the thin sections.

When some of the PLBs 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* (Figs 1-48 and 1-49), but the similarity ends there. Wimber followed standard scientific practice and provided full procedural details, included 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. He also cited all those whose techniques, media and research he used and/or benefited from.

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 KC medium 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; his dissertation 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 (all of which he failed to cite initially).

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 seedling 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 deserved. 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 J.A. (the letter is at the Singapore Botanic Gardens), 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 J.A. 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 the following.

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.
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 explants.
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; and (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 remember 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 mater (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 known and/or available. It is entirely possible that Knudson could have been successful in culturing these cells if he had vitamins, auxins, and cytokinins. 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 (1859–1924) 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 (1910–1996), 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., 1972*b*). *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 reviews see Churchill et al., 1973;

Arditti and Ernst, 1993; Arditti and Krikorian, 1996; Arditti, 2008; Yam and Arditti, 2009) 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 Miczycski, 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; see Chapter 3 for the latest information) 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, 2000*b*), *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 (see Chapter 3).

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 (non-orchidaceous) 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 PLBs 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 PLBs 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 PLBs 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 led to questions following their initial 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, 1979*b*), *Cymbidium* (Gopalan et al., 1992; Pindel and Miczycski, 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; Arditti and Ernst, 1993; Arditti and and Krikorian, 1996; Arditti, 2008; Yam and Arditti, 2009).

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 (for reviews see Arditti, 1977*a*, 1977*b*, 1978; Rao, 1977; Zimmer, 1978, 1980; Fast, 1979; Arditti and Goh, 1981; Czerevczenko and Kushnir, 1986; Arditti and Ernst, 1993; Arditti and and Krikorian, 1996; Arditti, 2008; Yam and Arditti, 2009).

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 Prefectural 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 (for reviews see Arditti, 1977*a*, 1977*b*, 1978; Rao, 1977; Zimmer, 1978, 1980; Fast, 1979; Arditti and Goh, 1981; Czerevczenko and Kushnir, 1986; Arditti and Ernst, 1993; Arditti and and Krikorian, 1996; Arditti, 2008; Yam and Arditti, 2009).

Inflorescences

In anointing "fathers" and giving credit 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; Park et al., 2002; for reviews see Arditti, 1977*a*, 1977*b*, 1978; Rao, 1977; Zimmer, 1978, 1980; Fast, 1979; Arditti and Goh, 1981; Czerevczenko and Kushnir, 1986; Arditti and Ernst, 1993; Arditti and and Krikorian, 1996; Arditti, 2008; Yam and Arditti, 2009).

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 J.A.'s undergraduate school professors; 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 constituted 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 attorneys, 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 Ernst, 1993; Arditti and Krikorian, 1996; Arditti, 2008; Arditti and Yam, 2009) 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, 1993). 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, Tennessee (the firm no longer exists) until about 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 around 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 [the McDades and their staff]. 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 (Easton, 2001) 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 who were not plant scientists (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 any plant science training) "made the first meristem cultures in 1950." And the second account (Easton, 2001) 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 (Easton, 2001) 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." These claims boggle the mind.

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 (Easton, 2001) "In November 1953, Everest was asked by Gordon Dillon (1912–1982), 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 earlier invited article which describes a revolutionary process would remain unpublished. Second, there are many orchid publications for growers, 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 the 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 on the island of Jersey 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. J.A. 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 visit by J.A., but never did. On a third visit J.A. 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 unethical 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 offspring 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; J.A. 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 (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 over-proliferated 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 over-proliferating 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 over-proliferation 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 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 Werkmeister (d. 1980) 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 (despite several searches we could not obtain a likeness and biographical information about him).

Robert Ernst (1916–2009; see Fig. 1-55), a surfactant chemist and manufacturer and Adjunct Professor of Biology at the University of California, Irvine, was the first to add charcoal to a practical seedling culture medium not long after P. Werkmeister

published his papers 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 reviews see Yam et al., 1990; Arditti and Ernst, 1993; Arditti and Krikorian, 1996; Arditti, 2008; Yam and Arditti, 2009).

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 in our laboratory, 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 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 and Appendix 8), may also prove useful.

KC 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 conditions is not more complicated than autoclaving the KC medium.

FIGS. 1-52–1-54. **52. Sharon Spencer (A) and Katie Thurston (B) on their graduation days (source: J.A.). 53. Jan Cooper on her wedding day (source: J.A.). 54. Marilyn Cvitanik (A) in New York and Kathy Hills (B) in Irvine [sources: (B) J.A.].**

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, 1919*b*) 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.

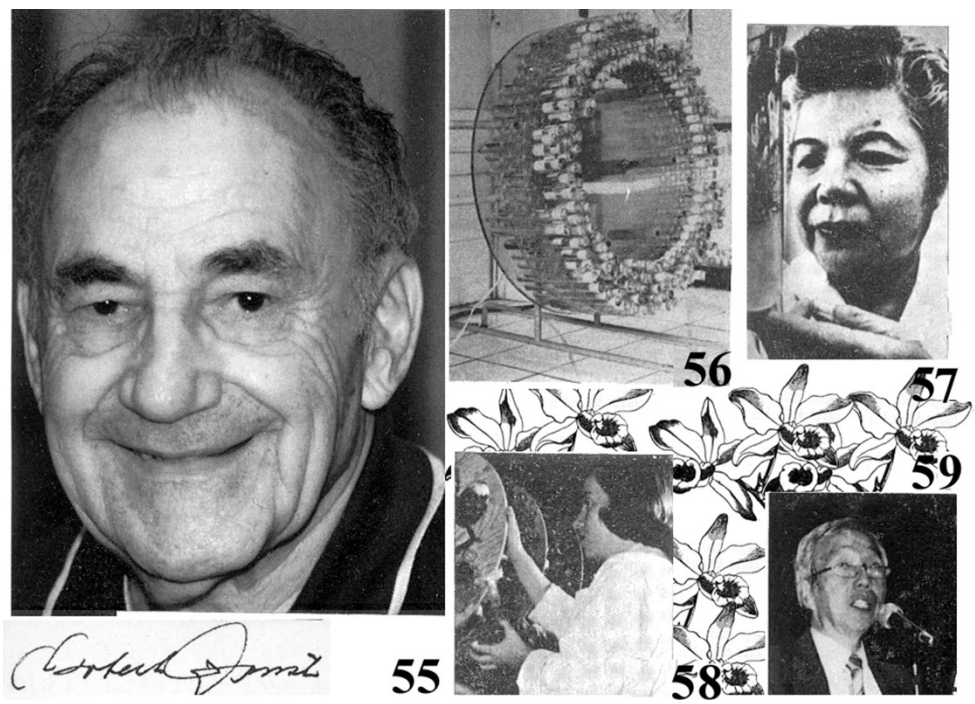
Knudson's experiments suggested the diffusion from roots of growth substances which the cells required, but this research was carried out (1) 8 years before the discovery of auxins (Went, 1928, 1990; Thimann, 1980); (2) about 20 years before it was demonstrated that vitamin B_1, niacin, and other factors 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 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 PLBs 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 (1913–1981; 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; Arditti, 2008; Yam and Arditti, 2009). *Cymbidium*



FIGS. 1-55–1-59. **Orchid tissue culture specialists and apparatus. 55. The late Professor Robert Ernst (photograph by J.A., signature from J.A.'s files which are now in the library of the Singapore Botanic Gardens). 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. The late Professor Yoneo Sagawa (Nagoya International Orchid Conference).**

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*). One suggestion is that the protoplasts were not isolated, fusion never took place, and the reports were erroneous. 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, 1986, 1989*a*, 1989*b*, 1990; Yasugi et al., 1986; 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; for reviews see Arditti and Krikorian, 1996; Arditti, 2008; Yam and Arditti, 2009).

Twenty years ago, 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 was also carried out by Professor Syoichi Ichihasi in Japan.

A direct electrical current pulse of 2500 V cm^{-1} of 2 ms 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 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 (for reviews and photographs see Arditti, 1984, 1990, 1992). This was in 1930. It may well have been one of the first reports of flowering in vitro of any plant. 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* (Figs 1-61, 1-62 and 1-63) at the Shanghai Institute of Plant Physiology (Wang, 1984, 1986, 1988*a*, 1988*b*, no date *a*, no date *b*; Wang et al., 1981, 1988; Wu Wang and Lin, 1987). Clonally propagated *Oncidium varicosum* was reported to flower in Brazil at the same time (Kerbauy, 1984). Next to flower were plantlets of *Phalaenopsis* hybrids in Japan (Tanaka et al., 1988*b*; Duan and Yazawa, 1994*a*, 1994*b*, 1994*c*, 1995*a*, 1995*b*). *Cymbidium ensifolium*, *Cymbium gyokuchin*, *Cymbidium kanran*, 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*). *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 free of worthwhile scientific content and devoid of 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 was prohibited (strange as it may appear this was indeed the case). 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 on the basis of the early and fragmentary information was that flowering of orchids in vitro could be spontaneous or induced by the state of the explant, nutritional conditions, medium components, intangibles and/or by environmental factors (Chia et al., 1999). And, indeed, transition of shoot apical meristems of seedlings of self-pollinated *Dendrobium* Madame Thong-In to inflorescence

FIGS. 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 J.A. which is now in the library of the Singapore Botanic Gardens). 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.**

primordia occurred in liquid modified KC medium (Sim et al., 2007). It did not take place on solid medium (Sim et al., 2007). Flowers were deformed in liquid modified KC. They developed normally when cultured on a two-layered system of liquid modified KC solution containing benzyladenine 22.2 $\mu mol\,l^{-1}$ poured over Gelrite solidified medium with or without the same level of benzyladenine (Sim et al., 2007).

Plantlets produced through micropropagation usually respond to culture conditions and media in the same manner as seedlings. Therefore it is reasonable to assume that at least some *Dendrobium* plantlets produced through tissue culture may also flower on liquid KC.

Reports which suggested that cytokinins, more specifically benzyladenine, N^6-(2-isopentenyl)adenine and thidiazuron, can induce flowering (for reviews see Chia et al., 1999; Chang and Chang, 2003) probably led to the research with *Dendrobium* Madame Thong-In (Sim et al., 2007). Use of benzyladenine 22.2 μmol l^{-1} to induce flowering of *Dendrobium* Chao Praya Smile (Hee et al., 2007) may also have been suggested by these reports. These flowers were pollinated and produced seeds which were germinated and produced seedlings that were germinated "and induced to flower in vitro again using the same procedure" (Hee et al., 2007). Further research (Sim et al., 2008) showed that flowering in *Dendrobium* Madame Thong-In seedlings is associated with increased endogenous levels of two cytokinins, N^6-(Δ^2-isopentenyl) adenine and N^6-(Δ^2-isopentenyl)adenosine.

Seedlings of other *Dendrobium* species were also induced to flower by benzyladenine. Seedlings of *Dendrobium lindleyi* (4–6 months old) with four to six leaves flowered in vitro on a medium containing benzyladenine 2 mg l^{-1} (8.9 μmol l^{-1}) plus picloram 0.5 mg l^{-1}, as well as on a medium containing benzyladenine 3 mg l^{-1} (13.3 μmol l^{-1}), naphthaleneacetic acid (NAA) 1.0 mg l^{-1} and kinetin 1.5 mg l^{-1} (7 μmol l^{-1}), a total cytokinin concentration of 20.3 μmol l^{-1}. Seedlings of *Dendrobium tortile* of the same age and with as many leaves flowered in vitro on similar media: (1) benzyladenine 3 mg l^{-1} (13.3 μmol l^{-1}), NAA 1.0 mg l^{-1} and kinetin 1.5 mg l^{-1} (7 μmol l^{-1}), a total cytokinin concentration of 20.3 μmol l^{-1}; and (2) benzyladenine 2 mg l^{-1} (8.9 μmol l^{-1}) plus NAA 0.1 mg l^{-1} (Bhadra and Bhowmik, 2005).

There is also a report that several months old seedlings with three to four leaves of another orchid, *Geodorum densiflorum*, on a medium containing benzyladenine 2.0–2.5 mg l^{-1} (8.9–13.3 μmol l^{-1}), NAA 1.0 mg l^{-1} and 0.1% activated charcoal started to flower after 3–4 months of culture (Bhadra and Hossain, 2003*a*).

A more recent review concluded on the basis of many studies and orchid species and hybrids that the factor which induces flowering in vitro "remains unclear" despite research which points to cytokinins, gibberellins, hormonal control, irradiance, reduced nitrogenous compounds, photoperiods, sucrose, and temperature (Teixeira da Silva et al., 2014). This review concluded (and thereby supported previous suggestions) "that no evident trend exists" and suggested "that many avenues of research are still available for exploration" and that "determined tissue culture scientists" will induce in vitro flowering and make possible the commercialization of an "in vitro bouquet" (Teixira da Silva et al., 2014). Along the way scientists will also discover the factors which induce and affect flowering in vitro.

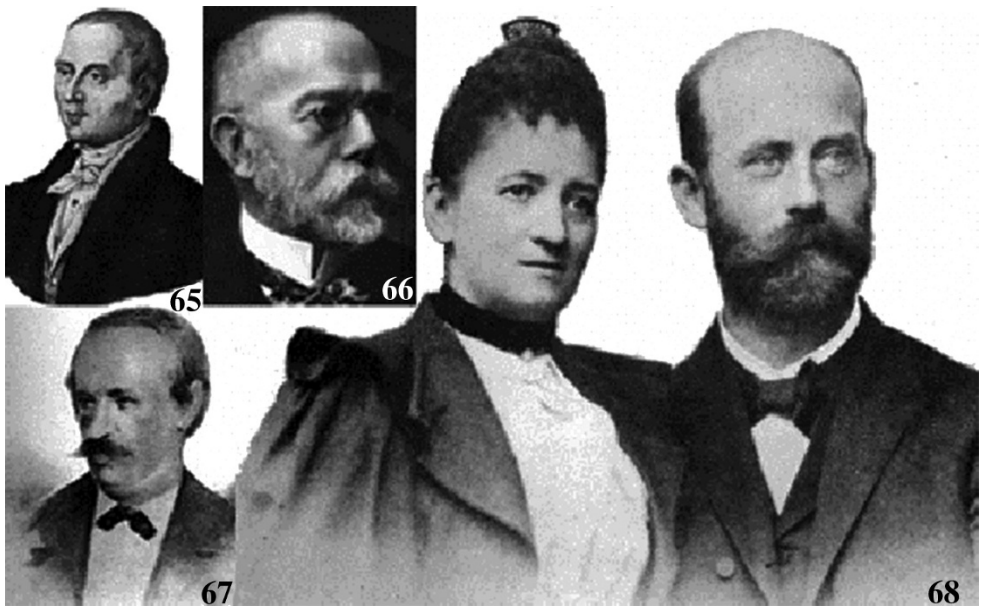
A method which can routinely induce flowering of orchids in vitro could prove to be of commercial importance. The findings with *Dendrobium* Madame Thong-In (Sim et al., 2005, 2008), *Dendrobium* Chao Praya Smile (Hee et al., 2007), *Dendrobium lindleyi*, *Dendrobium tortile* (Bhadra and Bhowmik, 2005), and *Geodorum densiflorum* (Bhadra and Hossain, 2003) could lead to the development of such a method. Guek Eng Sim may have carried out her research in the mid 1990s. It is a pity that enough information was not published in 1996 and that not all investigators were credited in the self-aggrandizing earlier article (Goh, 1996).

Solidifying Agents

Solidifiers are very important components of micropropagation media, but their history has not been reviewed in detail until recently when an excellent review (Cameron, 2008) called attention to many interesting but neglected facts. This section is based heavily on this review by Dr. Stewart I. Cameron (who is based at the Atlantic Forestry Center of the Canadian Forest Service in Fredericton, Canada) with his permission.

Microbiologists were the first to use solid or semisolid media in their work. Pietro (Pier) Antonio Micheli (1679–1737; Fig. 1-65) investigated fungal development as early as 1718 by brushing fungal spores onto freshly cut melons, pears and quinces and then transferring his fungi to new sections (Cameron, 2008). Bartolomeo Bizio (1791–1862), a botanist intent on finding the cause of "bloody bread" isolated *Serratia marcescens* in 1819 by using corn porridge as a medium (Hitchens and Leikind, 1939; Cameron 2008). He also studied "blood spots" on communion wafers in 1832 and isolated the same microorganism (Smith, 2008). In 1872 Joseph Shroeter (1837–1894) isolated discrete colonies of chromogenic bacteria on slices of cooked potatoes (Cameron, 2008) and also experimented with coagulated egg whites, flour paste, and meat (Hitchens and Leikind, 1939; Cameron, 2008).

Robert Koch (1843–1910; Fig. 1-66), of Koch's postulates fame, also used Schroeter's potato slices soaked in corrosive sublimate. Koch also used gelatin to thicken broth (Cameron, 2008). Gelatin was first used to solidify broth by Carlo Vittadini (1800–1865; Fig. 1-67) in 1852 (Hitchens and Leikind, 1939; Tseng, 1944). It has been suggested that the botanist and mycologist Julius Oscar Brefeld (1839–1925) proposed the use of



FIGS. 1-65–1-68. **Investigators who introduced solid supports into microbiology. 65. Pietro (Pier) Antonio Micheli (http://www.webalice.it/mondellix/images/Micheli.jpg). 66. Robert Koch (http://en.wikipedia.org/wiki/Robert_Koch). 67. Carlo Vittadini (http://en.wikipedia.org/wiki/Carlo_Vittadini). 68. Fanny Angelina "Lina" and Walther Hesse.**

gelatin to Koch. Gelatin, obtained from animal collagen, is readily available, easy to sterilize, transparent, and solid. However, it is solid only at temperatures of 20–25 °C and liquefies at 30 °C. Also, it can be degraded and liquefied by some microorganisms (Cameron, 2008). Despite being useful, gelatin is not an ideal solidifier. A different solidifier was needed and chance provided one in 1881–1882 (Hitchens and Leikind, 1939; Hesse, 1992; Anonymous, 2008*a*, 2008*b*; Cameron, 2008; Santos, -2008).

Agar

An ideal solidifier for microbiology and tissue culture must be non-toxic to the organisms and tissue cultured on it, resistant to destruction and liquefaction by microorganisms, and have a relatively low gelling temperature and high melting point. Agar gels at 30–50 °C and melts at 82–92 °C (Cameron 2008), is non-toxic and is resistant to degradation by organisms and tissue, and is thus an ideal solidifier. Serendipity introduced it into science (Hitchens and Leikind, 1939; Hesse, 1992; Anonymous, 2008*a*, 2008*b*; Cameron, 2008; Santos, 2008).

Walther Hesse

Despite being the descendant of a Saxon family from Bischofswerda and of two surgeons, one of them a military surgeon and the other a graduate of the Surgico-Medical Academy in Dresden, Friedrich Wilhelm Hesse was the first university-trained physician in the family. He received his medical degree from the University of Leipzig in 1842. Dr. Hesse was the *Bezikartzt* (country physician) in Zittau, a city in southeast Saxony, Germany in a district close to the current borders of Germany, the Czech Republic and Poland. He and his wife (she came from a cloth weaving family) had 12 children, 10 of whom (five sons and five daughters) survived to adulthood (Hesse, 1992). The daughters were all sent to a teacher's college with the idea of making them independent. Four of the brothers went into medical professions. Richard Hesse moved to Brooklyn and was a practicing physician there. Friedrich Louis Hesse became a dentist, came to the USA for 3 years to specialize, and founded the first university chair in dentistry in Leipzig, Germany. Georg Hesse was a surgeon and a director of a private hospital in Dresden (Hesse, 1992).

The third child, Walther, was born on December 27, 1846. He attended the *Kreuzschule Gymnasium* (high school), started to study medicine in 1866 at the University of Leipzig, and received his doctorate in 1870 (Hesse, 1992). After that he served as *Feldassistenarzt* or second lieutenant in the Saxon army and as such was a participant in the Franco-Prussian war from 1870 until 1871. He remained in the service until 1873 and also served as a staff physician in a private insane asylum and ship surgeon on a German passenger liner. In the latter capacity he visited New York several times. While there his brother Richard introduced him to the Eilshemius family.

Fanny Angelina "Lina" Eilshemius

Heinrich (Anglicized to Henry) Gottfried Eilshemius came from a family of Dutch descent which originated near the city of Emden in Frisia, Germany. At the age of 24 he immigrated to the USA in 1842 (Hesse, 1992) or 1818 (Hitchens and

Leikind, 1939), settled in Hoboken, New Jersey and in 1849 married Cecile Elise Robert, daughter of a French Swiss family from Lugano. Henry became so successful as an importer that he could retire at the age of 40. In 1860 he bought Laurel Hill Manor, a 70 acre property near the Passaic River in North Arlington, New Jersey. He and his wife had 10 children, but only five of them survived. His eldest daughter Fanny Angelina "Lina" Eilshemius was born in 1850 (Hitchens and Leikind, 1939), grew up and learned how to cook. At the age of 15 she was sent to finishing school in Switzerland. In 1872 (Hesse, 1992) or 1874 (Hitchens and Leikind, 1939), Fanny and her sister visited Germany where she met Walther Hesse again.

Walther and Fanny Angelina "Lina" Hesse and the introduction of agar into microbiology

Walter Hesse and Fanny Eilshemius (Fig. 1-68) were married on May 16, 1874[1] and settled in Zittau where Dr. Hesse practiced medicine and their first son, Friedrich Henry Hesse, was born in 1875. In 1877 Dr. Hesse was appointed county physician in Schwarzenberg im Erzgebirge which is located between Saxony and Czechoslovakia where he was responsible for 83 villages in a major mining area (Hesse, 1992). Many of the miners suffered from *Bergkrankheit* (mountain disease) which was later found to be lung cancer. Uranium and radium in the mines caused it, but this was not known at the time. Arsenic was considered to be the cause. Hesse wrote a comprehensive paper about the disease. He did not limit himself to being just a county physician and was also concerned with working conditions, child labor, environmental hygiene, public health, human environment, air quality, carbon dioxide levels, dust, and smallpox vaccinations (Hitchens and Leikind, 1939; Hesse, 1992). These interests led him to microbiology and a visit to Robert Koch's laboratory in 1881–1882 (Hitchens and Leikind, 1939; Hesse, 1992).

In many ways Lina Hesse was ahead of her time. Like a dutiful wife of that era she kept house and raised the children, but she did not limit herself to being a *Hausfrau* (Hitchens and Leikind, 1939). She familiarized herself with her husband's science and became his laboratory assistant as well as his scientific illustrator (Hitchens and Leikind, 1939; Hesse, 1992). A whimsical description of her role was that "she cooked not only the soup for her family but also bouillon for her husband's bacteria" (Hitchens and Leikind, 1939). But she was far more than that. She provided support, encouragement, love, respect, and a suggestion which made history (Hitchens and Leikind, 1939; Tseng, 1944; Hesse, 1992; Anonymous 2008*a*, 2008*b*; Cameron, 2008; Santos 2008).

Temperatures during the Schwarzenberg and Dresden summers were such that the gelatin used by Walther Hesse for his cultures melted. In his frustration he asked his wife why her puddings and jellies remained solid in these temperatures whereas his cultures did not. Her reply was "agar-agar" (Hesse, 1992). She learned about it from her mother who obtained the information from Dutch friends (Hesse, 1992),

[1] For those interested in coincidences: Joseph Arditti's son, Jonathan Omar Arditti, received his B.A. in psychology from the University of Southern California in Los Angeles (the university which granted his father a doctorate in 1965) on May 16, 2008.

or friends (Hitchens and Leikind, 1939) who immigrated to New York from Java, Indonesia which was a Dutch colony at the time (these immigrants could have been Dutch or Indonesians). Agar-agar is used in Indonesia to this very day as a thickening agent for cooking and the preparation of confectionery.

Walther started to use agar instead of gelatin and so did Koch. That is how agar was introduced into microbiology. And this raises a question and creates a mystery which is stated very well by Dr. Stewart I. Cameron, who is quoted here verbatim with his permission:

> It has been speculated that the agar-agar used by the Hesse, Koch and others actually may not have been agar at all. Tseng (1944) has suggested that true agar was a rare commodity (discovered in Japan in 1658, and produced only there as a foodstuff called "kanten") from *Gelidium amansii* in the 1800's (Armisen 1995), and probably not readily available for cooking in Europe. "Agar-agar" (the Malay name for jelly), is extracted from edible red algae, in particular the species *Eucheuma spinosum* which was more widely available than the Japanese agar. The gel produced by *E. spinosum* "is one of the carrageenans (the *iota*-form), which are related to agar but differ chemically" (Cameron, 2008).

Unless a sample of Hesse's agar-agar can be found and analyzed we may never know.

