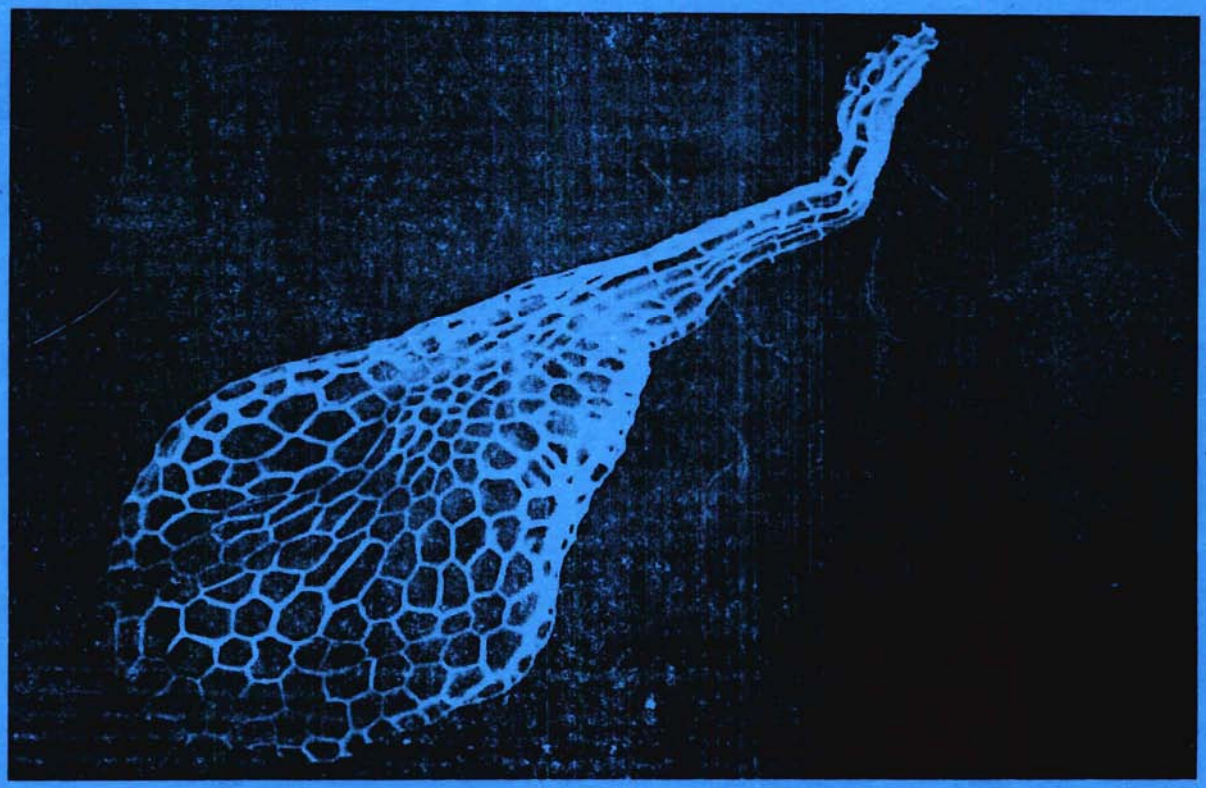
Asymbiotic Technique of

Orchid Seed Germination



by Aaron J. Hicks

Edited by Robert Huber

Orchid Seedbank Project

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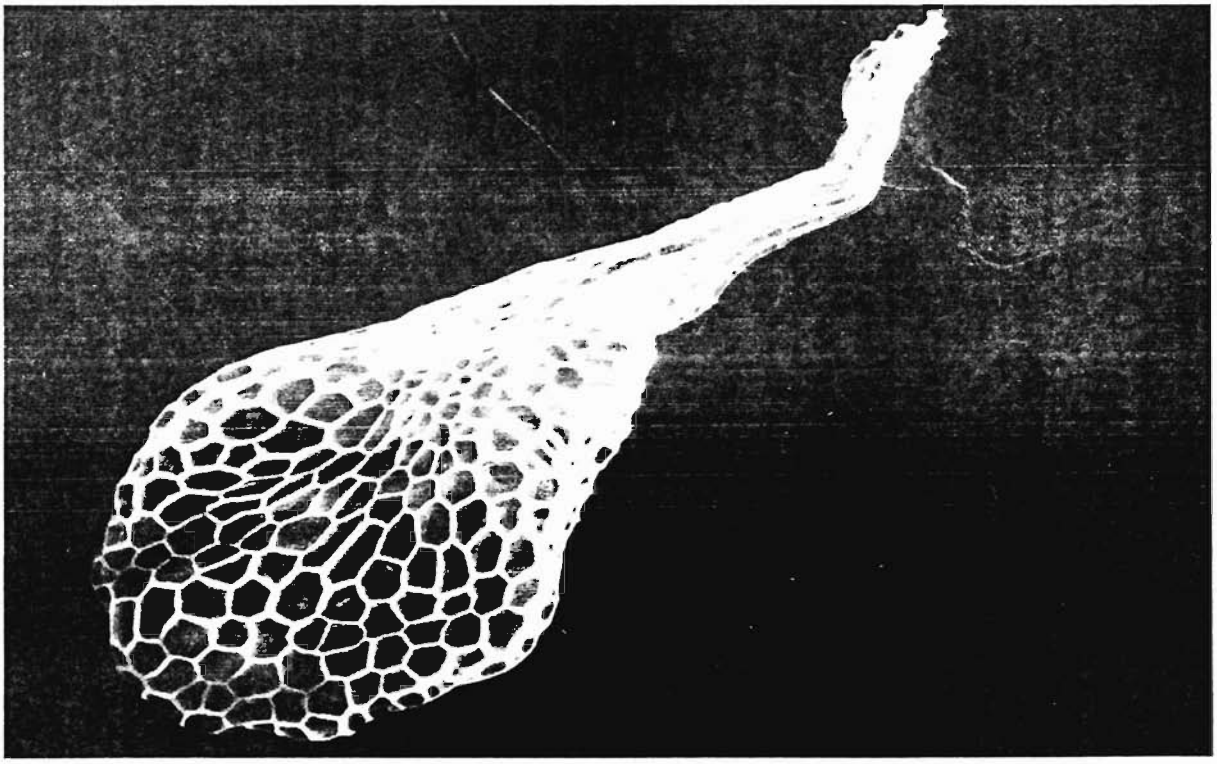

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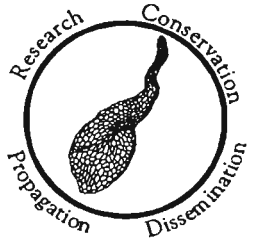

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Dedicated to conservationists everywhere.

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Special thanks to those that have gone far out of their way with respect to their efforts in conservation, including Bob Ferry, whose photomicrographic talents have made for a start in cataloging the structure of orchid seed. Thanks are due to Karl Siegler for his macrophotographic skill and artistry, and Ray Gabaldon for his input and his sacrifice of flowers for Karl to photograph. Most importantly, the donors and recipients of seed via the Orchid Seedbank Project should receive the attention they are due for their efforts. While the rest of the world is slated to be paved over, there are still growers who care enough to perpetuate the species of orchids.

The guy who lets me pay my bills, Kirk Yeager, deserves recognition for his understanding and encouragement, particularly in light of the fact that, deep down inside, he knows full well I am a lunatic.

Thanks to Marilyn Light for valuable comments and suggestions. Guido Braem deserves thanks for his contributions, for his wisdom in different techniques as well as editorial comments. Many more thanks are due to those that are convinced I do everything the hard way. You're absolutely correct.

Bob Goebel made this book possible, and his constant goading is the only reason you're reading this. Bob Huber dedicated many hours to editing this book, making it a first for the both of us. Any problems found in last minute changes are due to my errors, and not his. Yvette Justice, in her disbelief at my capacity for work, should know that she is just one more reason that I can continue to do so much. Most of all, Lisa deserves recognition for her patience. When the kitchen is the lab, the living room harbors the HEPA filter, and it's orchid seeds under refrigeration and not food, it takes a woman of remarkable character, not to mention humor, to understand.

I have done my best to assure that the information contained within is correct. If any attributions are incorrect or incomplete, please inform me.

Author's Note

Often regarded as a technique to be reserved for the laboratory, producing orchids from seed is something that can be performed in almost any home, using products that are readily available or can be ordered inexpensively by mail. Orchids are produced from seed not just by experts, but by hobbyists all around the world: people working in their kitchens, the hobby lab, and even in relatively crude setups in remote jungle locations.