Noël Bernard introduced the use of agar with orchids in his experiments with fungi in orchid seed germination (Bernard, 1902, 1909; Cameron 2008). Lewis Knudson used it first in his work on the toxicity of galactose on pea and vetch germination (Knudson, 1915) and later in the development of an asymbiotic medium for orchid seed germination (Knudson, 1921, 1922). Gavino Rotor (1949), not Georges Morel (1960), was the first to use agar in orchid micropropagation (for reviews see Arditti and Krikorian, 1996; Arditti, 2008; Yam and Arditti, 2009).

Gellan Gum

Agar is not the only solidifier which can be used in tissue culture (Cameron, 2008). However, for all practical purposes, the only reasonable alternative or subtitute for agar is gellan gum (see also Chapters 2 and 4).

Bacteria produce protective coatings known as capsular heteropolysaccharides (Cameron, 2008). The Kelco Division of Merck, a pioneer in the development of bacterial polysaccharides, has isolated many gum-forming bacteria. Gums produced by these bacteria are of scientific interest, but most have no application value. One notable exception is gellan gum, a gelling polysaccharide that can be used in a variety of commercial products, microbiology, and tissue culture. Gellan gum is a bacterial exopolysaccharide produced by *Sphingomonas elodea* (previously called *Pseudomonas elodea*). The monosaccharide building units of gellan gum are glucose, glucuronic acid, and rhamnose in the molar ratio 2 : 1 : 1. The primary structure is a tetrasaccharide repeating unit. There are approximately one and a half *O*-acyl groups per repeating unit. Originally the *O*-acyl substituent was thought to be *O*-acetyl. Thus the various forms of gellan gum are referred to as high-acyl and low-acyl (Fig. 1-69). Recent studies suggest that gellan gum contains both *O*-acetyl and *O*-L-glyceryl substituents on

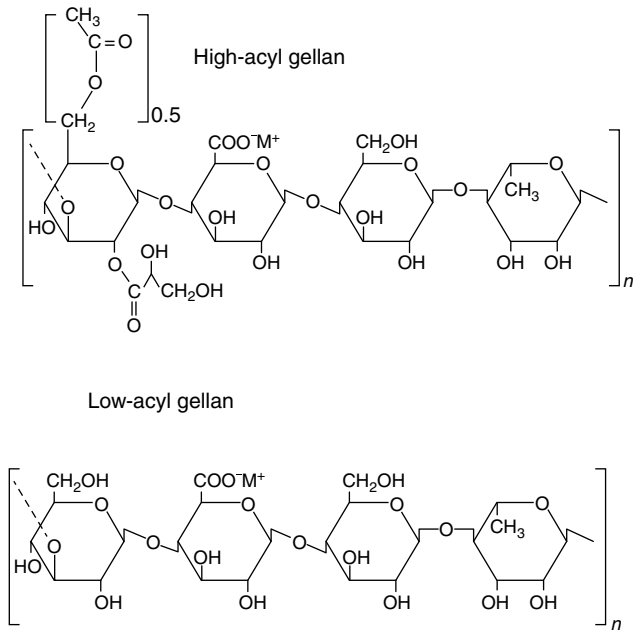


FIG. 1-69. **Gellan gum.**

the 3-linked glucose unit, the former tentatively assigned to the 6-position and the latter to the 2-position. Gellan gum forms an extended intertwined, three-fold, left-handed, parallel double helix. Molecular shape in the solid state is usually an indicator of how molecules associate in solution. The mechanism whereby gellan gum molecules associate in solution is believed to involve ion-mediated aggregation of double helixes.

Gellan gum is produced by inoculating an appropriate fermentation medium with *Sphingomonas elodea*. The medium contains a nitrogen source, sugar as carbon source, and inorganic salts. Fermentation takes place under sterile conditions and strictly controlled aeration, agitation, temperature, and pH. A viscous broth produced by the fermentation is pasteurized to kill all viable cells. After that the polysaccharides are recovered from the broth as either fully acylated or deacylated gum. Gellan gum for microbiological media is the low-acyl form. A major advantage of gellan gum is that it is a fermentation product and can be produced in large amounts and consistent quality.

At high temperatures the gellan chains exist as random coils. These coils form double helixes when the solution cools. In the presence of high cation concentrations the double helixes form a gel (Fig. 1-70) (Cameron, 2008). An excellent explanation of the function of cations provided by Stewart I. Cameron in his very enlightening review of gelling agents (Cameron, 2008) is reproduced here with permission:

> Helix formation is enhanced by divalent cations such Ca^{2+} and Mg^{2+} because divalent cations locate between opposing carboxyl groups within the helices, by attracting the carboxyl groups to the common divalent ion between them, i.e.,

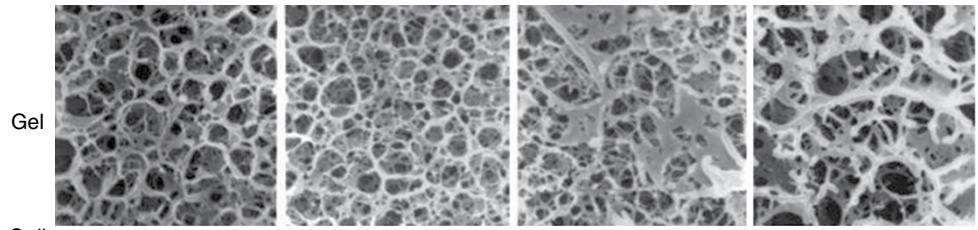


FIG. 1-70. **Effects of Ca^{2+} ions on gellan gum gels.**

COO^-—Ca^{2+}—COO^-. Monovalent ions such as K^+ and Na^+ will also cause gellation (see Fig. 2 in Kang et al., 1982), but a 40-fold concentration increase compared to divalent cation concentration is required to produce the same gel strength. This is because the monovalent ion bond, rather than being a single molecule, is a COO^-—K^+—water—K^+—OOC^- strcture which is both weaker and requires more K than Ca per helix bond that is formed (Chandrasekaran et al., 1992). Not only the strength of the bond within the helixes but also individual helix length is increased depending on the dominant ion, in the order: none < Na^+ < K^+ < Ca^{2+}, which is the same order as for these ions' effects on gel stiffness (Nakajima et al., 1996).

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 never be possible. Still, several predictions are possible regarding orchid micropropagation. They must be considered in the context of the prognostications made in the first and second editions.

The first prediction in the previous editions 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 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, 1999*a*) 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 usable and practical procedures for *Paphiopedilum* and *Phragmipedium* (procedures for these species exist at present, but they are not being used extensively).

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, if any, future with orchids is electrophoretic insertion of genes (Griesbach, 1994) despite multiple and repetitious publications from a single laboratory by the same author (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. (For excellent reviews of the history of plant biotechnology, see Sussex, 2008; Vasil, 2008. Indra Vasil's review is especially interesting because it provides biographical and personal information about several major historical figures.) 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 the excellent work by Professor Wen-Huei Chen and Hong-Hwa Chen and their associates in Taiwan (Chen and Chen, 2007, 2011).

Recently Phlaenopsis (Mishiba, Chin and Mee, 2005) and Cymbidium (Chin, Mishiba and Mii, 2007) were transformed genetically through the use of Arobacterium. At the 11th Asian Pacific Orchid Conference held in Okinawa, Japan (2013), several genetically modified Phalaenopsis plants bearing blue flowers were exhibited by Dr. Masahiro Mii of Chiba University, Japan (Chang and Wang, 2014). Indonesian orchids were also transformed by Agrobacterium (Semiarti et al., 2011).

"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; Kuhn, 1981*a*, 1981*b*; Tisserat and Vandercook, 1985, 1986; Hew et al., 1987; Okamoto, 1996; Paek et al., 2001). New and improved such methods will be developed in the future.

Investigations on low temperature storage of orchid callus (Sivasubramaniam et al., 1987) and PLBs suggested to us that germplasm, seedlings and tissues may be stored

cryogenically. This did happen, but is not covered in this book due to space limitations.

Isolated orchid petal cells have been used in plant physiology research (Hew and Yip, 1987). This suggests that in time isolated orchid organs, tissues, and cells will be used as model systems for research on plants. Not much of this has happened since the second edition, but it may happen in the future.

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

Perhaps the safest prediction for this, the third edition of a book first published in 1993 (Arditti and Ernst, 1993) with a second edition in 2008 (Arditti, 2008), is that Joseph Arditti (82 as this revision nears completion) will not participate actively if at all in a possible fourth version because of age or involuntary departure to the Big Orchid Garden in the Sky.

Kilo, the muse of history, contemplating an orchid flower (with the aid of 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 appropriate equipment, certain skills and some 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), second (Arditti, 2008) and present edition 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; Prakash and Pierik, 1993; Vij, 1993; Ichihashi, 1997; Prakash et al., 1996; Kishi and Tagaki, 1997*a*, 1997*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, individual 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 for 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. Some spply houses (see Appendix 2) may still issue informative and detailed hard copy (free on request, but usually mailed by bulk and/or surface mail) and online catalogs. Therefore requests for hard copy 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.

Micropropagation of Orchids, Third Edition. Tim Wing Yam and Joseph Arditti.

All suppliers now have well-illustrated web sites (see Appendix 2) and detailed online catalogs that simplify ordering and eliminate the wait for mailed information. Shipping can be faster too, but there can be 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 and tightly closed containers under dry conditions, or even in a freezer if possible. 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 or made of metal. The illumination itself is without effect on most chemicals. 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

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	Pt	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

[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 and meet the demands or preferences of the explants or tissues being cultured.

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 values 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, but an equal amount of phosphate (PO_4^{2-}) and half as much hydrogen (H^+). Levels of phosphorus and potassium must be considered in substituting KH_2PO_4 and K_2HPO_4 for each other. However it is not necessary to consider the levels of hydrogen.

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: When $MgSO_4$ [magnesium sulfate, usually $MgSO_4 \cdot 7H_2O$, formula weight (FW) 246.47] is replaced with anhydrous $MgSO_4$ (FW 120.37) or vice versa only the molarity of magnesium sulfate must be considered. The waters of hydration are not relevant, but must be conserved in terms of formula weight. Either of the two magnesium sulfate salts 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. It is important to note that substitutions of nutrients 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 and their concentrations can change. However, differences will be minor. Still, caution is needed to prevent supraoptimal levels of any one element. Waters of hydration must be taken into consideration when considering formula or molecular weights. Again, substitutions must be made on a molar, not weight, basis of the main component(s).

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

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

Compound	Molecular or formula weight	Number of atoms or ions[b]		Amount added per liter			
				Original		Substitute[d]	
		Cation	Anion	Weight	Moles[c]	weight[e]	Remarks[f]
Aluminum							
Aluminum chloride, $AlCl_3$	133.34	1*	3*	31 µg	0.23 mmol	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 mmol	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 original 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		

(*Continued*)

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

Compound	Molecular or formula weight	Number of atoms or ions[b]		Amount added per liter			
				Original		Substitute[d]	
		Cation	Anion	Weight	Moles[c]	weight[e]	Remarks[f]
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: original wt. × 149.89/166.01
Iodine	253.81	1*					Calculate as: original 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		

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	

(*Continued*)

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

Compound	Molecular or formula weight	Number of atoms or ions[b]		Amount added per liter			Remarks[f]
				Original		Substitute[d]	
		Cation	Anion	Weight	Moles[c]	weight[e]	
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 nitrate may introduce toxic levels of sodium and magnesium						
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 (1/1000 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.

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 µg
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 mmol	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 mmol	–	–
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	$MgSO_4 \cdot H_2O$[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 mg
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 mmol	16.357 mg
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 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.

($1SO_4^{2-}$ 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 ferrous chloride and 7 in ferrous sulfate) are irrelevant in this instance. A simple formula for calculation of equivalent molarities is:

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

where:

MWO = molecular weight of the original substance;
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).

Ammonium nitrate

There are reports that ammonium nitrate has been, or is about to be, banned in some countries and/or is hard to find or purchase because it can be used in the manufacture of explosives. The best (but perhaps illegal in some countries) solution for this problem (suggested by Patrick Sweetman, sweetmanpc@WOOSH.CO.NZ) is as follows.

1 Calculate the required molar concentration of the ammonium nitrate stock solution.
2 Dilute aqueous nitric acid (HNO_3) to this molarity.
3 Dilute aqueous ammonia (NH_4OH) to the same molarity.
4 Slowly neutralize the aqueous nitric acid with aqueous ammonia. The reaction is:

$$NH_4OH + HNO_3 = NH_4NO_3 + HOH$$

It is very important to keep in mind that ammonium nitrate is explosive and must – *absolutely must* – be handled with great care. Also, producing it by any means may not be legal and perhaps even may carry very severe penalties (long prison terms and even execution) in countries where the salt itself has been outlawed. Therefore, it is extremely important to be thoroughly familiar with and follow/obey local laws when using and/or preparing ammonium nitrate. In some cases it may be wise to check with local authorities.

Both nitric acid and ammonia are dangerous and corrosive. Both can cause severe injury when spilled on skin and blindness should they splatter into eyes. Therefore all steps of this procedure must be carried out by very experienced laboratory personnel wearing protective goggles, face masks, clothing, and gloves. The two solutions must be mixed in Erlenmeyer (i.e., narrow opening) heat-resistant (Pyrex or similar) glass containers placed in an ice bath to prevent possible overheating.

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 the concentrations of the substances in the solution. 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 "macro," "major," "micro" and "minor" refer not to their importance, but on the amounts, large or small, which are required; see Box 2-1). The reasons for this are (1) utilization of existing formulations; (2) imprecise and/or incomplete and/or anecdotal 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 10-fold (i.e., an order of magnitude), which is major]. Such a large increase can result in toxicity. Microelement stock solutions can be stored and thawed like those of macroelements. Also, like macroelements, all microelement salts are heat-stable and can be autoclaved. Given the current political climate in many countries, it is highly advisable to determine which macroelement and microelement salts may be illegal.

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 Na_2EDTA 37.3 mg l^{-1} and $FeSO_4{\cdot}7H_2O$ 27.8 mg 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…" was unelucidated in 1980 (Singh and Krikorian, 1980) and still is not. 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.

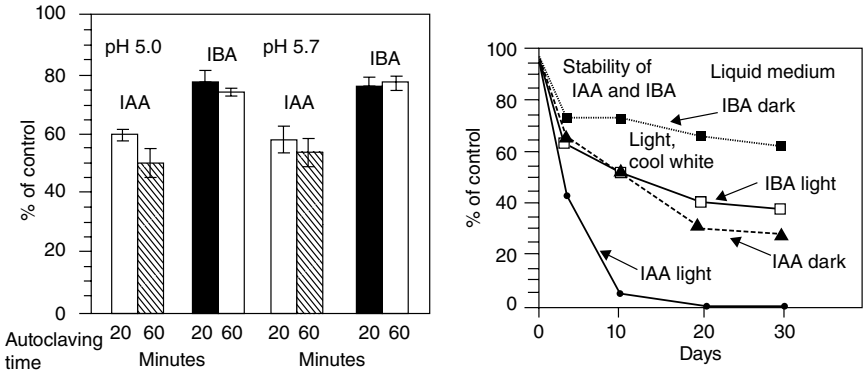
Auxins and Anti-auxin

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 (see figures in Table 2-3). Auxins should never be substituted and their concentrations should never be changed without prior tests.

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

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

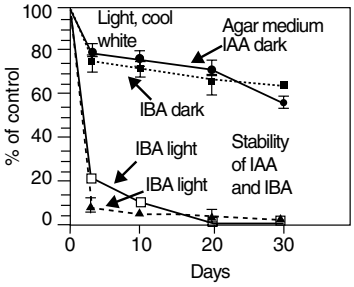
Hormone	Molecular weight	Concentration[a]	
		Weight (mg l^{-1})	Molarity (μmol l^{-1})
Anti-auxin			
trans-cinnamic acid	148.20	1.5	0.01
		15	0.10
		150	1.01
Auxins			
2,4-Dichlorophenoxyacetic acid (2,4-D), synthetic	221.04	1	4.52
		2	9.04
		5	22.60
		10	45.24
Indoleacetic acid (IAA), naturally occurring	175.19	1	5.71
		2	11.42
		5	28.54
		10	57.08
		25	142.70
		50	285.40
		100	570.81



Effects of autoclaving and pH (left) and storage in liquid (right) on stability of IAA and IBA (Nissen and Sutter, 1990)

(*Continued*)

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

Hormone	Molecular weight	Concentration[a]	
		Weight (mg l^{-1})	Molarity (μmol l^{-1})
Indolebutyric acid (IBA), synthetic	203.24	1	4.92
		2	9.83
		5	24.60
		10	49.20
		25	123.01
		50	246.02
		100	492.03
			
Effects of storage on stability of IAA and IBA in agar medium (Nissen and Sutter, 1990)			
Naphthaleneacetic acid (NAA)[b], synthetic	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
Thidiazuron (*N*-phenyl-*N'*-1,2,3-thiadiazol-5-yl-urea, 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 may 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 = 0.22 mg. 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 or effectiveness 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.

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 adsorb 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 its 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.

Chitosan

Deacylated chitin is chitosan. It has been reported to accelerate the growth of explants and protocorm-like bodies (Nge et al., 2006).

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 is 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-yl-urea) 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 0.01 N KOH or NaOH (forming a light-tan-colored solution), 0.5 N 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. comms). 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. comms), 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. However, if in some procedures media containing gibberellins are autoclaved, the published protocol should followed.

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 (Anonymous, 2003).

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 cannot 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 (filtration 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). There is no certainity that *myo*-inositol is required by explants, but it should not be removed from a medium without prior testing. The function of inositol was not clear for a long time. More recently it has been suggested that inositol is a component of cellular signaling molecules (Dotzauer et al., 2010). Inositol is usually sterilized by autoclaving. Inositol pills which are sold in pharmacies or health food stores should not be used because they may contain additives that may be toxic to tissues. 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.

Phloroglucinol

A degradation product of phloridzin, phloroglucinol can promote growth, stimulate rooting, control hyperhydricity, and improve the recovery of cryopreserved *Dendrobium* protocorms (Vendrame and Faria, 2011; for a review see Teixeira da Silva et al., 2013).

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.

Herbicides

Several weedkillers are used in a few media. One example is the benzoic acid derivative (3,6-dichloro-2-methoxybenzoic acid, MW 221.04) Dicamba (also sold as Banvel, Oracle and Vanquish). When used, herbicides must be added to media exactly as listed in the medium or media formulation. They must not be interchanged or substituted by other herbicides. Some of the herbicides used in media can be irritating, noxious, and even toxic to humans, pets and domestic animals. Therefore they must be used with caution and appropriate protection (gloves, goggles, respirators, and protective clothing inside hoods).

Vitamins

Niacin (nicotinic acid), pyridoxine (vitamin B_6), and thiamine (vitamin B_1) are most commonly added to orchid culture media as part of several media including the 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 described as not being heat resistant and it is common to read that they should not be sterilized by autoclaving (ten Ham, 1971). However, media that contain vitamins are routinely heat-sterilized without any ill effects. 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(s) suitable without autoclaving the vitamins, tests should be carried out before switching to large-scale heat sterilization. In practical applications it is enough to just follow instructions.

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 without prior tests. 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.

Coconut Water

Erroneously called "coconut milk" (which derives its name from the milky appearance of a liquid obtained by squeezing or grating fresh coconut "meat" or copra), coconut water (CW), the clear 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, 1974, 1993) CW is not used extensively in orchid seed germination and seedling culture.

François Mariat (1921–2003)

As an additive to culture media CW has beneficial effects on some orchid explants. Some explants require CW and will neither grow nor proliferate without it. CW can also increase proliferation without causing mutations. Because of these effects CW is incorporated in several media.

The most commonly used and recommended CW is that from green (unripe) nuts. Food stores usually remove the outer green part of the coconut husk. Therefore green (unripe) nuts in grocery stores are white in appearance. CW from ripe nuts can also be used. Such nuts are usually dehusked leaving only the internal hard shells which are brown in color. Dehusked nuts have three darker depressions at one pole. These are the "eyes" one of which is soft, very easy to penetrate and when broken through become the openings through which CW is drained.

It is easiest and best to collect CW from nuts by poking holes through the soft "eyes" and pouring the liquid into a clean container. Only water-clear liquid which is not discolored and does not have a bad and/or acidic smell (and preferably no pronounced smell at all) should be used. Several supply houses which specialize in culture media sell CW at present.

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

(*Continued*)

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
mg 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 g^{-1} tissue
"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 oxidase, carboxylase, catalase, invertase, lipase, oxygenase, phosphatase, peroxidase, polyphenoloxidase, protease

	Percent of N fraction of albumin-globulin protein
Amide nitrogen	10.13
Humin nitrogen	2.92
Monoamino nitrogen	37–64
Non-amino 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		
Tyrosine	0.072		
Valine	0.17		
	Each amino acid as percent of total protein nitrogen in ripening fruit		
Day:	0	6	12
α-Alanine	10	8.9	9.6
Arginine	8.0	6.8	8.9
Aspartic acid	9.0	13.5	9.4
Cystine	tr		
Glutamic acid	10.4	11.0	10.8
Glycine	9.0	7.1	8.6
Histidine	3.8	5.3	3.3
Hydroxyproline	tr	1.3	1.1
Leucines	18.0	13.9	15.1
Lysine	6.3	8.5	8.6
Proline	3.4	5.0	4.9
Serine	6.4	7.1	8.6
Threonine	5.3	4.4	4.8
Tyrosine	2.8	2.9	2.8
Valine	7.4	13.9	15.1
	Ninhydrin reactive substances		
	Percent of total amino acids		
Alanine	<5%		
α-Aminobutyric acid	5–10%		
Asparagine	>15%		
Arginine	<5%		
Aspartic acid	5–10%		
Glutamic acid	<5%		
Glutamine	10–15%		
Glycine	<5%		
Histidine	10–15%		
Leucines	<5%		
Lysine	<5–15%		
Pipecolic acid	5–10%		
Proline	<5%		
Serine	<5%		
Threonine	<5%		
Tyrosine	<5%		
Valine	<5%		
Total	*Alcohol insoluble protein hydrolysates* 3.13 mg g^{-1} FW		
	Percent of total amino acids		
Alanine	5–10%		
Arginine	<5%		
Aspartic acid	>15%		
Glutamic acid	>15%		
Glycine	5–10%		
Histidine	<5%		
Leucines	>15%		
Lysine	5–10%		

Component	Concentration
Methionine sulfoxide	Present
Phenylalanine	Present
Proline	5–10%
Serine	<5%
Threonine	<5%
Tyrosine	<5%
Valine	5–10%
Vitamins	
Vitamin A	131.0–131.43 IU mg^{-1}
Vitamin A	50–332 IU 100 g^{-1} pulp
Vitamin A	50–332 IU 100 g^{-1} edible portion
Vitamin A (equivalent)	1.6–2.03 mg g^{-1} b-carotene
Vitamin A	5.1% of USDA RDA/100 mg
Vitamin C	30 mg 100 g^{-1} edible portion
Vitamin D	0–0.2 IU g^{-1}
	100 g^{-1} pulp or edible portion
Biotin (vitamin H)	4.4 mg
Folic acid (vitamin B[c])	95 mg
Niacin	40–61 mg/0.7–1.8 mg
Pantothenic acid	70 mg FW/0.61 mg
Vitamin B_1 (thiamine)	34–48 mg/40–270 mg
Vitamin B_2 (riboflavin)	40–87 mg/87–180 mg
Vitamin B_6 (pyridoxine)	0.32 mg/0.5 mg
Vitamin C	6.86 mg/3–11 mg, 0.1 mg g^{-1}
Vitamin E (tocopherols)	poor source
Vitamin E	1.1 mg 100 g^{-1} edible portion
Vitamin K	none
Niacin	4.8% of USDA RDA/100
	Percent of USDA RDA in 100 mg
Vitamin B_1 (thiamine)	2.6%
Vitamin B_6 (riboflavin)	5.3%
Vitamin C (ascorbic acid)	20%
Growth promoting substances	
(No quantitative data in the original report)	
"Auxin-like"	
6-(a^6-Isopentenylamino purine)	
Ethylene (evolution)	
Of same general nature as in coconut water	
Purine	
Zeatin	
Zeatin riboside	
Pigments	
Carotenes	0.6–1 mg/g FW
a-Carotene	31% of carotenes
b-Carotene	28% of carotenes
b-Carotene	1.5–2 ppm FW
Lutein	33% of carotenes
Energy	
	285 kilocalories 100 g^{-1} edible portion

[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.

TABLE 2-5. **Partial composition of coconut water, copra, and coconut oil[a] (Raghavan, 1966, 1976; Woodroof, 1979; Arditti and Ernst, 1993; Ge et al., 2004, 2005, 2006; de Sousa et al., 2005; Anonymous, 2008*c*, 2008*d*, 2008*e*, 2008*f*)**

Substance	Content	Substance	Content
Coconut water		Leucine	22 μg ml^{-1}
Calories			1.95–4.18 %p
Total	46 per 240 ml	Lysine	150 μg ml^{-1}
From fat	4 per 240 ml		1.95–4.97 %p
Ash	0.62 mg 100 g^{-1}	Methionine	8 μg ml^{-1}
	0.45%	Ornithine	22 μg ml^{-1}
Water content		Phenylalanine	12 μg ml^{-1}
Water	95.5%	Pipecolic acid	Present[b]
	125–205 ml per nut	Proline	97 μg ml^{-1}
Moisture, unripe nut	95.01%		1.41–4.12 %p
Moisture, ripe nut	91.23%	Phenylalanine	1.23 %p
Acidity		Serine	111 μg ml^{-1}
pH	5.5–5.9		0.59–0.91 %p
Solids		Threonine	44 μg ml^{-1}
Total	4.71 mg 100 g^{-1}	Tryptophan	39 μg ml^{-1}
Inorganic ions	*mg per 100 g or mg per 100 ml or percent (%)*	Tyrosine	16 μg ml^{-1}
			2.83–3.0 %p
Calcium	10.7–23.7 mg 100 ml^{-1}	Valine	27 μg ml^{-1}
	29.8–33.2 mg 100 ml^{-1}	*Lipids*	
	29 mg 100 g^{-1}	Fat	0.12%
	6% in 240 ml		<0.1%
Calcium oxide	0.69%		15.9 mg per nut
Chlorine	183 mg 100 g^{-1}	Total fat	1%
Copper	0.04 mg 100 g^{-1}	Saturated fat	2% of fats
Iron	0.10–0.5 mg 100 g^{-1}	Cholesterol	0
	0.7–0.9 mg 100 ml^{-1}	*Enzymes*	
	4% in 240 ml	Acid phosphatase	Present[b]
Magnesium	30 mg 100 g^{-1}	Catalase	Present[b]
	5.0–8.8 mg 100 ml^{-1}	Dehydrogenase	Present[b]
	10.2–11.4 mg 100 ml^{-1}	Diastase	Present[b]
Magnesium oxide	0.59%	Peroxidase	Present[b]
Manganese	0.1–0.5 mg 100 ml^{-1}	RNA polymerases	Present[b]
Nitrogen	33.2–34.8 mg 100 ml^{-1}	*Organic acids*	*meq ml^{-1}*[d]
	0.05%	Citric acid	0.37
Phosphorus	37 mg 100 g^{-1}	Malic acid	34.31
	14–15.2 mg 100 ml^{-1}	Pyrrolidine carboxylic acid	0.39
Phosphoric acid	0.56%		
Potassium	312 mg 100 g^{-1}	Shikimic and quinic acids, etc.	0.57 mg ml^{-1}
	318–326 mg 100 ml^{-1}		
	6.6%	*Fiber*	
Sodium	105 mg 100 g^{-1}	Dietary	3 g in 240 ml
	252 mg in 240 ml	*Vitamins*	*mg ml^{-1} or μg ml^{-1} or see footnotes to this table*
	11% in 240 ml		
Sulfur	24 mg 100 g^{-1}	Vitamin A	0 in 240 ml
	4.5–5.0 mg 100 ml^{-1}	Biotin	0.02 mg ml^{-1}
Zinc	0.004–0.018 mg 100 ml^{-1}		0.02 μg ml^{-1}
Nitrogenous compounds	*μmol ml^{-1}*	Vitamin C	10% in 240 ml
Ethanolamine	0.01	Folic acid	0.003 mg ml^{-1}
Ammonia	Present[b]		0.003 μg ml^{-1}
Protein	0.1%	Niacin (nicotinic acid)	0.64 mg ml^{-1}
	2 g in 240 ml		0.64 μg ml^{-1}
Amino acids and related substances	*μg ml^{-1} or percent of total protein (%p) or see footnotes to this table*	Pantothenic acid	0.52 mg ml^{-1}
			0.52 μg ml^{-1}
Alanine	312 μg ml^{-1}	Riboflavin	0.01 mg ml^{-1}
	2.41 %p	Pyridoxine	Trace[b] mg ml^{-1}
β-Alanine	12 μg ml^{-1}		*Trace* μg ml^{-1}
γ-Aminobutyric acid	820 μg ml^{-1}	Thiamine	Trace[b] mg ml^{-1}
Arginine	133 μg ml^{-1}		*Trace* μg ml^{-1}
	10.75 %p	*Carbohydrate*	
Asparagine and glutamine	60 μg ml^{-1}	Content	4.0%
Aspartic acid	65 μg ml^{-1}		9 g in 240 ml
Cystine	0.97–1.17 %p		3% in 240 ml
	0.97–1.17[c]	*Sugars*	*Percent or mg ml^{-1}*
Dihydroxyphenylalanine	Present[b]	Fructose	5.25 mg ml^{-1}
Glutamic acid	240 μg ml^{-1}	Glucose	7.25 mg ml^{-1}
	9.76–14.5 %p	Sucrose	9.18 mg ml^{-1}
Glycine	13.9 μg ml^{-1}	Total, reducing	1.5–3.1%
Histidine	Trace[b]	Reducing	0.8 mg 100 g^{-1}
Homoserine	5.2 μg ml^{-1}	Total	3.1–3.9%
Hydroxyproline	Trace[b]		2.08 mg 100 g^{-1}
Isoleucine	18 μg ml^{-1}		6 g in 240 ml

TABLE 2-5. (***Continued***)

Substance	Content	Substance	Content
Sugar alcohols	*Percent*[b]	Myristic	High
Mannitol	0.8	Palmitic	High
Sorbitol	15[e]	Stearic	High
myo-Inositol	0.01	Oleic	High
scyllo-Inositol	0.05		
Plant hormones		**"Meat" or kernel**	
Auxin	0.07 mg ml^{-1}	*Fiber*	
1,3-Diphenylurea	5.8 mg l^{-1}	Crude, from ripe nuts	3.3%
Cytokinin	Present[b]	*Carbohydrate*	
Cytokinins	$\times 10^{-3}$ *µmol*	Unripe nut	6.30%
Benzylaminopurine	Below 0.010	Ripe nut	11.29%
Dihydrozeatin	0.14	*Water*	
Dihydro-transzeatin-*O*-glucoside	46.6	Moisture, unripe nut	90.8%
		Moisture, ripe nut	46.30%
Dihydrozeatin-*O*-glucoside	Present	*Lipids*	
		Oil	45–50%
Isopentenyladenine	0.26	Fat, unripe nut	1.4%
Kinetin	0.31	Fat, ripe nut	37.29
Kinetin riboside	0.33	*Protein*	
ortho-Topolin	3.29	Unripe nut	0.90%
ortho-Topolin riboside	Below 0.017	Ripe nut	4.08%
trans-Zeatin	0.09		
trans-Zeatin-*O*-glucoside	48.7	**Coconut oil**	
		Acids	*Percent*
trans-Zeatin riboside	76.2	Capric	7
Zeatin-*O*-glucoside	Present	Caprylic	8
Dihydro *trans*-zeatin riboside	Below 0.013	Lauric	48
		Linoleic	1
Gibberellin	Present[b]	Myristic	19
Miscellaneous		Oleic	6
Leucoanthocyanin	Present[b]	Palmitic	7
Phyllococosine	Present[b]	Stearic	4
		Crude	*ppm*
Copra		Ketones:	
Protein	*Percent*	Heptan-2-one	40
Crude	20.30	Nonan-2-one	73
	22.89	Pentadecan-2-one	65
Extracted	20	Tridecan-2-one	138
	25	Undecan-2-one	290
Fiber		Lactones:	
Crude	8.53–12	Decalactone	97
Lipids		Dodecalactone	60
Crude fat	2.76–7.4	Hexalactone	20
Oil	63–65	Octalactone	51
Water		Tetradecalactone	30
Moisture	4.84–11.14	*From fresh nuts*	*ppm*
Nitrogen-free: extract	29.15–47.46	Ketones:	
Ash	6.82–6.88	Tridecan-2-one	138
Amino acids		Undecan-2-one	290
Histidine	Deficient[b]	Lactones:	
Methionine	Deficient[b]	Decalactone	88
Lysine	Deficient[b]	Dodecalactone	65
Tryptophan	Deficient[b]	Hexalactone	27
Saturated acids	*Percent*	Octalactone	64
Lauric	High	Tetradecalactone	20

[a]Values in this table differ quantitatively and in the manner in which they are presented. This is because they were obtained from a multitude of sources. The accuracy of any of the values could not be determined. Some of the components probably do not contribute to the effects of coconut water in culture media.

[b]No units given.

[c]Units: grams per 100 g dried protein.

[d]meq ml^{-1} is milliequivalents per milliter.

[e]Units: mg ml^{-1}.

Some workers filter CW as it is being poured from a nut or after that. Standard laboratory filters or those used in coffee makers can be used for this purpose (two layers of white unscented paper towels have also been employed). Filtering is not necessary in most cases. Some users also deproteinize CW by autoclaving after pouring from a nut and then filtering the small amount of precipitate which forms due to the heating. This is also not necessary.

Occasionally CW may turn reddish even in a refrigerator. The color change not an indication of spoilage. Reddish CW can be used and is as effective as the clear kind unless it has become cloudy, brownish and/or smells acid or bad.

CW can be stored in a refrigerator, but may become contaminated after prolonged storage. Therefore it must be examined carefully before use if it has been stored for more than a few days. If frozen, CW can be stored for long periods without loss of activity or contamination. CW can be autoclaved.

It is possible and in some cases even desirable to combine CW with other additives such as banana homogenate, casein hydrolysate, vitamins, hormones, tryptone, polyols, and amino acids. However, such combinations must be made judiciously. CW is fully compatible with darkening agents (charcoal and graphite) and solidifiers (agar, gellan gum).

The reasons for the beneficial effects of CW are not clear. Plant hormones and related substances, hexitols (Pollard et al., 1961; Ge et al., 2004, 2005, 2006), and some or many other substances may be reasons for these effects (Table 2-5). A fortuitous combination or interaction between several of the different compounds in CW may also be a reason. Relatively recent work by Jean "John" Wan Hong Yong at the Nanyang Technological University in Singapore (Ge et al., 2004, 2005, 2006) has shown that CW contains at least 12 cytokinins and related substances (Table 2-5). These compounds possibly singly, perhaps all of them together or maybe a combination of only a few, could also be the reason(s) for the beneficial effects of CW.

Humic acid

A complex mixture of many different acids. This mixture is the major constituent of humic substances, which are the principal organic components in the soil. Humic acid enhances plant growth.

Hydrolysates and Autolysates

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

Sugars

Sucrose (β-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 (β-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 despite the fact that only the latter is added to most media. 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. Several suggestions on web sites, in discussion groups, and in several publications (mostly not peer-reviewed) that some orchid taxonomic groups exhibit preferences for specific individual sugars or sugar mixtures are anecdotal and are in need of confirmation.

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 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 glucose 20 g 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 and molarity in themselves are not the only relevant factors. 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 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.

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 edn, 1953, Difco Laboratories, Detroit, MI; H.W. Schoenlein, Difco Laboratories, pers. comm., 1957. Courtesy of E. McDonald, Technical Services, Difco Laboratories, Detroit, MI. 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.

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.

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]					

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:

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

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 of a substance, 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.

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 until seedlings or plantlets are large enough to be moved to community pots.

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 to J.A.; these letters are now at the Singapore Botanic Gardens library) and several antibiotics (Schaffner, 1954) were tested as additives for this purpose, but were found to be phytotoxic and unsuitable. In the late 1970s and early 1980s, several combinations were formulated (Table 2-8) following the screening of a number of substances (Thurston et al., 1979, 1980; Spencer et al., 1979/1980; Brown et al., 1982; Cvitanik and Arditti, 1984; the research was carried out entirely by several hard-working and brilliant 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. Some of the anticontaminants are antibiotics, which can affect humans and animals and should be used with care.

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

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
Gentamicin	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 autoclaving.
[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 + gentamicin + graphite[c]
2	Benlate + nystatin + penicillin G + gentamicin + sodium omadine + graphite[c]
3	Benlate + nystatin + penicillin G + gentamicin + amphotericin B + vancomycin + graphite[c]

[a]*Concentrations*: amphotericin B, 10 mg l^{-1}; benlate, 50 mg l^{-1}; gentamicin, 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, gentamicin, 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.

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 70% isopropanol (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 some explants. They are known to be suitable for *Cattleya* and *Stanhopea* seedlings (Thurston et al., 1979) and *Phalaenopsis* flower-stalk-node cultures (Spencer et al., 1979/1980). 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), gentamicin (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 (from orchids or other plants) in general may have similar or different effects on orchid seedlings and/or tissue cultures is not clear. 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). Of these rifampicin at 100 mg 100 ml^{-1} has been used to soak dawn redwood explants and was added to culture media at 50 mg l^{-1} to prevent contamination by endophytic bacteria. Silver nitrate ($AgNO_3$), 100–150 mg l^{-1}, can be added to media which do not contain amino acids or peptone for the same purpose (Rifat Tarik Yararbas on plant-tc@lists.umn.edu, August 6, 2007). Only a few of these substances have been 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 by external or endophytic contaminants was patented in the USA 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 (http://www.plantcelltechnology.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 (see Appendix 8).

The preparation is an amber-colored to clear liquid at pH 3.0–4.0 and has a mild inoffensive odor. It is relatively 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 (http://www.plantcelltechnology.com/ppm-msds/).

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		0, 2–3 days	U 500.0 mg	Penicillin-like
Cefotaxime	B	W		0, 2–8, 22 days	100.0–1000.0 mg	
Cephalothin	B	W	3	2–8	100.0 mg	
Chloramphenicol	B	E, W	5	2–8, 30 days	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		2–8, 30 days	10.0 mg	
Daconil						See Chlorothalonil
Desmel						See Tilt
Dihydrostreptomycin	B	W	5	2–8	100.0 mg	
Diniconazole					125.0 mmol 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 mmol 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		W		–20, 6 months	10–500 mg l^{-1}	
Gentamicin sulfate	B, M	W	5	2–8, 12 months	50.0 mg U 250 mg	T, CS
Hygromycin B		W		2–8, 6 months	100–200 mg	
Imaverol						See Imazalil
Imazalil						
Kanamycin monosulfate	B, M	W	5	2–8, 12 months	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, heat labile, light sensitive
Omadine, sodium					5.0 mg	T
Omnizole						See Thiabendazole
Orbit						See Tilt
Paromomycin sulfate	B	W	5	2–8	100.0 mg	CS
Pentachloronitrobenzene (PCNB)	F	E, O			100.00 mg	T
Penicillin G	B	E, W	3	2–8, 4 days	100.0 mg	T
					1000.000 u	T
PPM	B, F	W	Extended	Room	1–2.0 ml	T, proprietary mixture
Polymyxin B sulfate	B	W	5	2–8	50.0 mg	
Propiconazole						See Tilt
Puromycin HCl	B	W		0	10.0–100	
8-Quinolinol hemisulfate						
Quintozene						See PCNB
Radar						See Tilt
Ridomil						See Metalaxyl
Rifampicin	B	O		2–8, one day	10.0–50 mg	
Silver nitrate	B	W			100–150 mg	
Spectinomycin dihydrochloride	B	W		2–8	7.5–20 mg	
Spotless						See Diniconazole
Streptomycin sulfate	B	W	3	2–8, 30 days	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	F	O			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, 7 days	U 10.0–50 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, protocorm-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. Eng Soon Teoh 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); PhytoTechnology 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 (http://www.plantcelltechnology.com/ppm-msds/). 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 or 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 which was available on the Plant Cell Technology web site (http://www.plantcelltechnology.com/) 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 sucrose 20 g l^{-1}, thiamine 10 mg l^{-1}, pyridoxine 1 mg l^{-1}, niacin 1 mg l^{-1}, and *myo*-inositol 100 mg l^{-1} solidified with agar 8 g l^{-1} with and without PPM 2 ml 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 PPM 20 ml l^{-1} for 24–36 h on a rotary shaker and moving them after that without rinsing to half-strength MS containing PPM 2 ml 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 PPM 20 ml l^{-1} is 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; it may no longer exist) 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 PPM 4 ml 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."

PPM has been 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 http://www.plantcelltechnology.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 to 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. Several suggestions that animal and vegetable charcoals are equally suitable are anecdotal and if not ignored should be approached with caution and skepticism until proven experimentally.

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 positive effects. 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 (Werkmeister, 1970*a*, 1970*b*, 1971; for historical accounts see Arditti and Krikorian, 1996; Yam and Arditti 2009). 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 (from experiments based on Werkmeister's work, Werkmeister, 1970*a*, 1970*b*, 1971) led to the development of practical charcoal-containing media which gained widespread and rapid acceptance. In addition to being incorporated into media used for seedlings, charcoal is now added to many substrates

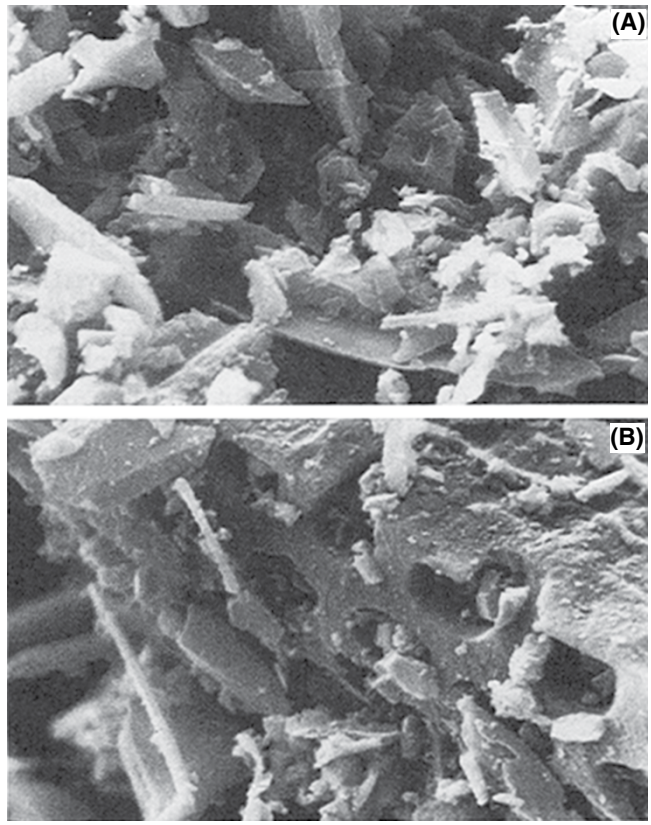


FIG. 2-1. **Scanning electron photographs of charcoal particles. A. Variation in size and shape of particles (×10,876). B. Xylem-like structure (×2175). Courtesy the late Dr. M.A. Weatherhead, Department of Botany, University of Hong Kong.**

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 et al., 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 (Pan and van Staden, 1998; Thomas, 2008).

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).

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

Element	Concentration[a]	Element	Concentration[a]	Element	Concentration[a]
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[e]	<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.

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). Darkening and slow release of beneficial substances have also been implicated in the beneficial effects of charcoal (Pan and van Staden, 1998; Thomas, 2008).

It is entirely possible, of course, that all 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 (99.6 mg 100 ml^{-1} vs. 99.8 mg 100 ml^{-1}) by charcoal (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 changed 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 pre-autoclaving 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 cannot be used, graphite 2 g 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.

Graphite has been added to orchid culture media (Thurston et al., 1979, 1980) in attempts to formulate culture media which do not require sterilization (Brown et al., 1982, 1984; Cvitanik and Arditti, 1984) and a comparison study with charcoal (Prizão

et al., 2012). The latter demonstrated that seedlings of *Cattleya bicolor* produced the largest number of buds on a medium containing graphite 6.0–7.5 g l^{-1} and the largest number of roots on a substrate darkened with activated charcoal 6 g l^{-1}. However, seedlings of *Brassolaliocattleya* Pastoral Innocence produced the largest number of both roots and buds on a medium containing activated charcoal at 4.5 g l^{-1} (Prizão et al., 2012). Altogether activated charcoal is a better darkening agent than graphite for orchid seedlings in vitro (Prizão et al., 2012) and probably also for tissue culture-derived plantlets. Graphite should be used only in special cases, as for example when the intent is to darken a medium with an agent which does not have the adsorptive capacity of charcoal (Thurston et al., 1979, 1980).

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/must not be used because they are toxic.

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 replacing agar and is being used in many formulations.

Agar

Several types of agar are available from commercial sources. They differ from each other in a number of characteristics (Table 2-11). For experimental purposes it is necessary to use reagent grade agar (Table 2-11) or gellan gum. Technical grade or kitchen quality agar can be used for practical micropropagation, but preliminary tests are advisable. They may contain additives which could interfere with cultures. Therefore agar or gellan gum which are not specially formulated for plant tissue culture should be used only after preliminary tests and with great caution.

A widely used procedure is to add the agar slowly with stirring to the medium at room temperature and 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 dispense the agar into a cold solution, disperse it thoroughly and dissolve it by autoclaving. Depending on the agar, gelling temperature can vary from 25 to 30°C.

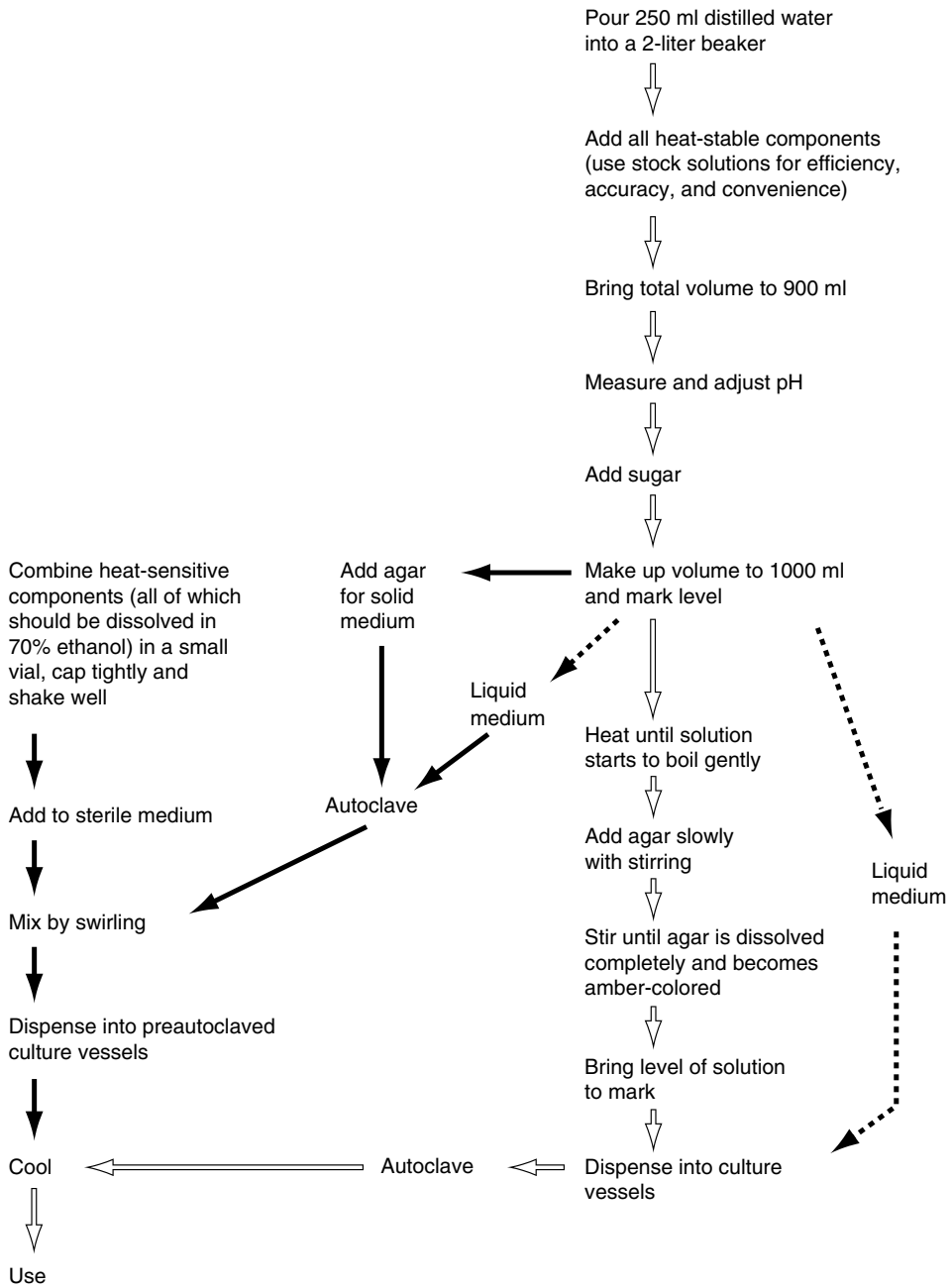


FIG. 2-2. **Preparation of culture media. Open arrows: solid media that contain only heat-stable components. Open arrowheads with dashed lines: liquid media that contain heat-stable substances. Solid arrows: solid media that contain heat-sensitive compounds. Solid arrowheads with dashed lines: liquid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

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.