With the explosion in biotechnology and the proliferation of hobbyists that grow orchids from seed and even clone them using materials and tools that are readily available, virtually anyone who wants to produce orchids from seed can do so. The home lab can be used to produce numbers of seedlings that are often limited only by space or time allowances, or by the amount of money one wishes to spend on equipment for yet more seedlings. But few orchid growers will wish to test these limits, and this book is for them.

Commercial growers often complain that the resources and information that exist on orchid seed germination is limited, or often scattered in publications that makes it hard to collect and comprehend. This text serves as a reference for those growers, consolidating concepts, techniques, and available methods by which orchids may be propagated by seed. Others wish to form their own labs to keep their hybrids "at home" and to keep down protocorm theft. This book is for commercial growers as well.

Researchers often have difficulty in determining what is the state-of-the-art, or current technique. Much of the existing literature is old and describes techniques that have been surpassed or use materials or equipment that is no longer available. A variety of techniques have been described within, and many roads to get to the same destination are given. If incapable of finding a brand-name product, a substitute can be found. These references should aid the researcher in their pursuits.

It's a litigious society out there, and more important, I don't want anyone to get hurt. If you have any doubts about any of the techniques in this book, consult an experienced chemist, biologist, or other person whose knowledge would lead you toward the correct answer. I've done my best to make certain everything is correct, checked and rechecked everything, but there is no possible way to consider all the possibilities involved.

Flasking at home is safe; this book recommends the use of chemicals available in every household: bleach, hydrogen peroxide, sugar, rubbing alcohol, and others. Nevertheless, they are to be treated with respect. Other compounds, such as Virkon S, agar, and nutrient media or its components, are safe enough to be purchased and used in the home, provided certain safety constraints are met. These chemicals, like those found in the home that are not for consumption, should be treated appropriately.

Wear your eye and skin protection, and please–be safe. Orchids are a hobby; getting hurt shouldn't be part of that.

All prices are in US dollars, from 1999, except where noted otherwise.

Once upon a time, orchids were regarded as plants that were impossible to propagate en masse. For many years, the only techniques by which orchids could reliably be propagated were vegetative, such as by division. Seed propagation only occasionally resulted in success, usually when seeds were dispersed upon the roots of established plants. By this means, the seed might become infected by the symbiotic fungus which permitted germination in the wild, allowing it to sprout and survive. This technique, although very easy, left much to be desired as was highly inefficient and unreliable.

Orchids are, by their very nature, inefficient plants with respect to their reproduction. Orchid seed capsules may contain hundreds of thousands, or even millions of live, viable seeds. If we were to observe a capsule as it first cracked and then split open, we would notice how the seeds slowly escaped to be dispersed by the wind. In order to survive and germinate, these seeds must find a suitable environment, which is not a simple proposition for the tropical epiphytic[1] species. Only a relatively small proportion of the trees in the tropics support orchids, where space is already at a premium, and there is aggressive competition for light, nutrients, water, and physical space. That a given orchid seed should escape its confines and land in a suitable aerie is unlikely at best; the odds that it should become infected with an appropriate fungus are not known, but certainly lessens the chance of survival.

Provided it successfully germinates, the seedling must survive predation by insects, pathogens, weather and climate changes, and perhaps most savage of all, the competition by other epiphytic plants. In our own terrestrial environment, we sometimes forget that competition for light in the epiphytic environment is a brisk business indeed. Assuming the supporting branch or tree does not fall before the orchid matures, dropping it to the ground to rot, it, too may have the opportunity to flower and produce a capsule, perpetuating the species in a similar manner.

As absurd as it may sound, let us examine the two extremes: compare, if you will, these processes with those of coconuts, and their few, very large seeds, which individually have a relatively high chance of survival. Still, we must remember that in theory, in a population with stable numbers of a given species, each member of the species only produces one plant to replace itself on the average. The coconut produces maybe several hundred seeds during its lifetime, one of which may replace the parent. At the opposite end of the spectrum, an orchid may produce a large number of seeds–perhaps millions–with the likelihood that any given seed will survive to reproduce being very low.

In the late 1910's, it was first determined that orchids may be grown from seed on nutrient agar, much in the same way that bacteria are cultured in the lab. These techniques slowly gained acceptance amongst horticulturists over the next few years despite initial intense criticism. The chancy and inefficient techniques of symbiotic orchid seed germination and plant division were replaced with a relatively reliable process by which orchids could be propagated in large numbers. Gone were the problems of plant identification caused when stray seed was dispersed in the greenhouse. Although orchid hybrids existed prior to this breakthrough, success was sporadic and the quantity was poor. Today, the number of plants produced from seed are primarily limited to space available and what the market will bear.