Gellan gum

Gellan gum is an agar substitute consisting of glucuronic acid, rhamnose, and glucose (Fig. 2-3A) produced through fermentation by *Pseudomonas elodea*. It was discovered by the Kelco Division of Merck and Co., in San Diego, California (O'Neil et al., 1983; Sanderson and Clark, 1983; Shungu et al., 1983; Kelco, 1985, 2002; Cameron, 2008). Gellan gum produces a high-strength, colorless, and clear gel which gels at 27–31°C.

The most commonly used concentrations for plant tissue culture range from 1.2–1.5 to 2–2.5 g l^{-1}. Phytagel and Gelrite require the presence of divalent cations for gelling. Most orchid culture media contain enough calcium and magnesium to make gelling possible. If more dilute media are used, higher concentrations of Phytagel or Gelrite may be required.

Gellan gums (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 solution, lumps will form and the medium will not gel even after autoclaving. Two methods can be used for the preparation of gellan gum-containing media depending on whether a medium is to be dispensed before or after autoclaving.

Unless otherwise indicated, the following steps should be followed if a medium will be dispensed after autoclaving.

Step 1 Prepare medium, but do not add gellan gum.
Step 2 Add the gellan gum slowly while stirring the medium continuously.
Step 3 Sterilize medium as required after gellan gum is completely and thoroughly dispersed.
Step 4 Add components which must be incorporated into a medium after autoclaving if any. Since media are very hot after autoclaving and can cause severe burns, this must be done with great care.
Step 5 Dispense medium while it is still liquid. Should a medium tend to form a precipitate, swirl or stir it often to disperse whatever precipitates. Hot media after autoclaving should be handled with great care because they can cause severe burns.
Step 6 Allow medium to cool and solidify.

In the event a medium is to be dispensed before autoclaving, the following steps should be followed unless there are different instructions in the relevant tables in Chapter 3.

Step 1 Prepare medium, but do not add gellan gum.
Step 2 Add the gellan gum slowly, stirring the medium continuously.
Step 3 After gellan gum is completely and thoroughly dispersed, heat the medium to a gentle boil until the solidifier is completely dissolved. Stir medium for a few minutes after removing it from hot plate.
Step 4 When gellan gum is completely dissolved dispense medium into culture vessels.
Step 5 Autoclave medium as required. Should a medium tend to form a precipitate, swirl or stir every vessel to disperse whatever precipitates. Media can cause

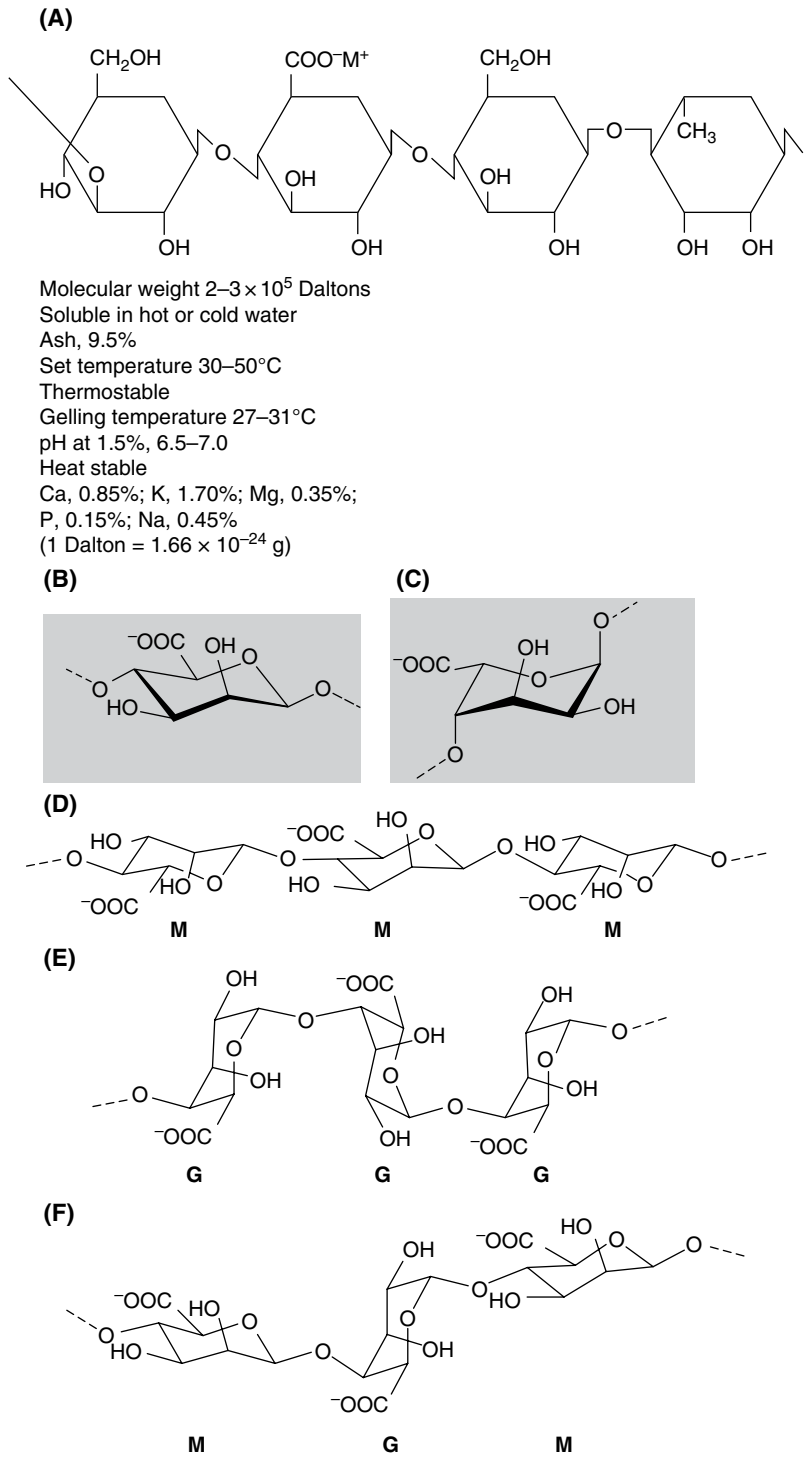


FIG. 2-3. **Gellan gum and alginate: A, gellan gum; B, 1,4-β-D-mannuronate; C, 1,4-α-L-guluronate; D, mannuronate polymer; E, guluronte polymer; F, mixed mannuronate/guluronate polymer. (*Source*s: www.fmcbiopolymer.com, www.kjemi.uio.no)**

severe burns after autoclaving because they are very hot and must be handled with great care.

Step 6 Allow medium to cool and solidify.

Agargel

A product which combines the positive attributes of agar and Phytagel (the Sigma brand name of gellan gum). Sigma-Aldrich sells a mixture of the two called Agargel (http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Plant_Biotechnology/Tissue_Culture_Protocols/Gelling_Agents.html). According to Sigma-Aldrich, Agargel can help control vitrification which may be a problem with Phytagel and serve as an economic substitute for agar. Depending on the required strength of a gel, Agargel should be used at 2.5–5 g l^{-1}. Agargel should be used in accordance with the instructions for procedures which require it.

Transfergel

Yet another specialty product by Sigma-Aldrich, Transfergel is a white powder (http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Plant_Biotechnology/Tissue_Culture_Protocols/Gelling_Agents.html). It is intended to be used as a carrier gel for micro cuttings and somatic embryos. Transfergel is added to media at 1.5–2% (w/v). It hydrates and thickens immediately on being added to media. Because of that it requires considerable and continuous stirring to ensure complete mixing and to get it to dissolve completely. Sigma-Aldrich describes the gel it forms as being "highly viscous (almost syrupy) in nature, which does not thicken significantly after autoclaving" and recommends that it "should be autoclaved in a container four times its volume (e.g. 500 ml in a 2 L flask) to avoid potential boil-over."

Alginic Acid

E.C.C. Stanford, a Scottish chemist, discovered alginic acid as a "jelly-like compound resulting from the sodium carbonate digestion of *Laminaria* seaweed" (Cameron, 2008). He called the compound "algic acid" and patented it in 1881, but could not make it a commercial success for a long time. In 1927 another Scotsman, F.C. Thornely "moved the technology" (Cameron, 2008) to San Diego, California, USA and formed the Kelco Company. By 1929 Kelco became well known as an alginate producer. It became part of Merck in the 1960s and Monsanto in 2000. It is now part of Huber (Cameron, 2008).

Alginate is a polysaccharide consisting of 1→4 linked mannuronic (M-blocks) and guluronic acid (G-blocks). The compounds can also alternate (Fig. 2-3B–F). It is used to prepare beads, which can contain cells, protoplasts, tissues, PLBs, and synseeds.

To prepare beads, alginate should be dissolved in accordance with the instructions for each procedure. Commonly used concentrations are 1.75–4.0% (w/v) in a buffered 2 m*M* calcium solution which contains an osmoticum. Alginate solutions can be sterilized through 0.45-µm filters or autoclaved. To form beads, the alginate–plant material mixture is dropped into a 50 m*M* $CaCl_2$ solution in osmoticum. To ensure

complete gel formation the beads should be allowed to remain in the calcium chloride solution for up to 45 min.

Following Instructions

The instructions given here are general and may not apply to specific situations. To ensure success it is important to follow the specific instructions associated with each procedure.

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 6–7 ($H^+ = 10^{-5}$ to 10^{-6}); pH 5–6 is mildly acid, and pH below 5 is increasingly acid; pH 7 ($H^+ = 10^{-7}$) is neutral and pH 7–8 ($H^+ = 10^{-8}$ 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 about 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 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 the pH of a medium is important because it affects the availability of nutrients. In addition, it 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. 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 a 0.1 N acid (hydrochloric, nitric, phosphoric, or sulfuric) solution. Should the pH be lower than required, the medium is too acid and must be adjusted up with a 0.1 N solution of 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. As indicated above, a suitable concentration of acid or base for adjusting pH is 0.1 N.

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 or names of the person or persons who made the solution.

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 70% 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 Dr. 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 5.2 ml of 95% ethanol (do not use methanol, methylated spirits or any form of denatured ethanol), and shake the flask gently. If the substance fails to dissolve, add a drop or two of dilute acid (for cytokinins) and shake again (for auxins, add sodium hydroxide). Should it be necessary, one or two additional drops may be added. After the substance has dissolved completely, add another 2.1 ml of 95% ethanol 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 or solid 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 ×

10^5 Pa which can also be expressed as 121°C and 1 atm, or 15–20 psi, or 10.5 kg · 6.5 cm^{-2}). Such conditions are generated in autoclaves. A large number of autoclave models and sizes are available (see Appendices 1 and 2). Several models are very complex and fully automatic, but there are also types which differ very little or not at all from a kitchen-type pressure cooker. Indeed, if an autoclave is not available, a kitchen pressure cooker can be used to sterilize media, tools, culture vessels, and other items. 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. Standard conditions for sterilization can be obtained automatically in autoclaves or very easily in pressure cookers, and the entire sterilization process is very simple.

Sterilization time depends on the volume per vessel which is being sterilized. Together with the time required for the medium to reach 121°C and 15 min at that temperature, the following guidelines suggested by Sigma-Aldrich and PhytoTechnology Laboratories should prove useful (for more information refer to Sigma-Aldrich (http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Plant_Biotechnology/Tissue_Culture_Protocols/Media_Sterilization.html).

Volume of medium per vessel, ml	Minimum autoclaving time, min
25	15–20
50	25
100	28
250	31
500	35
1000	40
2000	48
4000	63

It is important to keep in mind that prolonged autoclaving can break down or damage some media components. Therefore it is preferable to autoclave smaller volumes which require short sterilization periods.

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-volume filtration 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 W. 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-W 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."

Recommended microwave sterilization periods by PhytoTechnology Laboratories are as follows:

Volume of medium per vessel, ml	Minimum microwave time, min
25	4–6
50	6–8
100	8–10
250	10–12

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 listed below.

1. Completely sealed vessels such as screw cap jars or culture tubes must never be used 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.

A more recent report (Venturieri et al., 2013) suggests that plantlets of *Oncidium cebolleta* and *Phalaenopsis amabilis* plants grew better in microwave-oven sterilized

media. The research which developed this procedure used a Brastemp Jet Defrost, 27 L 950 W, which seems to be available only in Brazil. It is reasonable to assume that similar results can be obtained with other 950-W ovens. However, preliminary experiments to determine if other ovens are suitable and if adjustments are necessary must be carried out. In this procedure, 2 ml of 30% hydrogen peroxide are added to every liter of medium prior to sterilization in the microwave oven for 8 min. Media contained agar 8 g l^{-1}. Then, "Glass jars (500 mL), of the type commonly used in Brazil for commercial *in vitro* orchid cultivation, were each filled with ~70 mL of medium and were partially sealed with microwave-resistant plastic lids. Eight jars were placed in the microwave in a circle around a receptacle containing 500 ml of water (Fig. 1A, B), which served as an energy sink … During the sterilization process, the lids were left slghtly loose and were completely sealed only after solidification of the medium, in order to avoid explosions during [the] boiling phase and the entrance of contaminants due to the vacuum caused by cooling" (Venturieri et al., 2013). This seems to be a procedure worthy of consideration and testing.

(A) **(B)**

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. Methanol, 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, smok-free, 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% or more 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 be, and often is, 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.

Trace amounts of sodium hypochlorite may remain on the surface of the tissues which are sterilized with it. Water does not remove it (Abdul-Baki, 1974). In most instances these trace amounts of sodium hypochlorite will have no undesirable effects. Should it be necessary to remove the sodium hypochlorite, the tissues should be washed two to three times with 0.01 N HCl (Abdul-Baki, 1974), provided this weak acid will cause no harm. The acid washes must be followed by two to three washes with sterile distilled water.

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%. In the USA Clorox contains 8.3% sodium hypochlorite at present.

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 if any 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

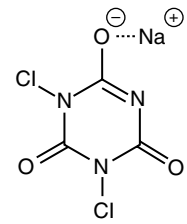
Ethyl alcohol (ethanol, drinking alcohol) and isopropyl alcohol (isopropanol/rubbing alcohol) can be used to sterilize work areas, tools, and outside surfaces of culture vessels by swabbing. These alcohols can be used in a concentrated form or as 70% aqueous solutions (70 ml alcohol made up to 100 ml with water). Methanol must be avoided since it is toxic to humans, pets, and livestock and may cause blindness.

Surface Decontamination

A number of methods are used for surface decontamination. In every 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.

Calcium and sodium hypochlorite are the most commonly used surface sterilants in micropropagation. Hydrogen peroxide (H_2O_2) is used rarely, but is being discussed online (plant-tc@lists.umn.edu). The general opinion seems to be that it is not as effective as the hypochlorites (Robert M. Hamilton, bob@eecs.berkeley.edu; Marni Turkel, marni@marniturkel.com). However, hydrogen peroxide can be used to save contaminated flasks: "If [contamination] is caught early, I scoop out the spot including a good amount of clean agar around it with a small [sterile] spatula. I use a [preferably sterile] pipette to put 5 or 6% H_2O_2 in the cavity. Be very careful not slop it out of the cavity or the plants [will] dies. It works for me in over half of the attempts. Sometimes I will also scrape the uncontaminated seed or protocorms from the mother [flask] onto another flask" (Marni Turkel, marni@marniturkel.com). The reference here is to flasks which contain seedlings, but this method should work with flasks which contain PLBs or plantlets obtained through tissue culture. However the extra effort may be worth while only with very valuable plants or when there are only very few flasks of specific clones.

Sodium dichloroisocyanurate (NaDCC) is used for water purification in swimming pools and as a routine disinfectant. It is used rarely for surface sterilization of orchid seeds and tissues, but has been discussed online (plant-tc@lists.umn.edu). There is also an anecdotal (Aaron J. Hicks, ahicks51@cox.net) and only partially susbstantiated (Parkinson et al., 1996; Niedz and Bausher, 2002) claim that it may be more effective that hypochlorites or at least less damaging to tissues (for the



Sodium dichloroisocyanurate

purpose of this book "anecdotal" means that statement is not based on a report published in a peer reviewed journal). This claim is based on the fact that the pH is NaDCC is slightly lower than that of hypochlorites which is highly alkaline. However the currently available evidence, both published (Parkinson et al., 1996; Niedz and Bausher, 2002) and anecdotal (plant-tc@lists.umn.edu) does not seem to justify the use of NaDCC except in very special cases and perhaps not even then. Benefits of NaDCC, such as they are, or if they exist at all, do not seem to justify the extra work and bother. The only exception may be the saving of contaminated seedlings or plantlets: "If your seedlings [or plantlets] are big enough and you … detect contamination [early] you may use 1 g/l NaDCC to sterilize. Add 5 ml [of] 1 g/l NaDCC enough to cover all surface for 2 hours, then withdraw liquid. Leaves may burn a little, but seedlings [and probably plantlets] survive. Link: https://picasaweb.google.com/knguyen101/RescueOrchidContamination#" (Kinh Nguyen, knguyen1001@gmail.com).

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[1] (which usually contains 5–6% sodium hypochlorite) in a glass desiccator, 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 (30 min is suitable for taro seeds). After treatment the container should be opened carefully to allow gas

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

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 (a 1-ml pipette can be used three times, but this should be avoided). 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 it has 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 and glass 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 vessels 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. Sometimes the cost of washing vessels may be higher than buying new ones.

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.

Specially designed and manufactured plastic covers with built-in filters, which allow gas exchange but prevent entry of microorganisms, are available and should be used when possible.

Tightly fitting bungs made of non-adsorbent cotton can also be used to cover containers (if the empty container can be lifted by holding the bung, it is tight enough). Both rubber stoppers and cotton bungs 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 bung 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 bungs 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, 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 (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 and are yellow and spindly. When moved to light such plantlets turn green and become normal in appearance. 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, with durations that can range from a few hours a day to continuous (24-h) illumination. 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

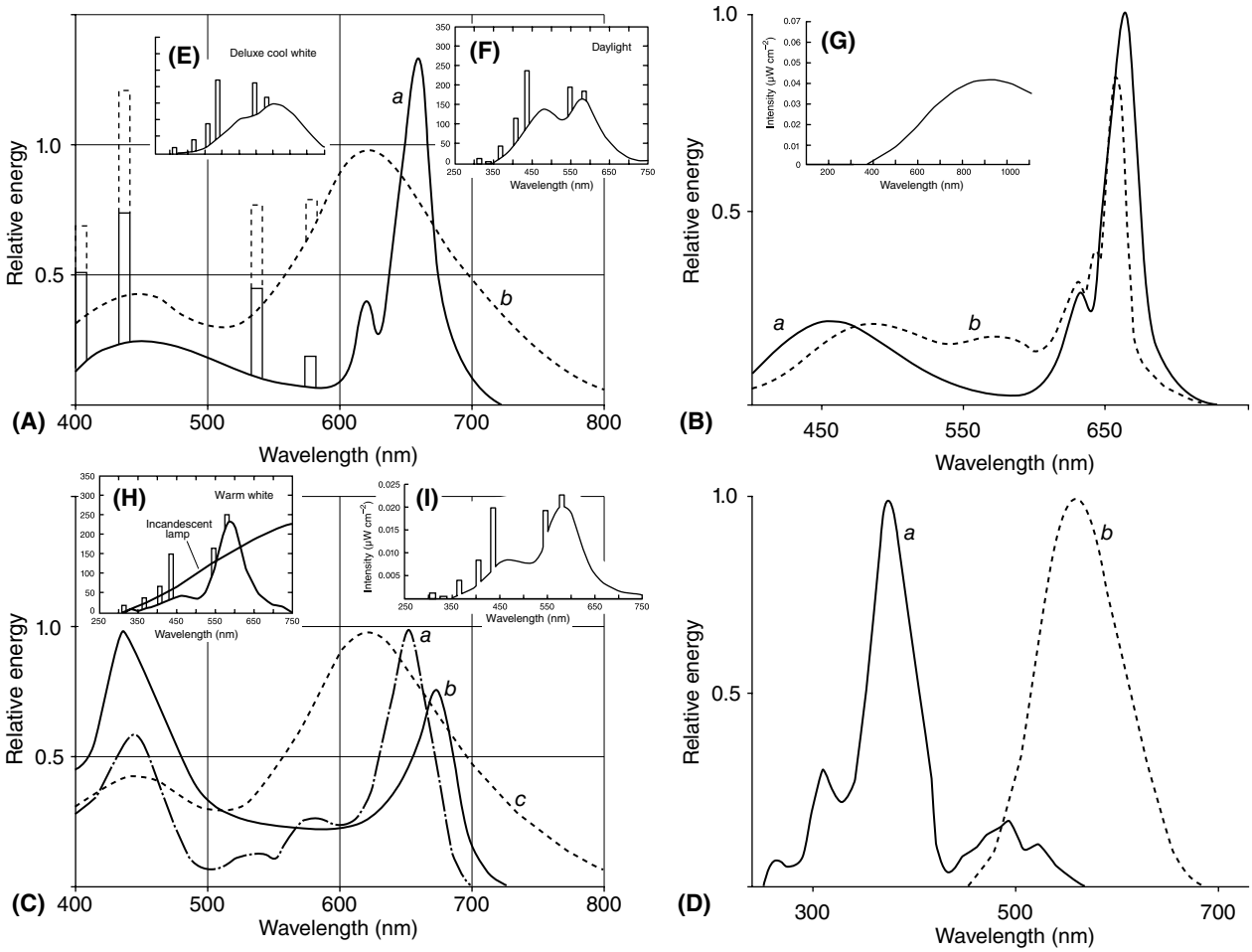


FIG. 2-4. **Light emission and action spectra. A. (*a*) Standard and (*b*) wide spectrum Gro-Lux (SGL and WSGL, respectively). B. Relative spectra of (*a*) American and (*b*) European fluorescent plant grow lights. C. (*a*) Chlorophyll synthesis and (*b*) photosynthesis action spectra compared with (*c*) 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.) Specification and models change constantly. Therefore the information here should be used only as a general guide.**

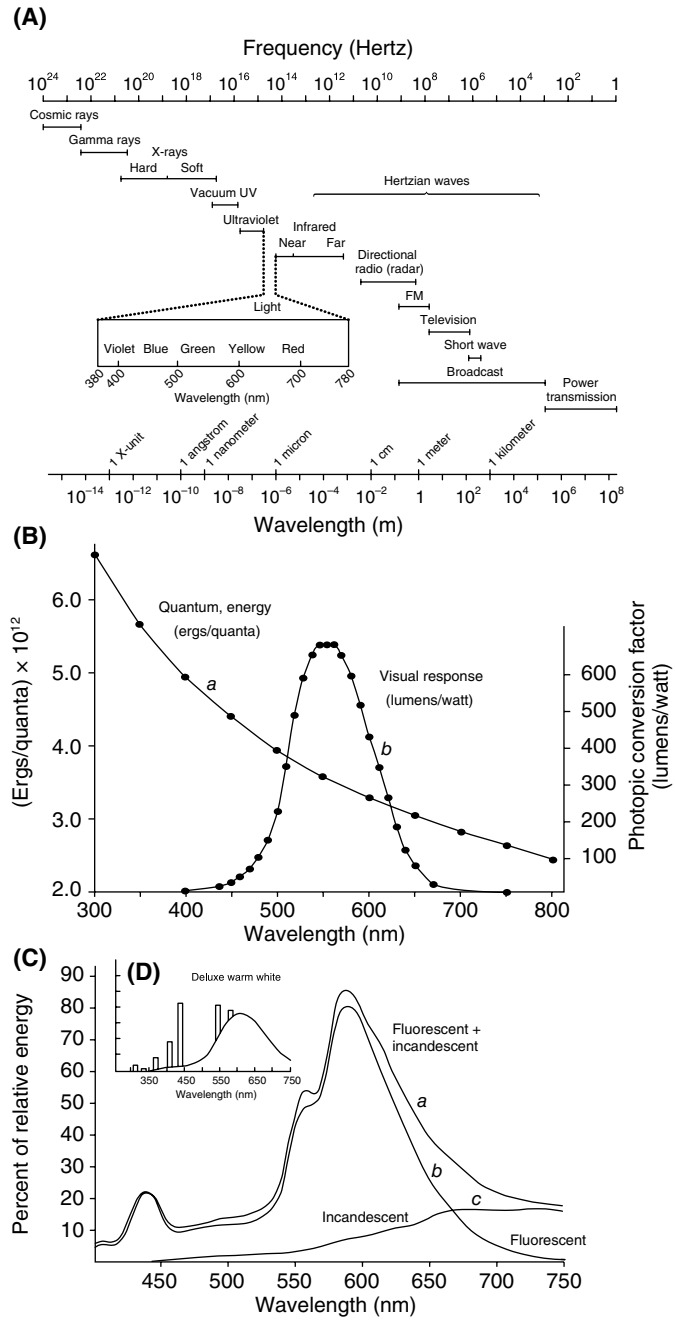


FIG. 2-5. **Light spectra and energy distribution. A. The electromagnetic (radiant energy) spectrum. B. Quantum energy (*a*), wavelength, and the human visual response (*b*). The sensitivity of the human eye differs from the action spectrum of photosynthesis (see Fig. 2-4C). This is why units used to 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.**

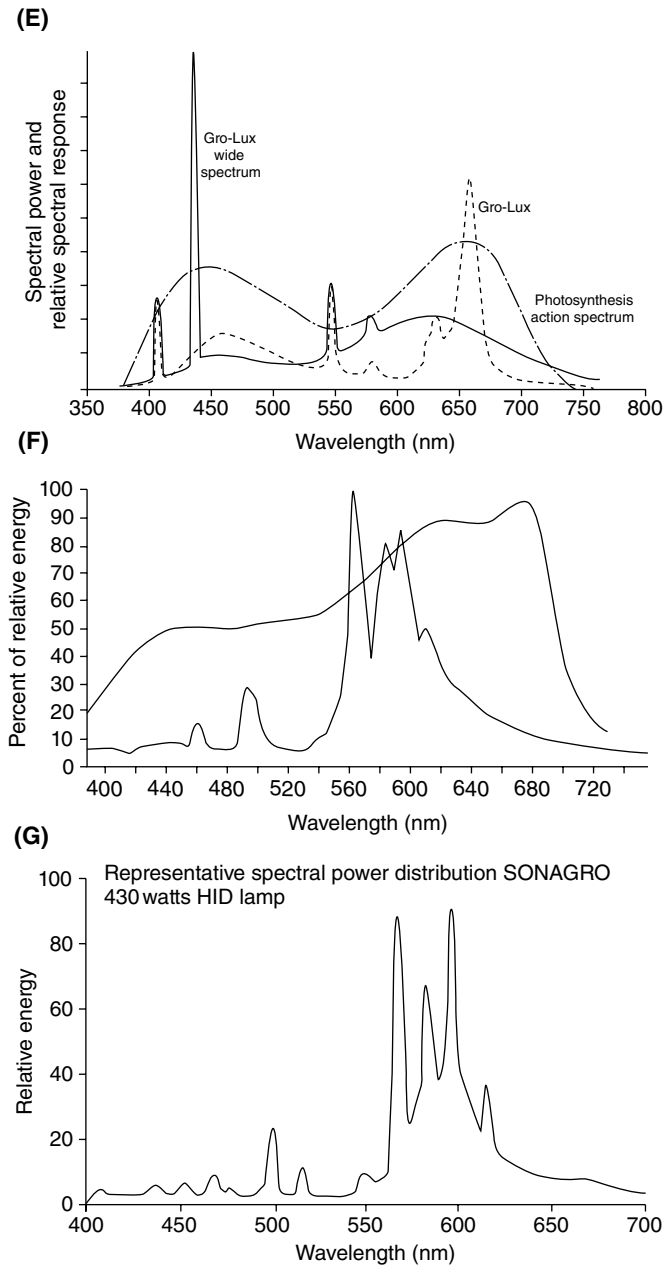


FIG. 2-5. (***Continued***) **E. Photosynthesis action spectrum and emission spectrum of Gro-Lux tubes. F. Hortilux spectral distribution and plant sensitivity curve. G. SON AGRO 430-W high intensity discharge (HID) tube. Specification and models change constantly. Therefore the information here should be used only as a general guide.**

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.

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. Abbreviation of candela is cd.

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 = 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 1 m^2. 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).

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_v) is the luminous flux or quantity of light that falls on a unit area. The unit is the lux and the expression is E_v = lumens/area, for example lm/m^2 or lm m^{-2}.

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

LED means light emitting diode.

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 (lm·s), **lumen minute** (lm·m), and **lumen hour** (lm·h) are the quantity and duration of light produced by a source.

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. Also see Thimijan and Heins, 1983.

[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.

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

	W cm^{-2} per foot candle	
Source	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. Also see Thimijan and Heins, 1983.

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 micromoles of photosynthetic illumination per square meter per second (μmol m^{-2} s^{-1} PAR) for several light sources (Hartman et al., 1988)[a]**

	Multiplication factors for conversion to[b]			
	W m^{-2} PAR		μmol m^{-2} s^{-1} PAR	
Light source	ft-c	lx	ft-c	lx
Average daylight	22.9	247	5.0	54
Cool white fluorescent tubes	31.5	340	7.0	74
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, mmol 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. Also see Thimijan and Heins, 1983.
[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 mmol 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 mmol m^{-2} s^{-1} PAR.

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

Lamp	Input (W)	Output in 400–700 nm wavelength (W)	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. Also see Thimijan and Heins, 1983.

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 (mmol m^{-2} s^{-1}) to foot candles	Foot candles to PAR (mmol 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]	10,020[b]	2000	13,740
Low-pressure						
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. Also see Thimijan and Heins, 1983.
[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 mmol m^{-2} s^{-1} × 5.01 = 10,020 ft-c. Multiply foot candles by the conversion factor to obtain PAR (or PPF). For example, 10,020 ft-c × 0.2 = 2000 mmol m^{-2} s^{-1}. Also see Thimijan and Heins, 1983.

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

	Conversion factors[b]			
			Photometric	
Light source	Radiometric, W m^{-2}	Photosynthetically 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. Also see Thimijan and Heins, 1983.
[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, s	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 lm·h = 60 lm·m.

Luminance (L_v) is candela per square meter. The unit is sometimes called "nit." Abbreviation is cd/m^2 or cm m^{-2}.

Luminous efficiency or **efficacy** (lm/W), lumens per watt is the ratio between luminous flux and the absorbed power.

Luminous emittance (M_v) is the light emitted from a surface. The unit is lm/m^2 or lm m^{-2}.

Luminous energy (Q_v) is lumens per second. The unit is sometimes called "talbot." Abbreviation is lm·s.

Luminous flux or **luminous power** (f or *F*) is the total quantity of light emitted per second by a light source. The unit is the lumen (abbreviation lm).

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 distributed uniformly over an area of 1 m^2; 10 lux equal 1 ft-c (i.e., to convert foot candles to lux it is necessary to multiply by 10; for example 200 ft-c = 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 1 m^2 at a distance of 1 m from the candle.

Lux second (lx·s) 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.

Photosynthetic photon flux density (PPFD) is the photon irradiance, expressed in moles of photons per square meter per second. See also photosynthetically active radiation and PAR.

Photosynthetically active radiation (PAR) is illumination in the wavelengths used in photosynthesis (400–700 nm). Light sources differ in the production of PAR (Table 2-15) and several conversion factors must be used (Tables 2-12–2-15). PAR

is expressed as μmoles (photons) per square meter per second (μmol m^{-2} s^{-1}). One mole of photons consists of 6.0222×10^{23} photons (i.e., Avogadro's number of photons).

PPFD see Photosynthetic photon flux density.

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).

Additional information on light units, their interconversion, and emissions by sources of illumination can be found in Thimijan and Heins (1983), http://www.autogrow.com, http://sunmastergrowlamps.com, and www.orchidlight.com. Statistical techniques for the evaluation of light quality on growing characteristics of *in vitro* cultures were developed by Chen and Hsu (2009).

Quality

This term refers to the spectra of light sources (see 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:

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

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.

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 about 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

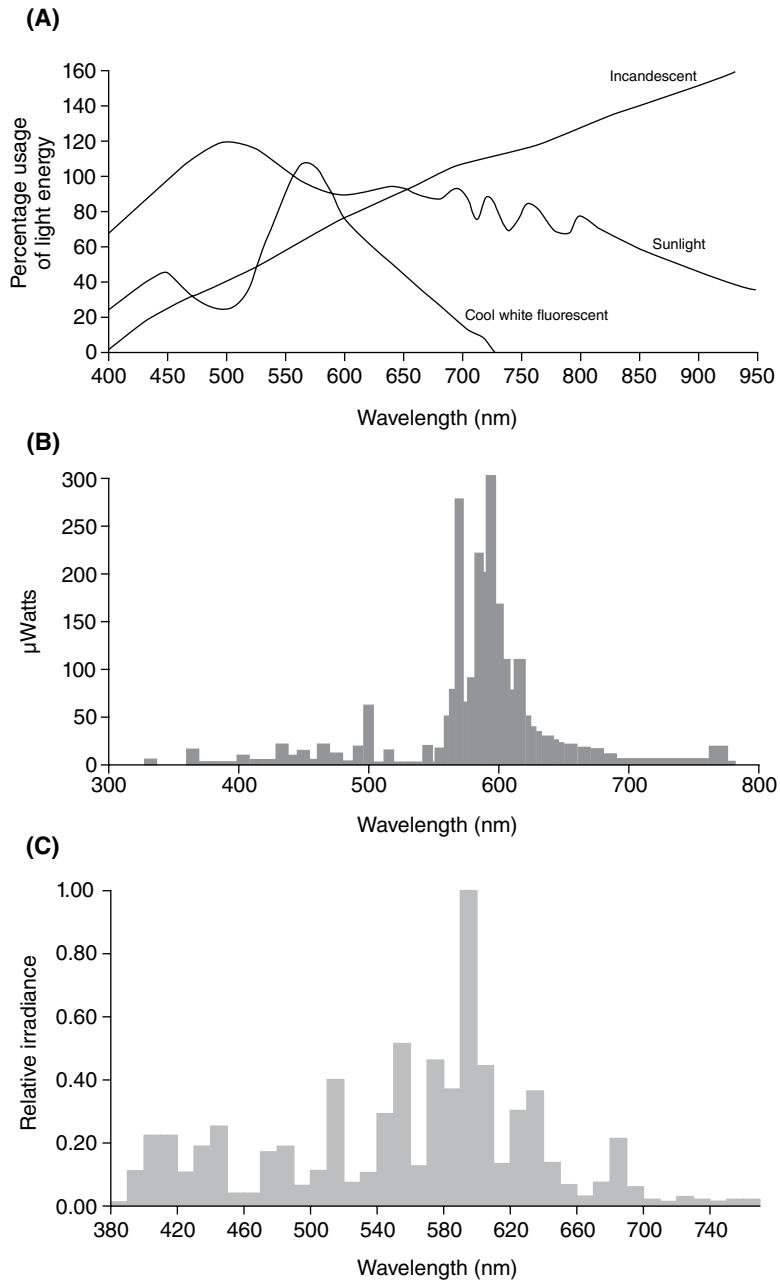


FIG. 2-6. **Light emission by several sources, and wavelength utilization by plants. A. Blue and red are the most important parts of the spectrum for photosynthesis. Flowering, germination, stem elongation, and aspects of growth and development are affected by red and far red light. Blue light affects 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). Specification and models change constantly. Therefore the information here should be used only as a general guide.**

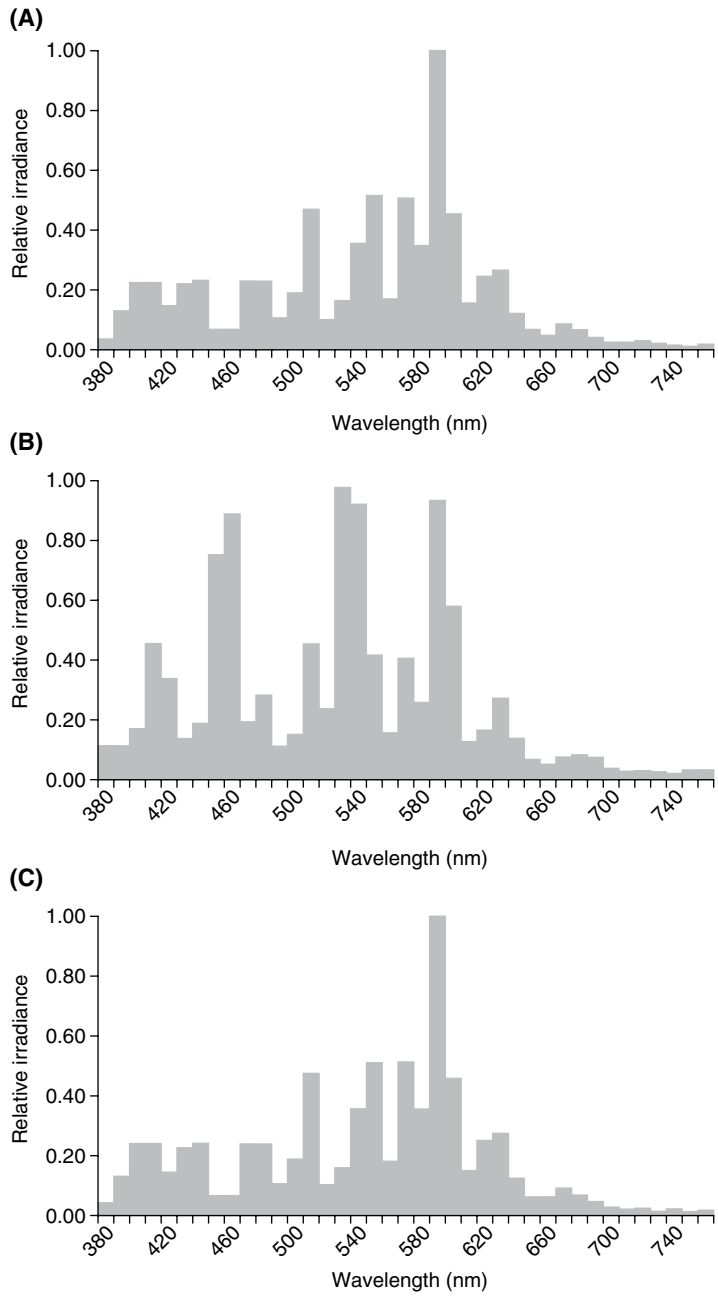


FIG. 2-7. **Light emission by metal halide bulbs. A. SunMaster Neutral Deluxe metal halide lamp. B. SunMaster Cool Deluxe metal halide lamp. C. SunMaster conversion lamp, high pressure sodium to metal halide. (*Source*: www.hydroponics.com.) Specification and models change constantly. Therefore the information here should be used only as a general guide.**

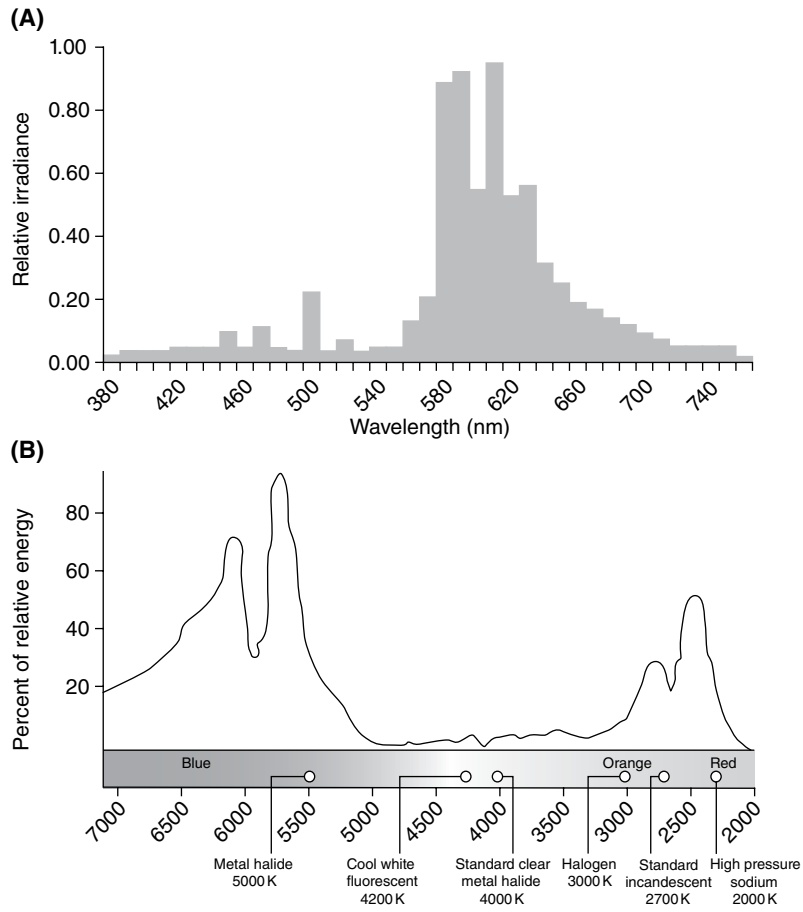


FIG. 2-8. **Light emission and temperature. A. SunMaster High Pressure Sodium Deluxe lamp (*source*: www.hydroponics.com). B. Light absorption by chlorophyll (*y*-axis and shaded areas), light temperature in Kelvin (*x*-axis), and several light sources (*source*: www.kingswoodorchids.com).**

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 and 2-7).

Increased availability, reduced prices, reduced use of energy, and longevity are making LEDs increasingly attractive as sources of illumination for tissue culture.

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,

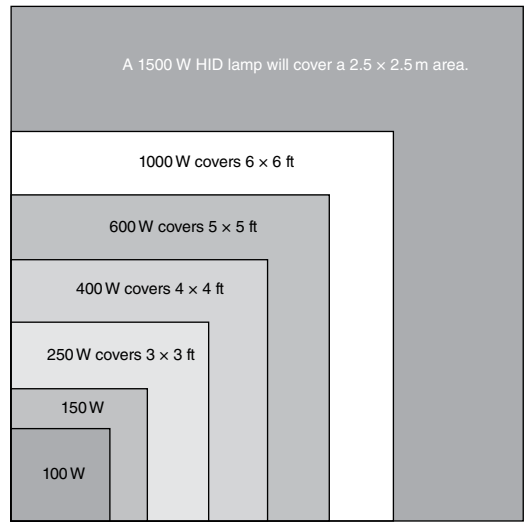


FIG. 2-9. **Areas of illumination. HID, high-intensity discharge. (*Source*: www.kingswoodorchids.com)**

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^{-2} 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.

Studies with red and blue light-emitting diodes (LEDs; Fig. 2-10) have shown that red light promoted leaf growth in *Cymbidium* plantlets, but decreased chlorophyll

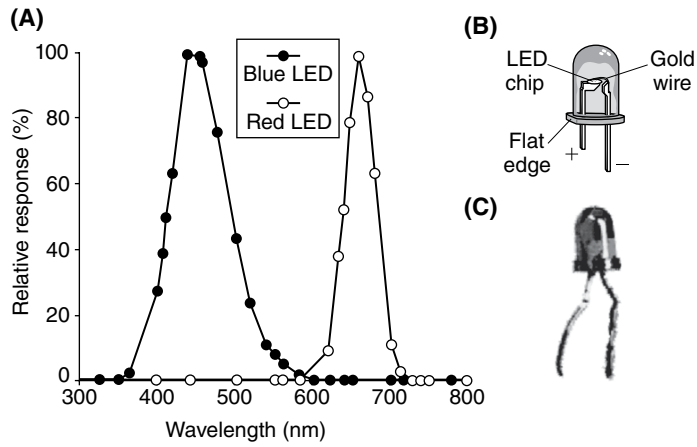


FIG. 2-10. **Light-emitting diodes: spectra (A), diagram (B), and photograph (C). Specification and models change constantly. Therefore the information here should be used only as a general guide.**

content. This was reversed by blue light. Growth under a combination of red and blue LEDs 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

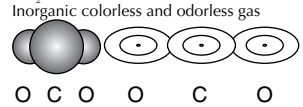
Despite its relatively low concentration in the atmosphere (Table 2-19), carbon dioxide (CO_2) plays a very important role in plant life. It is the source of carbon for photosynthesis, but the low atmospheric levels can be a limiting factor. Therefore higher levels of CO_2 can bring about increased growth 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).

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 *Cymbidium*, *Mokara*, and *Phalaenopsis*. Growth of *Mokara* Yellow was enhanced greatly 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

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	
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%: unconsciousness
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

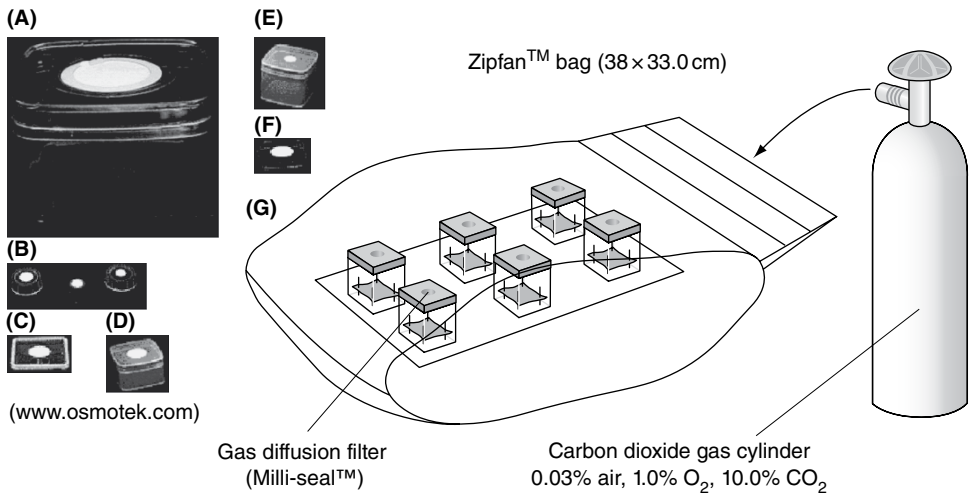


FIG. 2-11. **Carbon dioxide enrichment. A–F. Vented lids that allow for gas exchange, including the diffusion of carbon dioxide. G. A carbon dioxide enrichment system developed by Professor Choy Sin Hew (National University of Singapore) and Dr. John W.H. Yong (Nanyang Technological University, Singapore).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

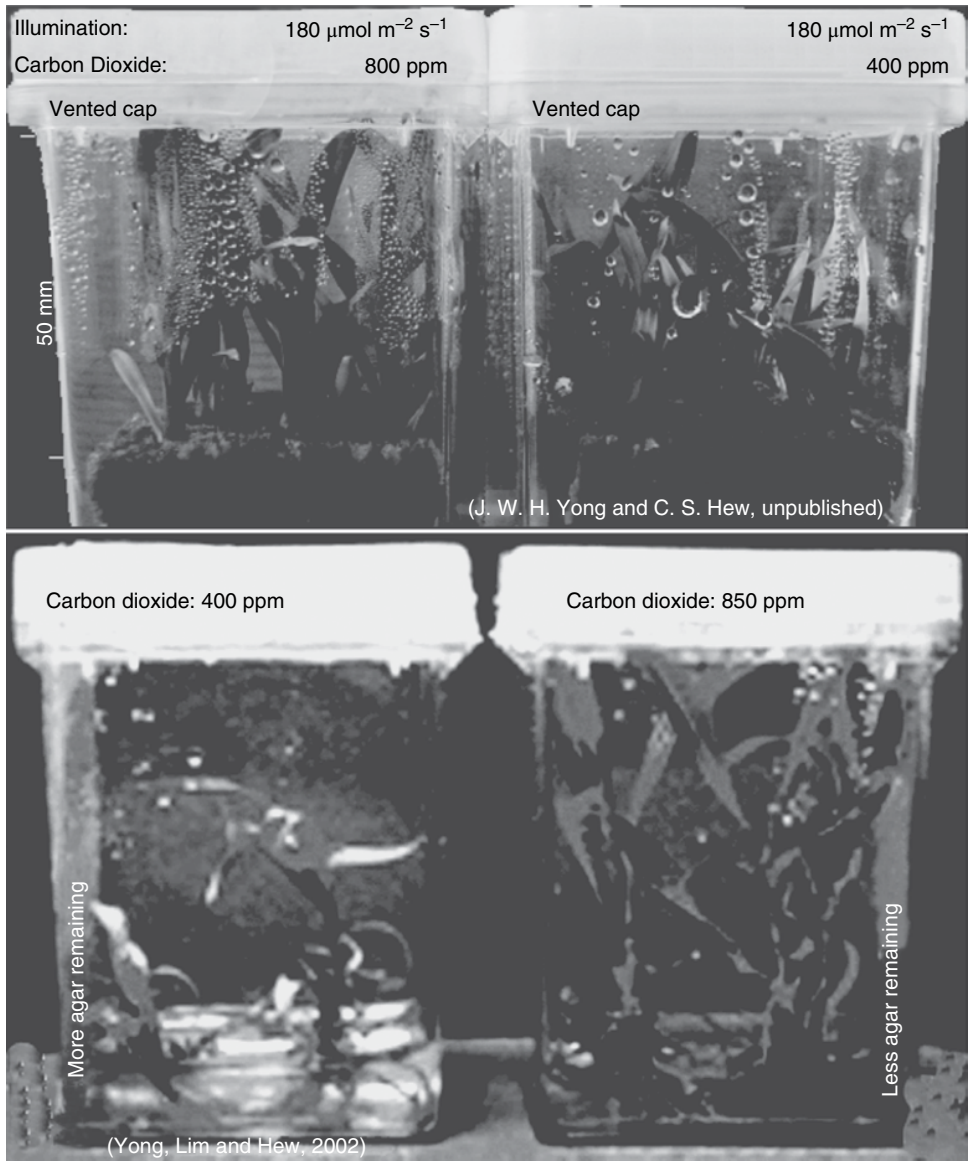


FIG. 2-12. **Effects of increased carbon dioxide levels (800 and 850 ppm) on the growth of *Cymbidium* plantlets. (*Sources*: Yong et al., 2002; Dr. J.W.H. Yong and Professor C.S. Hew, unpublished.)**

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, and www.unicornbags.com. However such vessels are not strictly necessary. Culture vessels (tubes, flasks) which are covered with cotton bungs 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.