Although there have been changes, modifications and improvements, *in vitro*[2] germination of orchid seed as performed today would be immediately recognizable by anyone who performed the work 10, 20, or even 50 years ago. Advances in plant cell culture in the past few years have made available a broad variety of products that enhance the ability of the "kitchen laboratory" flasker[3], as well as the small home lab, to efficiently produce results comparable to those of a much larger lab. In many instances, home labs can be assembled relatively inexpensively, and most growers that wish to dabble in flasking[4] just for the experience can perform it at home using canning supplies and other inexpensive substitutes for "traditional" laboratory equipment. Any reader familiar with microbiology and chemistry will be right at home with this manual.

This manual is designed with the novice in mind, having done my best to make it readable while presenting some technical information. It is printed with emphasis on techniques and apparatus suited to the home flasking environment, but also designed to accommodate those with home labs and other improvements above and beyond where many people may wish to start. The techniques by which orchids may be grown from seed are readily adapted for the small lab, and even the home, using tools that are readily available and chemicals that may be purchased via mail order from several different sources.

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1 Plants that live on other plants; with orchids, deriving their nutrition in a non-parasitic manner.

2 Meaning, literally, "in glass."

3 One who propagates orchids from seed *in vitro*.

4 The techniques by which orchids are propagated from seed *in vitro*.

Chapter I

1.1 The Orchid Seed: Germination

Orchid seed germination remains a "black box" to many orchid growers around the world. We send the flasking company the seed or capsules, and receive flasks full of hearty, well-grown seedlings a few months later. We then happily de-flask the seedlings with little or no knowledge as to the mechanics behind the entire flasking process. Indeed, this serves the majority of orchid growers everywhere. Flasking, quite simply put, is not for everybody: the process of germinating orchid seeds via the asymbiotic[5] route is more rigorous and involved than say, growing vegetables from seed in your garden. Some growers are curious, and just want to try flasking for the experience. This text is for them, but it is also intended to portray seed sowing in what, it is hoped, is a new light for established propagators. Many of them find a set of parameters that works well, a sort of "assembly line" for seed germination, by they potentially bypass or overlook techniques or products that may facilitate their efforts. Given the often secretive nature to which some labs subscribe, this is not unexpected, but it is probably not a good thing. More than one lab has had to reinvent the wheel to achieve success after others have undoubtedly failed at similar tasks. Labs are often unwilling to share ideas, perhaps rightfully so–but the extreme secrecy to which some labs have subscribed is a barrier to everyone's progress.

This text is meant to be instructional, not just a recipe book. It is hoped that it will clarify the entire flasking process for all readers, explaining the usefulness of certain media components, different disinfection agents, techniques, tricks of the trade, and other aspects of flasking. A number of different pathways to achieve the same result are presented, but we do not want to lose the reader the result. Horticulture is an art, as is flasking, but growing orchids from seed requires knowledge of technique as well as the science behind it. The information here is intended to give you that knowledge.

It is worth pointing that a more complex procedure may not necessarily replace or surpass one that is easier to perform; it is not required for the reader to go out and purchase elaborate, expensive equipment when the end product will not be significantly greater than that produced using cheap, readily available labware, or even kitchen equipment. Although its value may be recognized if one decides to expand into a laboratory facility, there is little or no requirement for elaborate setups, or expensive equipment. Make do with what you have, and if it does not suit your needs, scrounge equipment. Surplus stores and sales can turn up all kinds of valuable equipment, often at a significant price break. Brand-new lab instruments and glassware have an incredible markup, and should only be purchased as a last resort. The Internet has proven to be an ideal source of used lab equipment, and a quick search will yield several vendors specializing in used equipment, often at prices acceptable to the grower who is willing to search a little.

This manual will include instructions to make your own glove box, as well as how to make your own laminar flow hood for much less than commercially available models. Best of all, the home craftsman will be able to produce a product that can be customized to fit peculiar needs and unusual situations.

Orchid Mycorrhiza

It has long been known that orchids germinate in the wild only in the presence of one of a number of symbiotic fungi, called mycorrhiza[6], and substituting the nutrients normally provided by these fungi was the largest obstacle initially faced by Knudson and others with respect to the commercial propagation of orchids by seed (more on this in History, below).