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).

Tissue Explants

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.

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 and 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

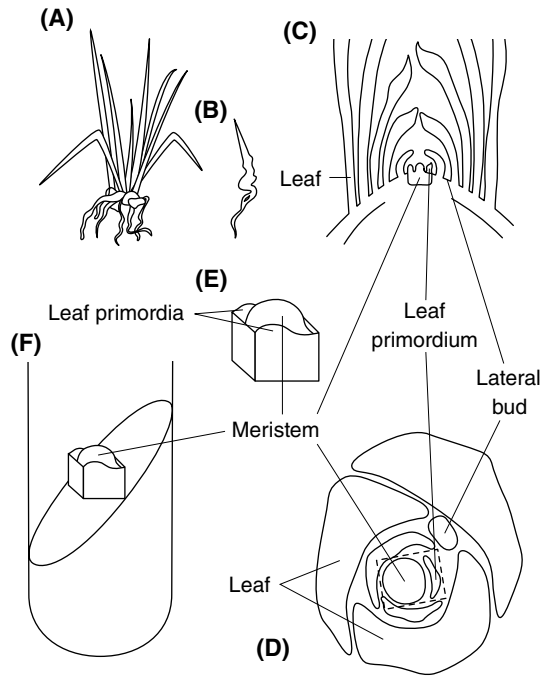


FIG. 2-13. **Excision and culture of a meristem. A. Mature plant; the meristem is located at the tip surrounded by leaf or petiole bases that must be removed to expose it. B. Excised shoot tip. C. Longitudinal section of excised shoot tip showing mature leaves, leaf primordial, lateral buds, and the 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.)**

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 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 hypochlorite in the brand being used (Table 2-20).

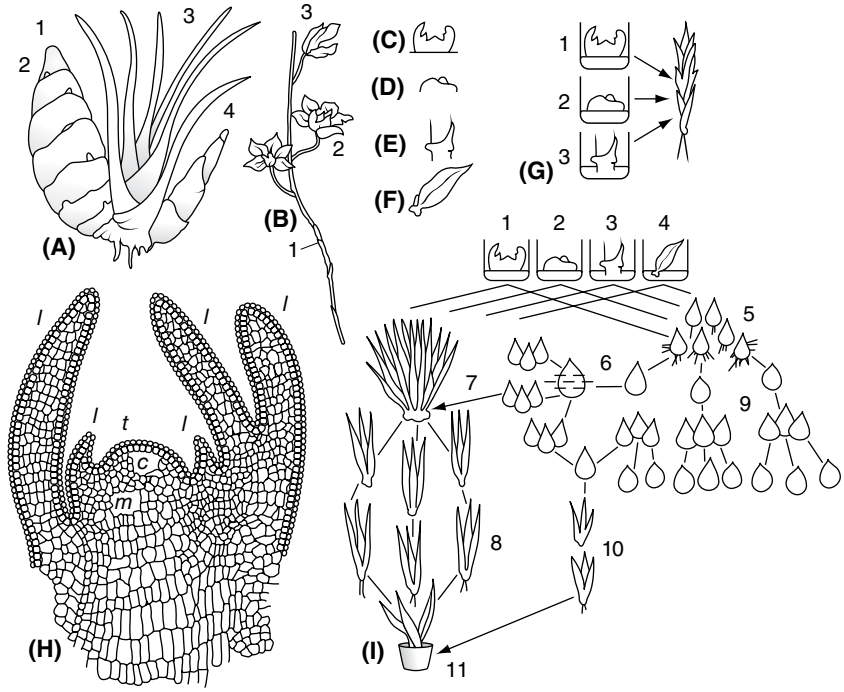


FIG. 2-14. **Sources, utilization, and development of explants. A. (1) Old, (2) young, and (3) new growth, and (4) pseudobulbs and axillary bud. B. Inflorescence with (1) axillary bud, (2) open flowers, and (3) bud in the process of opening. C. Apical meristem. D. Bud. E. Bud on a flower stalk. F. Young leaf. G. Shoots can be formed from (1) shoot tips, (2) buds, and (3) flower-stalk buds. H. Shoot tip (*c*, corpus; *l*, leaf primordia; *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.)**

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.

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 as horticultural plants) 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.

Thin layers from leaves, nodes, shoot tips, stems, and protocorms have been used for regeneration and transformation of orchids (Table 2-21; for a review see Teixeira da Silva, 2013*a*).

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

TABLE 2-21. **Use of thin layer explants for regeneration, organogenesis, embryogenesis, and transformation in orchids (modified from Teixeira da Silva, 2013*a*)**

Species	Source of thin layer	Response/ purpose	Reference
Aerides maculosum	Shoot tips	Organogenesis	Malabadi et al. (2009*a*)
Aranda Deborah	Shoot tips	Embryogenesis	Lakshmanan et al. (1995)
Coelogyne cristata	Protocorm-like bodies	Organogenesis	Naing et al. (2011*a*)
Cymbidium aloifolium	Protocorm-like bodies	Embryogenesis	Nayak et al. (2002)
Cymidium bicolor	Shoot tips	Organogenesis	Malabadi et al. (2008*a*)
Cymidium Sleeping Nymph	Protocorm-like bodies	Organogenesis	Vyas et al. (2010)
Cymbidium Twilight Moon "Daylight"	Protocorm-like bodies	Embryogenesis	Teixeira da Silva (2012*a*, 2012*b*); Teixeira da Silva and Tanaka (2006); Teixeira da Silva et al. (2005*a*, 2006*a*, 2006*b*, 2007*a*, 2007*b*)
Dendrobium candidum	Protocorm-like bodies	Organogenesis	Zhao et al. (2007)
Dendrobium draconis	Young stem	Organogenesis	Rangsayatorn (2009)
Dendrobium gratiosissimum	Protocorms, shoots, shoot tips	Organogenesis	Jaiphet and Rangsayatorn (2010)
Dendrobium nobile	Protocorm-like bodies	Embryogenesis	Nayak et al. (2002)
Doritaenopsis	Leaves	Organogenesis	Park et al. (2002*b*, 2006)
Eria dalzelii	Shoot tips	Organogenesis	Malabadi et al. (2008*b*)
Liparis elliptica	Shoot tips	Organogenesis	Malabadi et al. (2009*b*)
Paphiopedilum Armeni White	Flower bud	Organogenesis	Liao et al. (2011)
Paphiopedilum Deperle	Flower bud	Organogenesis	Liao et al. (2011)
Renanthera Tom Thumb	Leaf base	Organogenesis	Wu et al (2012)
Rhynchostylis gigantea	Stem and shoot tips	Organogenesis	Le et al. (1999)
Spathoglottis plicata	Nodes and leaves	Organogenesis	Teng et al. (1997)
Xenicophyton smeeanum	Shoot tips	Organogenesis	Mulgund et al. (2011)

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.

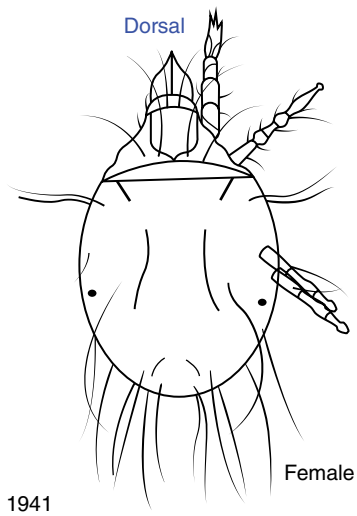
Internal Contaminants

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.

Mites and Thrips as Contaminants and Contamination Vectors

Mites (usually less than 1 mm in length) and thrips (ca 1.25 mm), being very small (Figs 2-15–2-18), can easily gain entry into culture vessels and move between them (Blake, 1988). The carry spores on their bodies and spread contamination. Both are common in greenhouses and whenever orchids are grown. They are carried into laboratories by laboratory personnel (on clothing and shoes, in hair, and on skin) and once there can easily enter into cultures. According to one estimate 50% of laboratories are contaminated by mites and thrips (Blake, 1988; van Epenhuijsen and Koolaard, 2004*a*). Mites multiply at phenomenal speeds (West and Preece, 2006) and can wreak havoc in laboratories or culture facilities within a very short time.

Tyrophagus perniciosus Zakhvatkin 1941

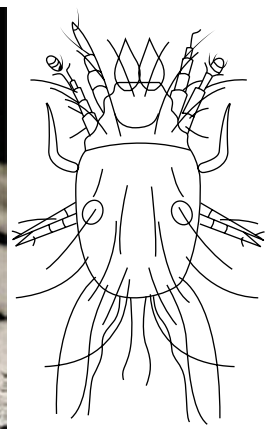


Species: *perniciosus*
Genus: *Tyrophagus*
Family: Acaridae
Order: Sarcoptiformes
Subclass: Acari
Class: Arachnida
Phylum: Arthropoda
Kingdom: Animalia
Size

	Length nm	Width
Female:	572	387
Male:	400	296

(ask.com)

FIG. 2-15. ***Tyrophagus perniciosus.***



Cheese mite
Species: *casei*
Genus: *Tyrophagus*
Family: Acaridae
Order: Sarcoptiformes
Subclass: Acari
Class: Arachnida
Phylum: Arthropoda
Kingdom: Animalia

Length: 450–700 nm

FIG. 2-16. ***Tyrophagus casei.***

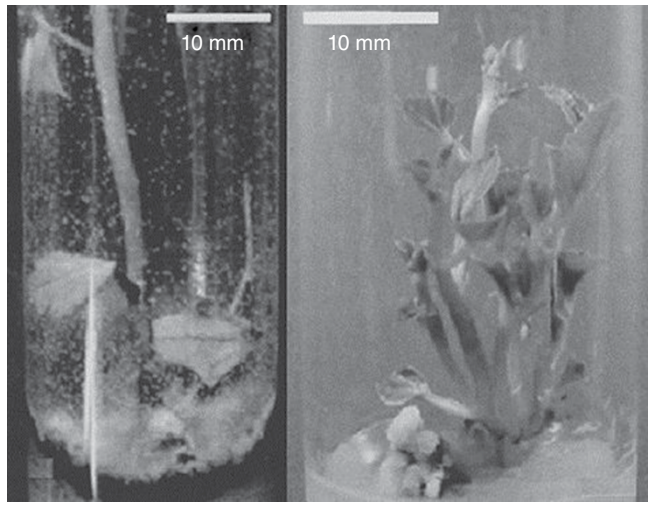


FIG. 2-17. **Cultures of *Hibiscus moscheutos* L. (left) infested and (right) free of mites. Courtesy Dr. Todd F. West and Dr. John E. Preece.**

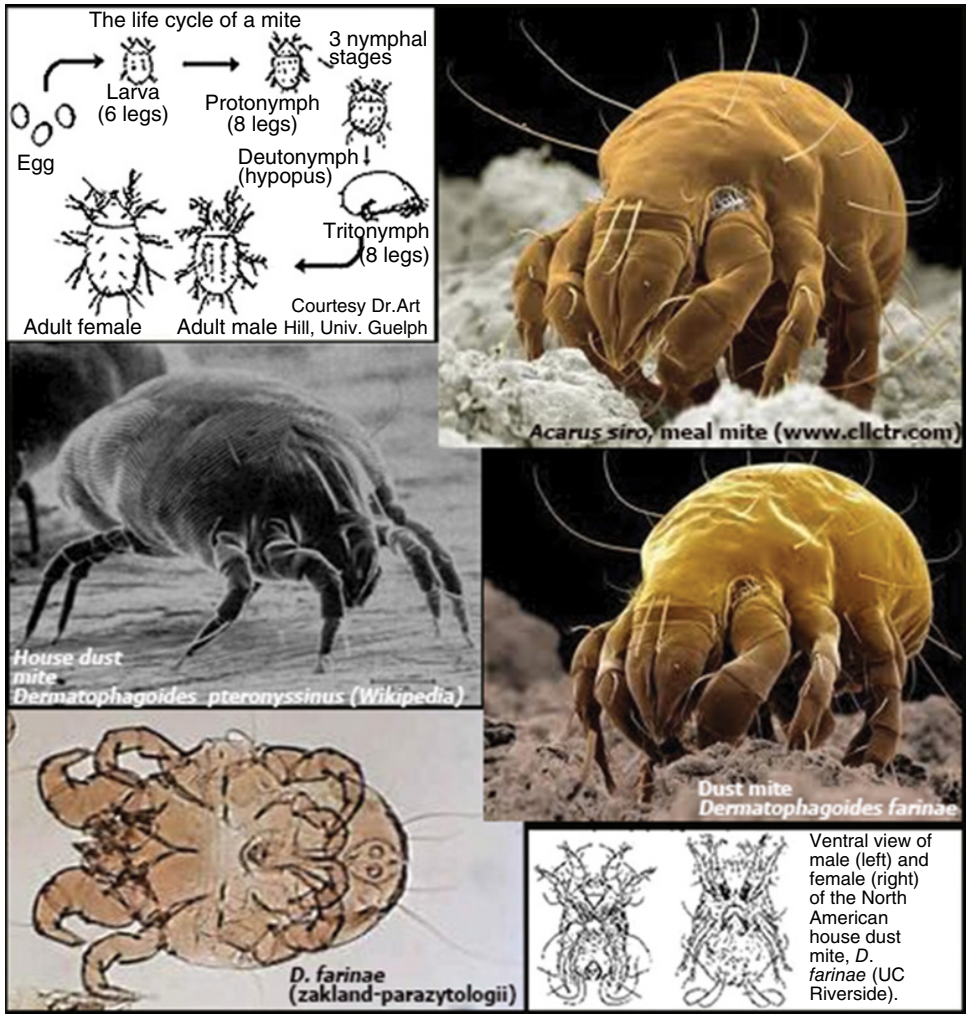


FIG. 2-18. **Several mite species. Life cycle courtesy M.E. Solomon, J.P. Melnik, A. Smith, C. Scott-Dupree, M.F. Marcone and A. Hill, Ontario Cheese Society.**

Several species, including *Tyrophagus perniciosus* (Fig. 2-15) and *Tyrophagus* (*Tyrolichus*) *casei* (Fig. 2-16) have been reported to infest tissue cultures. However since not much is known about the mites which infest orchid tissue cultures it is possible that other species may also be involved in infestations. In addition to being a problem themselves, the mites also spread fungal contamination as they move from flask to flask due to their attraction to sugar. Mites can usually be seen as small spots on the walls of culture vessels (Fig. 2-17).

Cultures which have been infected with mites for a longer period may also have webbing on the walls (Fig. 2-17). Both the mites and the webbing can be seen in more detail with a dissecting microscope or a magnifying glass (×15–25). Infections are hard to prevent and contamination is very difficult to combat let alone eliminate (Blake, 1988; van Epenhuijsen and Koolaard, 2004*a*, 2004*b*; West and Preece, 2006). As a result many countermeasures have been and are being proposed in the literature. Some are effective, others are not, and all are difficult, complicated and expensive. Many of them will be listed below in the hope that at least some may be effective under one set of circumstances or another. Like the propagation methods in Chapter 3 we have not tested any of the measures listed. Therefore those who may decide to use any of these practices must do so at their own risk. Preliminary testing is not only wise but strongly advised especially since the response of different orchids or their growth stages may vary.

Hygiene

Mites enter laboratories and culture rooms on the skin, hair, clothing, and shoes of workers and visitors, on tools and the outside of culture vessels as well as through any cracks (even very small ones) which are connected to the outsides, door frames, window jams, or air ducts. Damp walls, air blown by air conditioners or heaters or through wet mat of evaporative coolers, and any accumulation of dust and debris in a laboratory or culture room can also be sources of infestations. Plastic covering on shoes and hair as well as laboratory coats may reduce infestations, but cannot eliminate them because mites can be and are carried on the surface of the covers, protective clothing, hands, hair and faces. Sealing cracks, cleaning and filtering air ducts, and eliminating locations where mites can breed near or outside the laboratory are effective anti-infestation measures which must be attended to regularly. Cleanliness and prevention (quarantine) are crucial for the prevention of infestations. In the long run prevention is easier, simpler, less expensive, and more effective than combating or eliminating an infestation. Hygiene measures vary.

- Keeping all surfaces clean is imperative. This can be done by frequent washing of work surfaces and spraying them with 70% ethanol or isopropyl alcohol. The latter is inexpensive and easy to find in pharmacies as well as drug and hardware stores. However there is also a report that 70% alcohol may not be effective (van Epenhuijsen and Koolaard, 2004*a*).
- No rugs or door mats on floors because mites can breed on them.
- Preventing airborne contamination by filtering the air which comes into areas where cultures are maintained.

- Restricting access to laboratories only to individuals who should be there because mites can be carried on the clothing. Also those who come to a laboratory from where plants are being grown should cover their hair and wear clean laboratory coats over street clothes.
- Requiring persons who enter the laboratory to step on doormats treated with acaricides or alcohols because mites can be carried on shoes.
- Spraying or washing work and culture maintenance areas with acaricides. Surface areas should be allowed to remain in contact with an acaricide overnight. After that they should be washed with 70% alcohol. Proper protection (gloves, respirators, goggles) should be worn by those who apply acaricides since some of them can be toxic to humans (and pets). Mites can develop resistance to acaricides. Therefore acaricides should be changed as necessary. Availability, nomenclature, and licensing of acaricides can vary depending on country or area. The following compounds were or are used: Acephate (Orthene) at 10, 50 and 100 mg l^{-1} (West and Preece, 2006), Pirimiphos-methyl (Actellic), Dursban, Chlorocides, Kelthane, Murfit, Reldan, Sanmite, Tedion V-18, Telstar and Verimex (all available from agriculture and horticulture supplies dealers). To not run afoul of the law it is important to conform with local regulations and licensing.
- Washing clothing and laboratory regularly and/or placing clothing in a freezer at –20°C for 3–4 days. A temperature of –15°C for 60 min can bring about 100% mortality of mites (van Epenhuijsen and Koolaard, 2004*a*).
- Heating to 40°C for at least 48 hours can kill mites. It should not be difficult to raise the temperature in laboratories or culture rooms which are infested to this level and for that long. Temperatures of 55°C or higher for 30 min can cause 100% mortality of mites (van Epenhuijsen and Koolaard, 2004*a*), but raising temperatures in laboratories and culture rooms to this level even for a short time may not be easy.
- Checking cultures for infestation and removing infested ones.
- Discarding contaminated media and cultures.
- Wrapping contaminated cultures in a plastic film like Nescofilm or similar.
- Quarantining contaminated cultures or those being suspected of contamination if they cannot be discarded.
- Autoclaving contaminated media and cultures.
- Swabbing the outside of culture vessels with 70% alcohol or another dissinfectant.
- Placing Vapona strips in the laboratory or culture room.
- Painting walls, racks, cabinets, and other surfaces with Artilin (or another brand) acaricide paint.

Prevention

Physical barriers can be used to prevent entry of mites into cultures.

- Cultures can be placed on surfaces which are surrounded by oil (mineral or cotton seed), petroleum jelly (Vaseline), silicone grease, or water. This only prevents infestation by crawling mites and renders unpleasant the handling of cultures.
- Artilin 3A, an acaricide paint or bendiocarp (Ficam 10G; www.pestproducts.com/ficamw.htm) can prevent the spread of mites.

- Sealing cultures, especially with Parafilm M (www.sargentwelch.com) can prevent entry into cultures. Other films and tapes can also be used. However, sealing can also prevent aeration. Also, cracks may form in the film with time and mites can enter into cultures through them. Oil around caps has also been used.
- Covering or sealing cultures with sterile cigarette paper glued in place with copper sulfate glue (gelatine 20 g and copper sulfate 2 g in 100 ml water; gelatine can be found in food stores, copper sulfate can be purchased from www.sargentwelch.com) prevents entry by mites into culture without affecting air movement.
- Cotton plugs dipped in miticide or mercuric chloride can kill mites or prevent entry but these agents (specially the latter) are toxic to humans and should be avoided or if used handled with great care.
- Placing culture vessels in plastic bags that have tops which are folded but not sealed to allow for air exchange.
- Spraying with Actellic D may control mites, but this organic phosphate should be used with great caution because it is toxic to people and animals. This is an acaricide that will not affect thrips. An insecticide must be use instead or in addition.

It is important to keep in mind that mite infestations are not very common (in all the years we have worked on/with orchid seed germination and tissue culture we have neither experienced nor seen an infestation). The measures listed above are time-consuming and expensive and can be avoided by simply being careful, taking reasonable precautions, and keeping laboratories clean. However some or all of them may be necessary and should be used if there are reasons to believe that there is a possibility that mites will infest cultures, following an infestation which was eliminated.

An actual infestation is a serious problem which is hard, time-consuming, and expensive to overcome. One or more of the following measures should be used to clean up infested laboratories or culture rooms.

- Fumigation of infested rooms with appropriate fumigants. Such treatments can be very effective, but are extremely dangerous to humans, pets, and plants. That is why the fumigants have been banned in many countries and will not be mentioned here.
- Removal of all infested cultures from the laboratory and culture rooms and then autoclave and discard them.
- Spraying with 1.9% benzylbenzoate (available from several sources including, but not limited to, www.medical-and-lab-supplies.com/?gclid=CMS-jb-SzbECFUK4tgodlzsA1A), isopropyl alcohol (rubbing alcohol, i.e., 70% aqueous isopropanol), Acarosan (http://www.bissell.com/acarosan-dust-mite-spray/), bifenthrin (more of a repellant than a killer of mites; available as Talstar at http://www.domyownpestcontrol.com/bifenthrin-c-114_116.html, but there are other sources), azadirachtin (http://www.ozonebiotech.com/neem-extract.html), Omite, Kelthane, permethrin, acephate (West and Preece, 2006), or other acaricides. It is important to keep in mind that laws and regulations regarding pesticides may vary. Therefore it is advisable to check local regulations before using any of these compounds. Also, it is necessary to take proper precautions which ensure the safety of workers, visitors and pets.
- Dipping flasks in hydrogen peroxide and sealing them in plastic (polypropylene) bags.

- Cleaning with benzyl tannate or tannic acid.
- Placing paper towels or cotton balls or swabs soaked with *Eucalyptus* oil (which acts as a repellent) on shelves. The problem with this is that some people may object to the smell.
- Wiping infected areas with ethanol or isopropanol.

In general, mite infestation is not easy to eliminate and some of the above measures may have to be repeated several times. As is usually the case, prevention is much preferable to a cure.

Culture and Encapsulation Media

When added to culture media, acaricides or fungicides can prevent contamination.

- Acephate (Orthene) can be added to culture media to prevent contamination of *Simnondsia chinensis* (jojoba) cultures. This insecticide has not been tested with orchids. Therefore preliminary tests must be carried out prior to extensive use. Starting concentrations of acephate (Orthene) in such tests can be 10, 50 or 100 mg l^{-1}.
- A combination or acephate (Orthene) and the fungicide benomyl (no longer sold in the USA and may be hard to find elsewhere, and which must be used with great caution by all and especially by women who are, could be or may become pregnant because it is toxic and can cause severe birth defects including the birth of children without eyes; http://www.sweetbeet.com/growernet/Resources/Pesticides/Labels/benlate_SP_ca.pdf) when incorporated into alginate encapsulation medium at 10 mg l^{-1} of the former and 100 mg l^{-1} of the latter (Fig. 2-19) can eliminate contamination following storage at 5°C in the dark for 4 weeks.

Fumigation

Contamination can be reduced or eliminated through fumigation or treatment with several aerosols.

- Cultures can be placed within tightly closed enclosures in an atmosphere saturated with a miticide spray, camphor (www.herballoveshop.com, www.drugstore.com, www.iHerb.com among other sources) or paradichlorbenzene (PDB, which can be harmful and toxic to humans; it is available from chemical supply houses) to eliminate, reduce or control mites.
- Drops of Kelthane on cotton plugs in culture vessels placed inside a container can evaporate and fumigate the cultures.
- Aerosol of 16.7% ethyl formate (which according to some sources should be used with caution because it is volatile and highly flammable unless it is formulated with liquid CO_2 and used as the Vapormate brand which is available from a number of sources including, but not limited to, http://www.fabsurplus.com/sdi_catalog/salesItemDetails.do;jsessionid=B09F02AE01368AD1A072BB84F50AA510?id=16383 or http://eurosteam.com/catalog/index.php?cPath=49) can control mites.
- Mites are killed by an atmosphere which contains 65% or 85% CO_2.

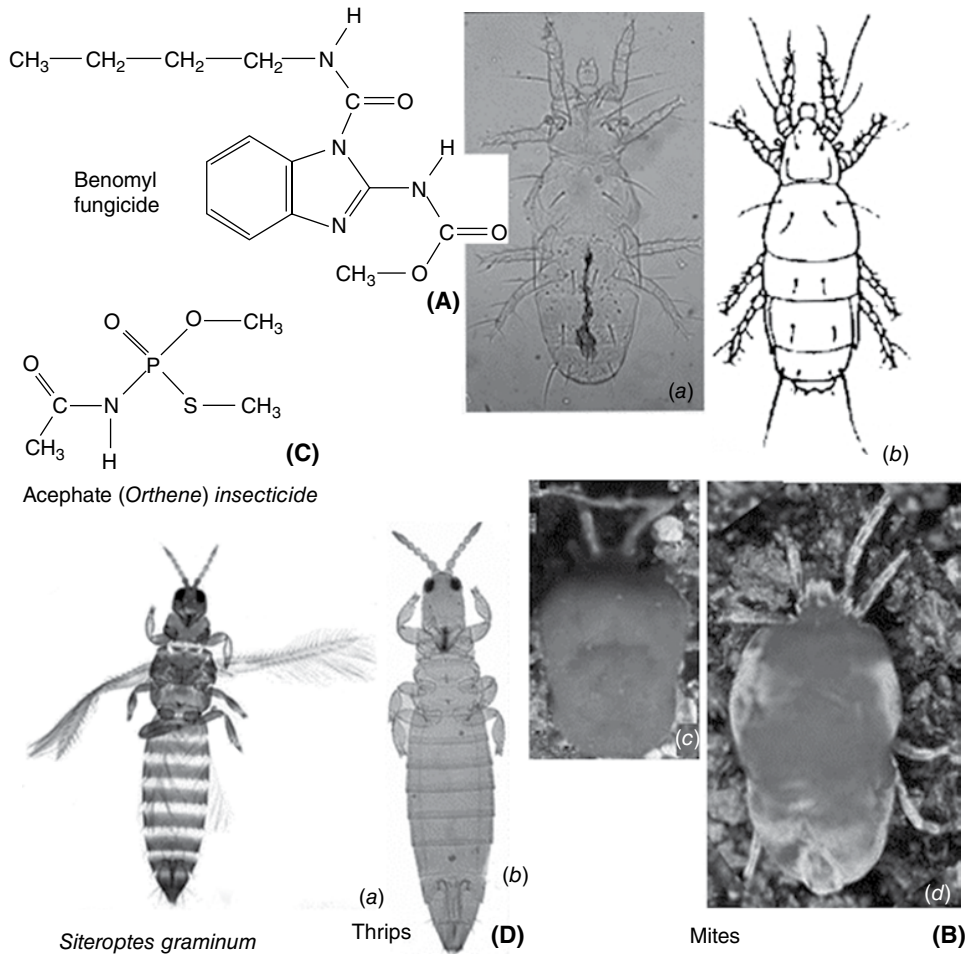


FIG. 2-19. **Structures of (A) acephate and (C) benomyl. (B) Mites (*a*, *c*, *d*, photographs; *b*, drawing). (D) Winged (*a*) and wingless (*b*) thrips. (*Sources*: http://www-vaxten.slu.se/marken/markdjur/siteroptes_graminum.htm, http://zooeco.com, www.alanwood.net/pesticides/acephate.html, www.alanwood.net/pesticides/benomyl.html, http://entoplp.okstate.edu/ddd/insects/thrips.htm)**

- Dichlorovos (a toxic organic phosphate which should be used with care; available from http://www.chemdacheng.com/pages/product481277_en.htm) when applied as an aerosol can kill mites.

Eradication

Solutions which contain 1% available chlorine can be poured into flasks for 20 min to 4 h or longer to kill mites and eliminate infestation. This is a drastic treatment which can work but may damage or kill plants.

None of these methods are inexpensive, simple, easy to use, and always effective. Therefore prevention and good hygiene remain the best anti-mite and anti-thrips measures.

Thrips

Thrips (Fig. 2-19) can also infest cultures. Measures similar to those employed against mites can be effective to counter thrips infestations.

Springtails

Although springtail (Fig. 2-20) infestations are rare, they can be devastating. The measures used against mites should work in combating springtails except that insecticides should be used in place of acaricides and *Eucalyptus* oil may not work as a repellent.

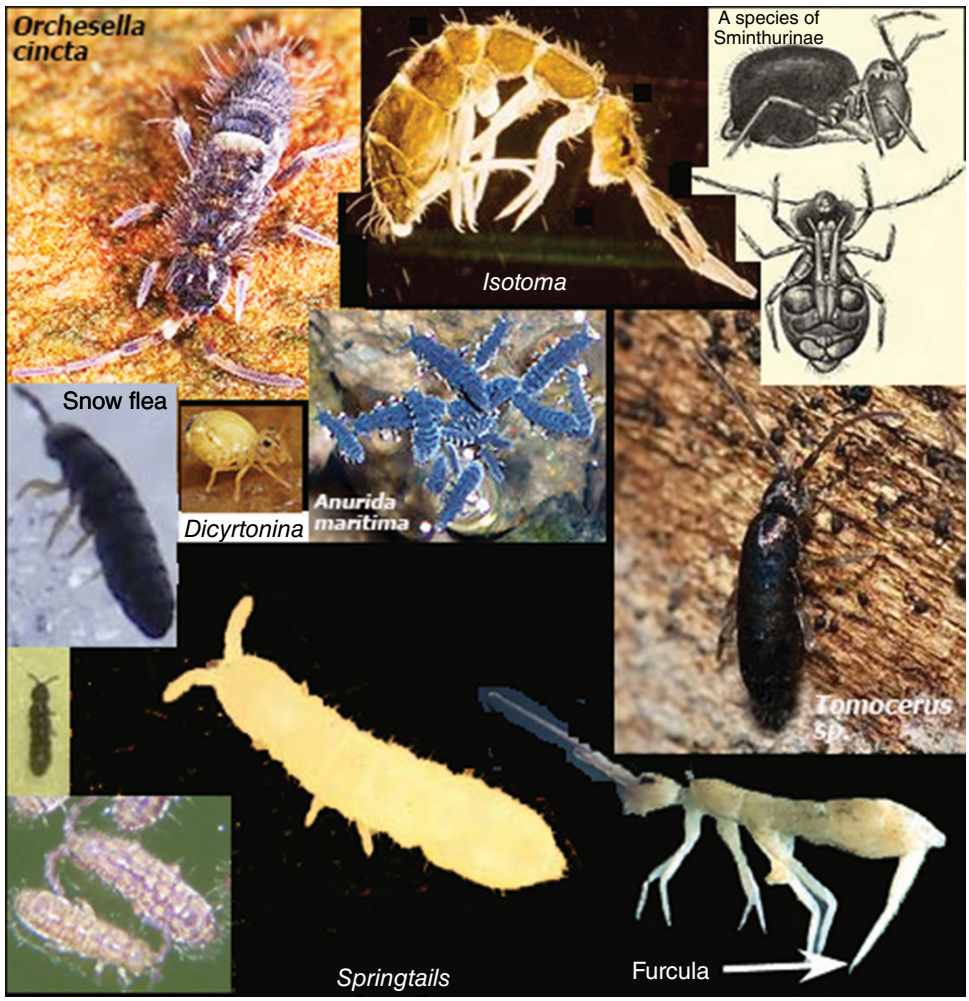


FIG. 2-20. **Springtails.**

Virus infections and testing

As indicated in Chapter 1, virus infections have become a more serious problem after the advent of micropropagation than before. Therefore it would be wise to determine if plants are virus-free before the removal of explants. Enzyme-linked immunosorbent assay (ELISA) and easy-to-use test strips, all specifically designed for orchid viruses, are available from Agdia, 30380 County Road 6, Elkhart, Indiana 46514, Telephone: 1-574-264-2615 or 1-800-62-Agdia (1-800-622-4342), FAX: 1-574-206-9360, e-mail: info@agdia.com, website: http://www.agdia.com.

Consideration of viruses which attack orchids is beyond the scope of this book and the expertise of the authors. Information about Agdia products (above) and the testing procedure (below) are presented to assist those who may wish to test their orchids. Neither the authors nor the publisher assume any responsibility for the use of these products, the results, and their interpretation and possible virus infections of plants produced through micropropagation. Those who may decide to use the Agdia ELISA or strip assay to test their orchids do so entirely and only at their own risk. This statement is made for legal reasons and due to the possibility that the assays may not be used properly. It should not be interpreted to suggest or imply that there are problems with the Agdia assays.

The Agdia assay is cleverly designed and very easy to use. A potential explant source infected (Fig. 2-21A) plant with virus spots (Fig. 2-21Av) on its leaves (Fig. 2-21Al) is tested by excising a leaf section, 2.5 × 2.5 cm in size (Fig. 2-21B), preferably one with a virus spot (Fig. 2-21Bv) in it. A plastic bag containing buffer (Fig. 2-21C) should be taken from the package. The leaf section should be placed inside the bag (Fig. 2-21D1, D2) and macerated (Fig. 2-21E, F) from the outside with an eraser, the dull end of a pen, or by gently hitting wth a hammer (taking care not to damage the container). A strip containing reagents (Fig. 2-21H) should be taken from its foil packaging (Fig. 2-21G) and dipped in the macerated leaf (Fig. 2-21I). Only the control line (Fig. 2-21J2) will indicate if the leaf is not infected. Two lines and the control will develop (Fig. 2-21J1) if the leaf is infected with Odontoglossum ringspot virus (ORSV) and Cymbidium mosaic virus (CymMv). Only one of the virus lines will show up in the case of a single infection.

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 Ichihashi 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.), 0.5% Driselase (Kyowa Hakko Kogyo Co., Ltd.), $Ca(NO_3)_2 \cdot 4H_2O$ 160 mg 100 ml^{-1}, KH_2PO_4 10 mg 100 ml^{-1}, 2-(*N*-morpholino)ethane sulfonic acid (MES)

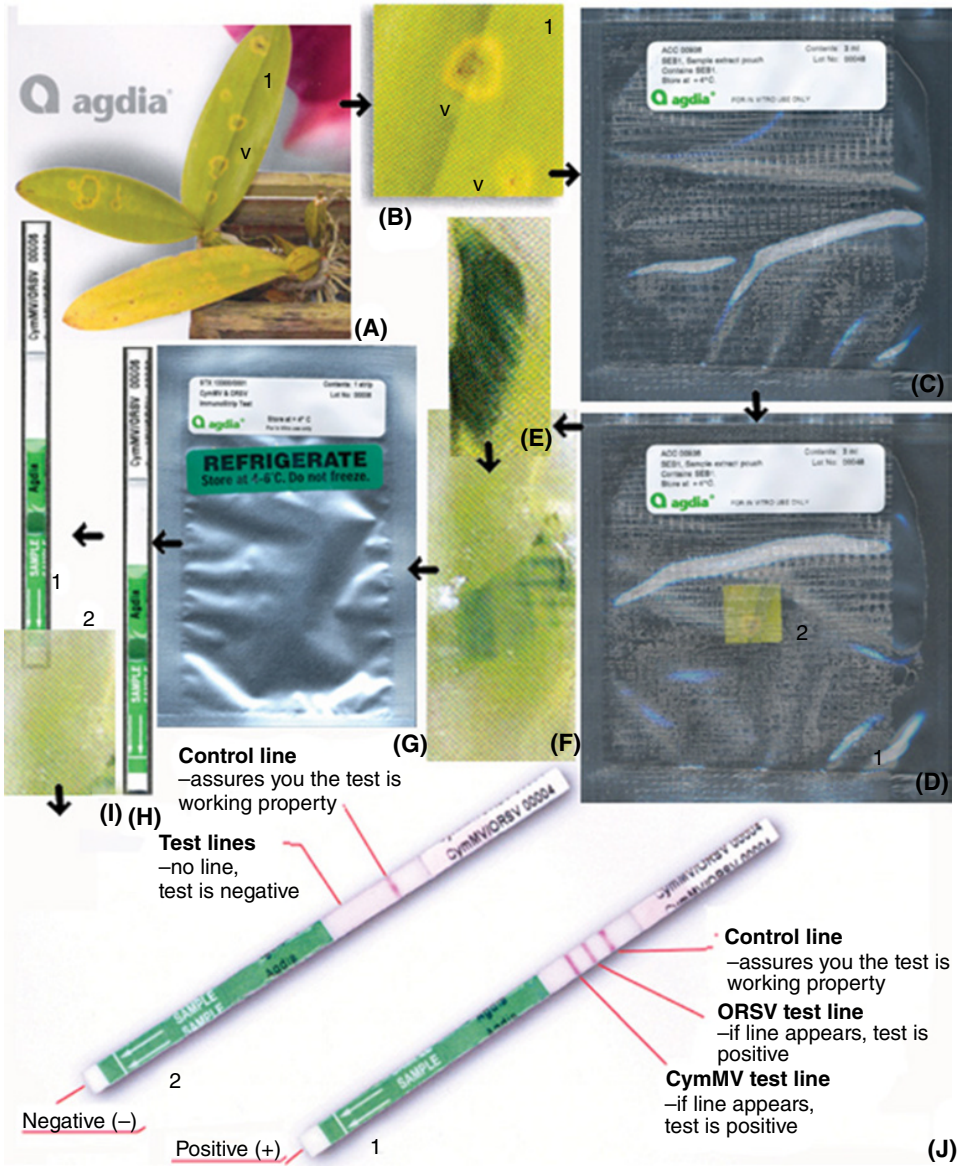


FIG. 2-21. **Virus testing with the Agdia immunostrip. A. Infected plants with virus spots (v) on its leaves (l). B. Leaf (l) section, 2.5 × 2.5 cm with a virus spot (v) of the kind that should be taken for a virus assay. C. A sample extraction pouch. (Label reads, line 1 "ACC 0093 Contents: 3 mL"; line 2 "SEB1, Sample extract pouch. Lot No. 00048"; line 3 "Contains SEB1"; line 4 "Store at +4°C"; bottom line "Agdia for in vitro use only".) D. Leaf section placed inside the bag. E. Tissue sample at the start of maceration. F. Macerated sample. G. Foil package. (Label reads, line 1 "STX 13300/0001 Contents: 1 strop"; line 2 "CymMV & ORSV Lot No. 00006"; line 3 "ImmunoStrip Test"; line 4 "Store at +4°C"; bottom line "Agdia for in vitro use only".) The second label is green and reads "REFRIGERATE" on the top line and "Store at 4–6°C. Do not freeze". H. Immunostrip. It has a white area on the bottom. Above it is a green area with two downward-pointing arrows above a horizontal line near the bottom. The word "SAMPLE" in white is above the arrows. The remainder of the strip is white. This is where the test and control lines appear. Near the top are the letters "CymMVORSV" and numerals (00004 in this case). I. Dipping the immunostrip in the macerate. J. Strips showing positive (1) and negative (2) results. This figure is a collage of illustrations taken from Agdia publications and a package containing an extraction pouch and an immunostrip, all courtesy of Lesley Staples, Internetopma; Account Representative, Agdia Inc., Marketing Division, lstaples@agdia.com, 3150 Windsor Court, Elkhart, IN 46514, USA.**

58.5 mg 100 ml^{-1}, and sorbitol 0.3 mol l^{-1}, pH 5.6. Professor Ichihashi's method for the isolation of protoplasts is now published in 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 bungs, following the introduction of tissues, and after replacing the bungs.

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.

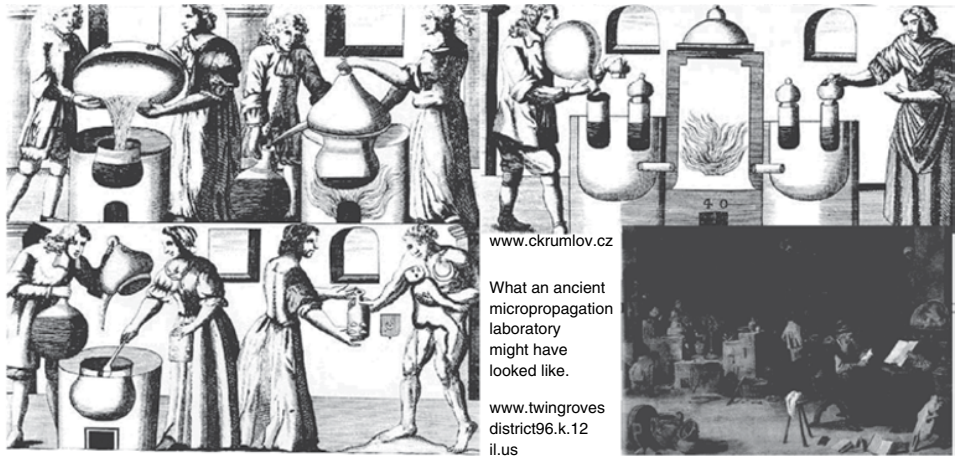
Washing Glassware

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.

Statistics

Research workers should subject their data to statistical analysis. Methods suitable for plant tissue culture data collection, analysis and presentation are available (Compton, 1994; Compton and Mize, 1999; Mize et al., 1999), but beyond the scope of this book. General statistics and graphing computer programs which can be used in micropropagation research are SigmaPlot (http://www.sigmaplot.com/products/sigmaplot/sigmaplot-details.php) and SPSS (http://www-01.ibm.com/software/analytics/spss/). Both provide free trial versions.

In Memoriam: Professor Adisheshappa Nagaraja Rao (1925–2014), friend, mentor, collaborator, author of the first review on orchid micropropagation.



What medieval micropropagation laboratories and technicians might have looked like.

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. Several *Saccolabium* species were/are sometimes classified as belonging to the genus *Acampe*.

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).

Micropropagation of Orchids, Third Edition. Tim Wing Yam and Joseph Arditti.

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, section 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.

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

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)	1.0	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.

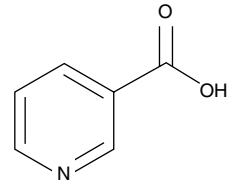
[d] Like other iron salts, this one is hard to dissolve. It is therefore preferable to chelate the iron. To prepare a stock solution of chelated iron dissolve 3.73 g chelating agent, Na_2EDTA, and 2.78 g of ferrous sulfate, $FeSO_4 \cdot 7H_2O$, in 1 l water. Stir and/or heat until both are completely 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 (known as nicotinic acid) is one of the most commonly used vitamins in orchid micropropagation. Pharmaceutical and/or nutritional supplement preparations should be avoided, or used only after prior testing because the tablets contain binders and/or other unknown additives.

***Acampe praemorsa*. (*Source*: *Annals of the Royal Botanic Gardens Calcutta*, 1891)**

Plantlet Regeneration from Leaf Tips of *Acampe rigida*

In Hong Kong *Acampe rigida* grows on rocks in sunny locations. 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.

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	25.0 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}		
(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}		
	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.

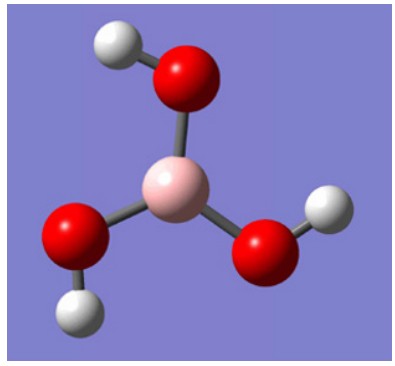
[f] If the cytokinin fails to dissolve add a few drops of dilute HCl.

[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.

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	39.1 g l^{-1}	10	
2	Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$[b]	828	82.8 g l^{-1}	10	
3	Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	172	17.2 g l^{-1}	10	
4	Potassium nitrate, KNO_3	747	74.7 g l^{-1}	10	
5	**Microelements**[c]				
(a)	Boric acid, H_3BO_3	1	100 mg l^{-1}		
(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}	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)	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.



Boric acid. (*Source:* Greeves, 2015. Reproduced with permission from N. Greeves and Oxford University Press.)

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 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 rigida*. [*Source*: Badlydrawnboy22 https://commons.wikimedia.org/wiki/File%3AAcampe_rigida_flower.jpg. Used under CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0).]**

***Acampe rigida*. (*Source*: King and Pantling, 1898.)**

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 Philips (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.

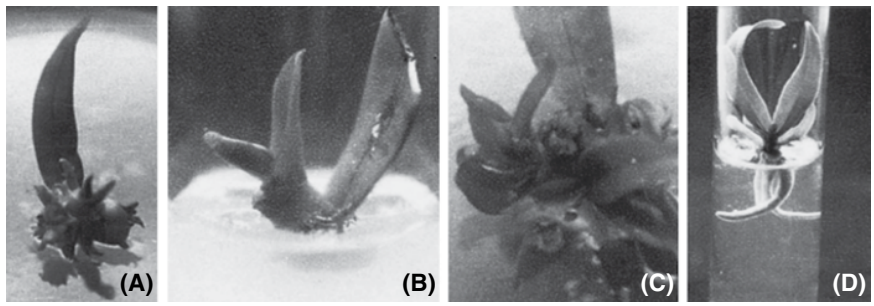


FIG. ACAMPE-1. **Micropropagation of *Acampe*. A. Entire leaf is inserted vertically in the medium. Multiple buds develop on its base after 10 weeks of culture on MS medium containing TDZ 1.0 mg l^{-1}. B. Only a single shoot developed from the base of a leaf (also inserted vertically) after 12 weeks on MS which contains NAA 1 mg l^{-1}. C. Multiple shoot formation after 12 weeks on MS supplemented with TDZ 1.0 mg l^{-1}. D. A rooted shoot on MS with IBA 2 mg l^{-1}. (*Source*: 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., 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}	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	83.0 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} 0.1 N 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.

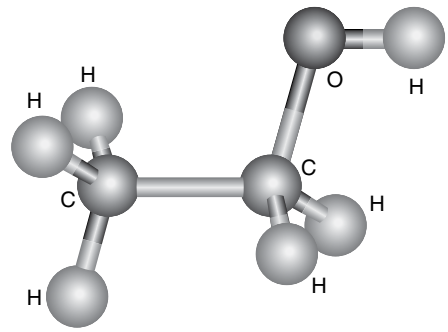
[d] Add all microelements to the same 1 l of distilled water, and stir and/or heat until all are dissolved. Dispense the volume indicated per liter of culture medium. The original recipe (Murashige and Skoog, 1962) lists $ZnSO_4 \cdot 4H_2O$ as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ 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 0.1 N 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.



Ethyl alcohol (ethanol). (*Source*: www.nyu.edu/pages/mathmol/library/hydrocarbons)

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}	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	83.0 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 0.1 N KOH or HCl respectively.
[g] A combination of NAA 1 mg l^{-1} and BA 10 mg 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.

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.

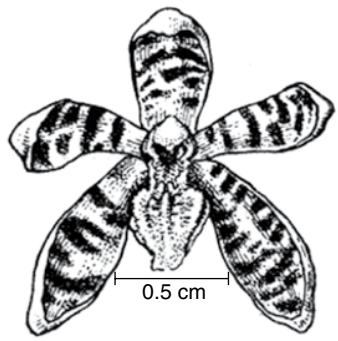
TABLE ACAMPE-6. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for rooting *Acampe praemorsa* shoots produced by leaf explants (Nayak et al., 1997*a*)**

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}	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	83.0 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 0.1 N KOH.
[g]A combination of NAA 1 mg l^{-1} and BA 10 mg 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.

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 *Acampe praemorsa* was also developed in India (Kanjilal et al., 2001).



***Acampe praemorsa* flower. (*Source*: Abraham and Vatsala, 1981)**

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 BA 1 mg 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 NAA 1 mg l^{-1} (Table Acampe-8). For callus formation followed by PLB production the explants should be cultured on MS with BA 1 mg l^{-1} and NAA 1 mg l^{-1} (Table Acampe-9). To induce shoot and root formation, the PLBs should be transferred to fresh MS containing BA 1 mg l^{-1} (Table Acampe-7).

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.

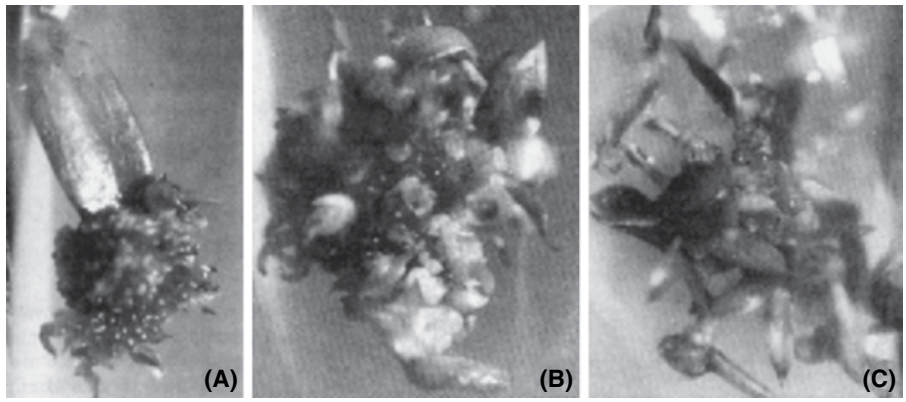


FIG. ACAMPE-2. **Leaf culture of *Acampe praemorsa*. A. PLBs on explant after 8 weeks of culture on MS medium containing BA 1 mg l^{-1}. B. Shoot on PLB after 12 weeks of culture (medium not indicated in caption for illustration in the original paper). C. Plantlets which formed after 12 weeks of culture (medium not indicated in original caption). (*Source*: Pyati and Murthy, 1999.)**

A bitter tonic called *Rasna* made from the roots *Acampe praemorsa* in India is used as a remedy for rheumatism, sciatica, neuralgia, syphilis, and uterine disorders. However, it has been reported to have no therapeutic value (Teoh, 2016).

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 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)	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}	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	83.0 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}	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	83.0 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.

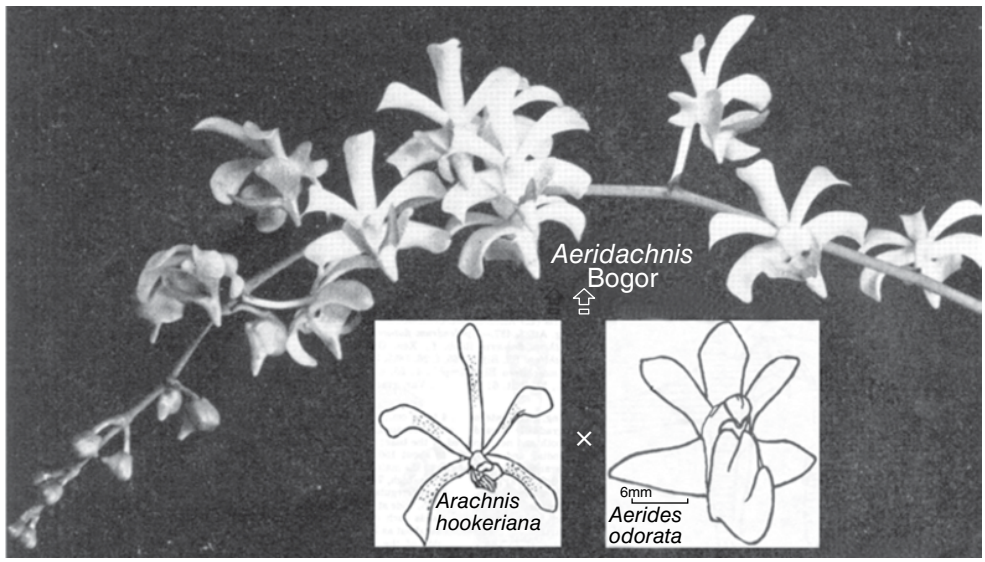
[g] Add items 1–7 and 9 to 900 ml of distilled water (item 16), adjust pH to 5.6, add sugar (item 15), and raise volume to 1000 ml with distilled water (item 16). Bring the solution to a gentle boil and add the agar (item 17) slowly while stirring. The agar can also be added to the cold water which is 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.

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 lawrenceae*) 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.



***Aeridachnis* Bogor. (*Sources*: main photo, Addison and Henderson, 1954; inset drawings, Holttum, 1964.)**

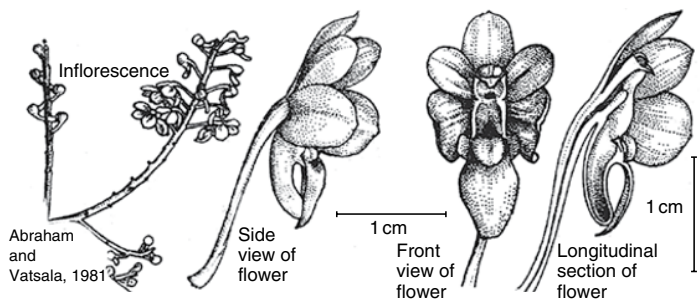
Aerides

An epiphytic genus, *Aerides* consists of approximately 20 species which are found in South East Asia.

Aerides was named by João de Loureiro (1717–1791) in his *Flora Cochinchinensis* (1790). The name is derived from the Greek *aer* (air) and *eides* (resembling) in allusion to the epiphytic habit of the plant: "They possess the power of living almost entirely upon the matters which they absorb from the atmosphere" (Schultes and Pease, 1963).

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).



***Aerides ringens* inflorescences and flowers. (*Source*: Abraham and Vatsala, 1981.)**

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.

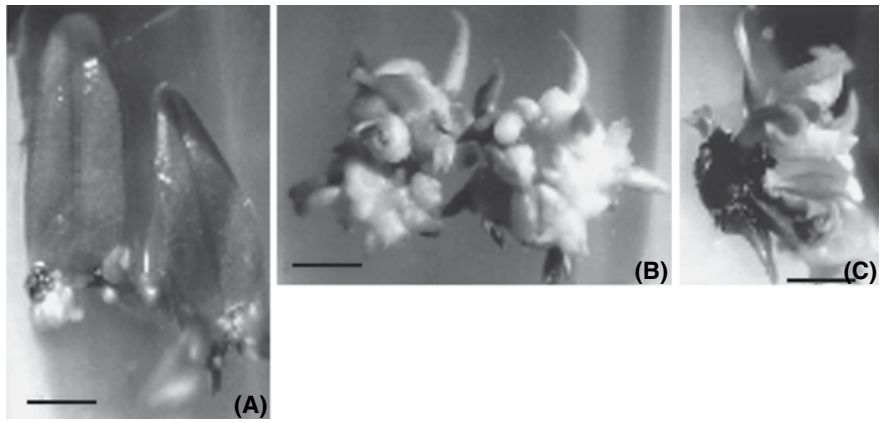


FIG. AER-1. **Culture of *Aerides maculosum* leaf explants. A. Leaf-tip explants inserted vertically in medium and developing PLBs on their bases. B. Increase in the number of PLBs. C. Development of shoots and roots. (*Source*: Murthy and Pyati, 2001.)**

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 PAR 40 µmol 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 BA 2.0 mg 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 (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 eighth 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.

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.

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.

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}	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[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.

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.

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}		
(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	
	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}	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	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)[b]	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 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	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	
	Cytokinin				
11	Kinetin (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.

Flower of *Aerides multiflorum*

Inflorescence of *Aerides multiflorum*

Micropropagation of *Aerides crispum*

A genus of approximately 20 species, *Aerides* is native to south-east and south Asia. Researchers in India and Korea (Sheelavantmath et al., 2005) developed a method for the micropropagation of *Aerides crispum*.

Plant Material. The original report states: "The 4 weeks old protocorm like bodies (PLBs)/protocorms and young leaves from in vitro grown 4 weeks old plantlets were taken as explants. Protocorms were segmented into two halves and each half was considered as an explant, the leaves were cut into 2–5 mm sections…" It is not clear from this statement if the "in vitro grown 4 weeks old plantlets" were seedlings or plantlets produced through tissue culture. The "protocorm like bodies (PLBs)/protocorms" phrase exacerbates the confusion because by definition PLBs are produced by explants whereas protocorms originate from seeds.

Surface Sterilization. Explants taken from axenic plants growing in vitro do not require surface sterilization.

Culture Vessels. Standard culture vessels are suitable.

Culture Conditions. The research cultures were maintained at 25 ± 2 C under 16-h photoperiods of 40 µmol m^{-2} s^{-1} (sources of illumination are not described) and 60% relative humidity. Standard culture room conditions should prove to be suitable.

Culture Media. Protocorm explants should be cultured on Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) with BA 1 μmol l^{-1} (Table Aer-6). Leaf explants should be cultured on MS with BA 2 μmol l^{-1} (Table Aer-7). PLBs should be cultured on basal MS (Table Aer-8) to bring about plantlet formation. The authors state throughout their paper that they used basal MS. The word "basal" regarding a medium usually refers to its inorganic components only. The assumption made here is that the use of "basal" in this instance refers only to the inorganics of MS. However, the usual MS vitamins, amino acid and polyol are included in all recipes here (Tables Aer-6, Aer-7, and Aer-8) because they will probably do no harm whereas their absence can result in failure. Adventuresome propagators may wish to try a version of each medium without the usual MS vitamins, amino acid and polyol (Tables Aer-9, Aer-10 and Aer-11 can be used as replacements for Tables Aer-6, Aer-7 and Aer-8, respectively). Plantlets should be potted in a mixture of sand, soil, brick pieces, and charcoal (1 : 1 : 4 : 4).

Procedure. PLBs and leaf explants should be cultured on media containing, respectively, BA 1 μmol l^{-1} (Table Aer-6 or its alternative Table Aer-9) and BA 2 μmol l^{-1} (Table Aer-7 or its alternative Table Aer-10). PLBs produced on these media should be cultured on basal MS (Table Aer-8 or its alternative Table Aer-11) for plantlet production. Plantlets should be removed from this medium, washed three times to remove all agar (in the original research they were washed with sterile distilled water which seems unnecessary because neither the potting mix nor the horticultural conditions under which the plants are grown are sterile) and potted.

Developmental Sequence. Explants on one of the BA-containing media (Tables Aer-6, Aer-7 or their alternatives Tables Aer-9 and Aer-10, respectively) will swell 3 weeks after the start of culture and develop PLBs in another 2 weeks. Well-differentiated PLBs will develop after a total of 8 weeks. The PLBs should produce plantlets after 6–8 weeks on basal MS (Table Aer-8 or its alternative Table Aer-11).

General Comments. The lack of clarity regarding the explant sources and the media which were used suggests that the editors and reviewers of *Scientia Horticulturae* leave much to be desired in terms of their editorial and peer-review standards. This is not the only paper in need of more stringent reviews and editing we have seen in this journal. The authors should be commended for subjecting their findings to statistical analysis.

TABLE AER-6. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of protocorm-like body/protocorm explants of *Aerides crispum* (Sheelavathnath et al., 2005)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared 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	Benzyladenine (BA)	0.225	22.5 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 lists $ZnSO_4 \cdot 4H_2O$ (FW 251.50) as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ (FW 287.54) 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 1–13 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. When the agar is completely dissolved distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

A more recent name for this species is *Aerides crispa* Lindl (Teoh, 2016).

TABLE AER-7. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of leaf explants of *Aerides crispum* (Sheelavathnath et al., 2005)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared 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	Benzyladenine (BA)	0.451	45.1 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 lists $ZnSO_4 \cdot 4H_2O$ (FW 251.50) as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ (FW 287.54) 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 1–13 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. When the agar is completely dissolved distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

***Aerides crispum* Warneri painting by John Nugent Fitch in the *Orchid Album*, volume VII, 1888**

TABLE AER-8. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for plantlet production from protocorm-like bodies of *Aerides crispum* (Sheelavathnath et al., 2005)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared 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,f]	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[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. The original recipe lists $ZnSO_4 \cdot 4H_2O$ (FW 251.50) as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ (FW 287.54) at the same concentration. The difference will probably have little or no effect.
[e] Keep frozen between uses.
[f] 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. When the agar is completely dissolved distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.
[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.

***Aerides crispum* painting from *Flore des Serres et des Jardins de l'Europe*, volume 5, 1849. (*Source*: *Flore des Serres et des Jardins de l'Europe*, 1923. Digitized by Peter H. Raven Library, Missouri Botanical Garden.)**

TABLE AER-9. **Basal Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of protocorm-like body/protocorm explants of *Aerides crispum* (Sheelavathnath et al., 2005)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared 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}		
	Cytokinin				
8	Benzyladenine (BA)	0.225	22.5 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]	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 lists $ZnSO_4 \cdot 4H_2O$ (FW 251.50) as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ (FW 287.54) 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 1–8 to 900 ml of distilled water (item 10), adjust pH to 5.6, 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. When the agar is completely dissolved distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

***Aerides crispum* inflorescence**

TABLE AER-10. **Basal Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for the culture of leaf explants of *Aerides crispum* (Sheelavathnath et al., 2005)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared 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}		
	Cytokinin				
8	Benzyladenine (BA)	0.451	45.1 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]	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 lists $ZnSO_4 \cdot 4H_2O$ (FW 251.50) as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ (FW 287.54) 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 1–8 to 900 ml of distilled water (item 10), adjust pH to 5.6, 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. When the agar is completely dissolved distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

***Aerides crispum* flower**

TABLE AER-11. **Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) modified for plantlet production from protocorm-like bodies of *Aerides crispum* (Sheelavathnath et al., 2005)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[a]	Stock solution (concentrate prepared 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}		
	Sugar				
8	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
9	Water, distilled[g]	To 1000 ml			
	Solidifier				
10	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 lists $ZnSO_4 \cdot 4H_2O$ (FW 251.50) as the source of zinc, but many subsequent recipes use $ZnSO_4 \cdot 7H_2O$ (FW 287.54) at the same concentration. The difference will probably have little or no effect.
[e] 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. When the agar is completely dissolved distribute the medium to culture vessels and autoclave. Agar is not added to liquid media.

***Aerides crispum* painting from *Flore des Serres et Jardins de l'Europe*, volume 1, 1845. (*Source*: Warner and Williams, 1882–1897.)**

Propagation of *Aerides multiflorum* through Encapsulated PLBs

PLBs encapsulated in alginate capsules, known as synthetic seeds or synseeds, can potentially be a very useful orchid propagation system. Professor S.P. Vij and his associates have developed several synseed procedures including one for *Aerides multiflorum* (Fig. Aer-2; Sembi et al., 2006).

Plant Material. Leaf explant-derived PLBs, probably produced through the method outlined in the section "Micropropagation of *Aerides multiflorum* through Leaf Segments" (Tables Aer-3 and Aer-4), were used in the original research. PLBs produced through different methods can also be used.

Surface Sterilization. PLBs taken from axenic cultures in vitro do not require surface sterilization.

Culture Vessels. The vessels should be those used to produce the PLB.

Culture Conditions. PLBs and synseeds can be cultured under the conditions used to produce them or in a standard culture room. Similar conditions can be used to culture synseeds. The synseeds should be stored at 4°C.

Culture media. PLBs should be produced as described in the section "Micropropagation of *Aerides multiflorum* through Leaf Segments" (Tables Aer-3 and Aer-4). Prior to encapsulation PLBs should be cultured on basal Mitra, Prasad and Roychowdhury (MPR) medium (Mitra et al., 1976; Table Aer-12). Synseeds should be grown on the same medium (Table Aer-12) or one with activated charcoal (AC) containing medium (Table Aer-13).

Encapsulation Solutions. Sodium alginate for encapsulation is available from http://www.sigmaaldrich.com/catalog/search/ProductDetail/ALDRICH/W201502 and other sources. It should be dissolved (3.5%) in liquid basal MPR medium (Table Aer-14). Calcium chloride ($CaCl_2 \cdot 2H_2O$), 100 mmol (Table Aer-15), should be used to precipitate the alginate.

Procedure. PLBs should be cultured in the MPR medium (Table Aer-12) for 4–8 weeks (the original report suggests mild dehydration but it does not describe how to accomplish the "mild dehydration" so this step can be omitted) and then suspended in the sodium alginate solution which must be sterile. For encapsulation the PLBs which are suspended in the sterile liquid medium (Table Aer-13) should be dropped with a sterilized 10-ml wide-mouth pipette into magnetically stirred autoclaved calcium chloride solution. The alginate beads should be allowed to complex for 30 min, taken out and washed several times with sterile distilled water. Should the beads be tacky they should be treated with sterilized laboratory-grade talcum powder (http://www.sigmaaldrich.com/catalog/search/ProductDetail/FLUKA/86257), $3MgO \cdot 4SiO_2 \cdot H_2O$. Cosmetics-grade talcum powder should not be used because it may contain additives which could harm the PLBs.

Developmental Sequence. When stored at 4°C, 7.4% of the beads retain viability for up to 75 days. If stored at 25°C, 79% of the beads germinate after 15 days but viability decreases to 52.8%, 27.2%, and 6.4% after 30, 45, and 60 days, respectively. Synseeds on the AC-containing medium (Table Aer-13) produce hairy roots and callus.

General Comments. Synseeds can facilitate shipping and commerce.

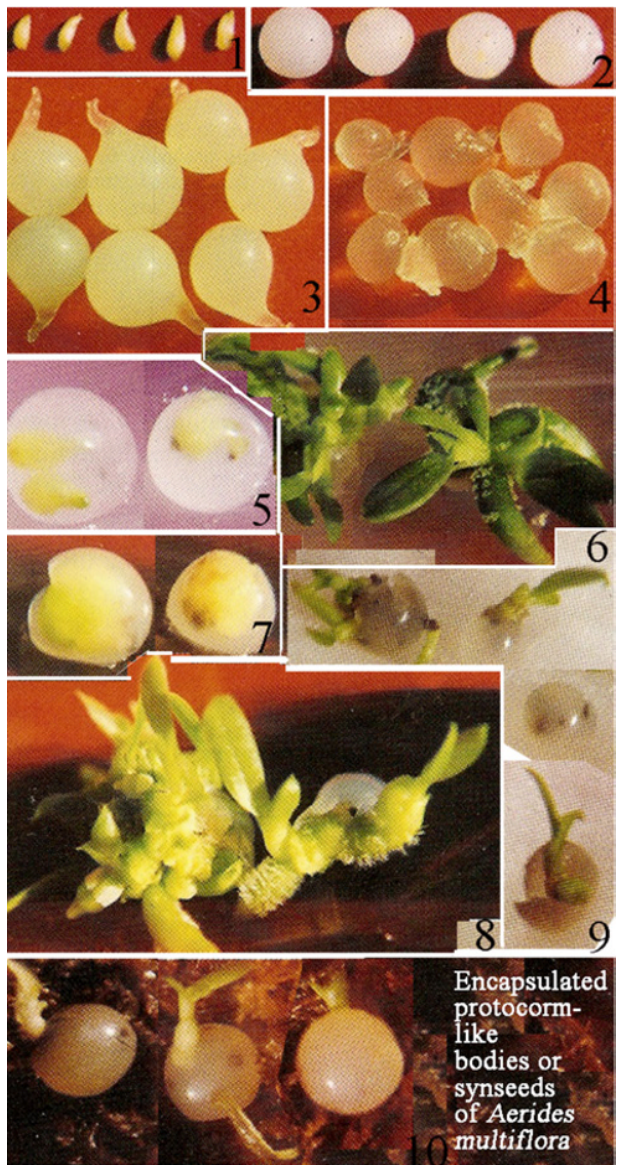


FIG. AER-2. **1. Protocorm-like bodies used for encapsulation. 2. Firm synseeds. 3. Synseeds with beaks. 4. Soft disfigured synseeds. 5. Seeds starting to break on culture medium. 6. Plantlets. 7, 8. Germination of synseeds and production of plantlets with hairy roots on AC-containing medium. 9, 10. Impaired germination on liquid medium. (*Source*: Sembi et al., 2006. Reproduced with permission from Professor P. Pathak.)**

TABLE AER-12. **Mitra, Prasad and Roychowdhury medium (Mitra et al., 1976) for the culture of protocorm-like bodies of *Aerides multiflorum* prior to encapsulation and of synseeds (Semdi et al., 2006)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[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	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	
	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 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.sigma-aldrich.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 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.6, 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 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).

***Source*: Director General of Posts, India**

TABLE AER-13. **Mitra, Prasad and Roychowdhury medium (Mitra et al., 1976) for the culture of encapsulated protocorm-like bodies (synseeds) of *Aerides multiflorum* (Semdi et al., 2006)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[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	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	
	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
	Darkening agent				
17	Activated charcoal[g]	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.sigma-aldrich.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 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.6, 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. When the agar is completely dissolved add the darkening agent (item 17) slowly with vigorous stirring. After the charcoal (item 17) 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).

[g] Only activated pure vegetable charcoal should be used. One possible source is www.sigma-aldrich.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 lampblack 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 AER-14. **Sodium alginate containing Mitra, Prasad and Roychowdhury medium (Mitra et al., 1976) for the encapsulation of protocorm-like bodies of *Aerides multiflorum* (Semdi et al., 2006)**

Item number	Component	Amount per liter of culture medium (final concentration in culture medium), mg[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	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	
	Sugar				
14	Sucrose	20.0 g	No stock	No stock	Weigh
	Solvent				
15	Water, distilled[f]	To 1000 ml			
	Alginate				
16	Sodium alginate[f]	35.0 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.sigma-aldrich.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 1–13 to 900 ml of distilled water (item 15), adjust pH to 5.6, 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 sodium alginate (item 16) slowly while stirring. When the alginate is completely dissolved pour 500 ml of the solution into each of two 1-l Erlenmeyer flasks 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 AER-15. **Calcium chloride solution for the encapsulation of protocorm-like bodies (PLBs) of *Aerides multiflorum* (Sembi et al., 2006)[a,b]**

Component	Amount
Calcium chloride, $CaCl_2 \cdot 2H_2O$	14.7 g (100 mmol)
Sucrose	20.0 g (2%)
Distilled water	To 1000 ml

[a] Mix well to ensure that all components are dissolved and autoclave.

[b] The original report does not state that sucrose was added to this solution, but it seems advisable to do so. If this is not done when the PLB is placed in the Mitra, Prasad and Roychowdhury medium, the sugar concentration may suddenly be reduced by half. The shock caused by such a sudden and drastic reduction in osmolarity may be deleterious to the PLB and the synseeds. Sucrose is added to the calcium chloride solution used for the encapsulation of *Dendrobium densiflorum* PLBs (Vij et al., 2001).

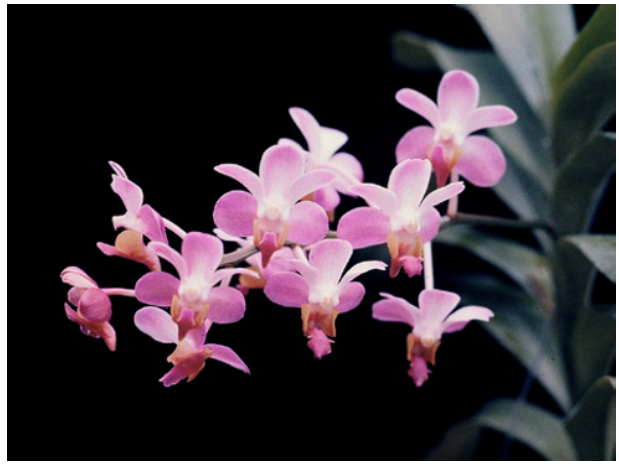
Aeridovanda

Aeridovanda is an artificial hybrid genus. The first reported hybrid between an *Aerides* species and a *Vanda* is *Aeridovanda* Mundyi (*Aerides vandarum* × *Vanda teres*), produced by Sir Jeremiah Colman in 1918 (Sanders, 1946). *Aeridovanda* Ruth (*Aerides crassifolia* × *Vanda cristata*) is the second hybrid, produced by S.E. Gillmar in 1944 (Sanders, 1946). However a compilation of hybrid and generic names of orchids (Garay and Sweet, 1974) has the following entry:

The *Aerides vandarum* × *Vanda teres* hybrid is not considered to be an *Aeridovanda* by taxonomists at present due to the transfer of *Vanda teres* and *Aerides vandarum* to the genus *Papilionanthe* as *Papilionanthe teres* and *Papilionanthe vandarum* respectively. Orchid nomenclature and its vagaries being what they are, there is no telling how, when and if these species will be reclassified yet again and perhaps several times after that. Any reclassification(s) will bring about additional changes and confusion in the determination of which of the initial hybrids is the first *Aeridovanda*.

Given the taxonomic and nomenclatural considerations, *Aerides vandarum* × *Vanda stangeana* is not an *Aeridovanda*. It is *Papilionanda* because the hybrid is *Papilionanthe vandarum* × *Vanda stangeana*. But, then, the International Registrar of Orchid Hybrids does accept some taxonomic name changes, but not others. Therefore *Aerides vandarum* × *Vanda stangeana* is an *Aeridovanda* according to them. Regardless of these nomenclatural problems, in this book this hybrid will be treated as *Papilionanda*.

Aeridovanda in Gard. Chron. ser 3, 63:93, 1918
Aerides × *Vanda*
1st hybr. A. Ruth
Parentage: *Aerides crassifolia* × *Vanda cristata*
Observation: For the hybrid *Aeridovanda* Elizabeth Young see *Eupapilio* and for *Aeridovanda Mundyi* see **Papilionanthe**



***Aeridovanda* Mary Wilson**

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 that it should be done as *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, pers. comm., 1974), only two to three 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 for *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				
1	Ammonium nitrate, NH_4NO_3[b]	1.65 g	165 g l^{-1}	10	Or weigh
2	Potassium nitrate, KNO_3[b]	1.9 g	190 g l^{-1}	10	Or weigh
3	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	
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}		
(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}	10	One solution[d]
(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}		
	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	
	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.