The relationship between the plant and the fungus is, as with most symbiotic relationships, a tenuous one; it is essential that the plant not be parasitized or unduly harmed by the presence of the fungus, and that the fungal symbiont is kept in check. Moreover, germination itself requires that the orchid seed land such that it may be infected in the first place. That orchids must produce thousands or millions of seeds to simply perpetuate the species is to be expected, given that the chances that each of the several conditions required for germination will be met are certainly very small.

Since the introduction of asymbiotic germination of orchid seed in the 1920's, there has been relatively little interest in the esoteric field of the respective symbionts; all the same, their presence is an important one, both from a natural history perspective, as well as that from subsequent growth of seedlings and mature plants. Research in other,

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[5] In the absence of symbiotic mycorrhizal fungi.

[6] Literally: fungus-root, these symbiotic fungi reside in association with orchid roots furnishing nutrients and their presence is generally considered to be a requirement for the germination of the orchid seed in the wild.

more lucrative commercial crops has shown enhanced nutrient uptake on the part of plants that have been infected with the appropriate mycorrhizal fungi, versus their "sterile" counterparts. Still, it is not known exactly what the fungus extracts from the plant in terms of gain; the plant is provided nutrients, but does little in terms of returning something to the fungus. In effect, the plant is living parasitically off of the fungus, which Rasmussen (1995) recognizes as a mycotrophic relationship, rather than symbiosis.

The fungi that exist in mycorrhizal relationships with orchids are mostly members of the Basidiomycota. These basidiomycete genera include *Ceratobasidium, Rhizoctonia, Sebacina, Thanatephorus,* and *Tulasnella* (Hadley, 1982). Others report the presence of species of *Corticium, Armillaria, Fomes,* and *Hymenochaete* present in the wild, and that species of *Phytophthora, Penicillium, Aspergillus,* and *Trichoderma* have been "reported to initiate and promote germination" (Arditti, 1967). Peterson et al. (1998) note that *Ceratorhiza, Epulorhiza, Moniliopsis, Russula, Tomentella, Erythromyces,* and *Armillaria* have been found in relationships with orchid roots. Several species have been found in the absence of orchids, and therefore may be pervasive under the correct conditions or with other hosts. Further, several species that are known to be symbionts with orchids are serious pathogens in other plant families. The extent to which these fungi support the mature orchid is extremely variable; some mature plants may be free of any fungi, while some achlorophyllous (lacking chlorophyll) species are entirely dependent upon the fungus for their survival. Seedlings may germinate in the presence of one fungus, then turn to favor a different symbiont later in life, and then possibly abandon any such relationship when more mature.

It was originally thought that a given fungus would be specific to a given plant, or a "one host-one fungus" relationship; this type of dependence was eventually disproved. This discovery undoubtedly lent success to early endeavors by which horticulturists could produce limited numbers of seedlings by sprinkling seeds on live orchid roots, which was probably first met with success sometime in the early 1850's (Arditti, 1967). All the same, germination and production were very much hit-and-miss propositions, and very inefficient.

In the mature plant, the role of the fungus is obscure and varies between not only species and genera, but individual plants as well. It is generally recognized that many (if not most) plants are infected once they have left sterile culture and are brought into contact with other plants. Blowers and Arditti (1970) suggest that "some growers reduce considerably their losses of *Odontoglossum* and *Paphiopedilum* seedlings" if they are inoculated with the proper organisms when de-flasked. They go on to suggest that some orchids, such as cymbidiums and cattleyas, are affected somewhat less by the presence of mycorrhiza. Obviously, achlorophyllous species require the presence of a mycorrhizal symbiont, and it has been suggested by different growers that many species of *Cypripedium* do poorly or perish in their absence (although it has been noted by several researchers that no symbionts have been recognized for temperate *Cypripedium* species).

For a more comprehensive view of orchid mycorrhiza, the reader should examine Arditti's book two, which has an excellent section by Hadley on the subject. Rasmussen's text has a great deal of information on this subject, particularly with respect to terrestrial species. Those interested in the symbiotic germination in terrestrial orchids would be well-advised to read chapters 5 and 8 in her book, which include excellent sections on isolation and culture with the symbionts. It is worth noting that there is mounting evidence that symbiotic propagation of terrestrial orchids greatly enhances nutrient uptake and seedling growth rates (Rasmussen, 1995), and these techniques should be explored further. This text deals exclusively with asymbiotic technique as a function of both simplicity for the reader and the author's experience.

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Chapter II: A Brief History of Orchids from Seed

2.1 History

"Nothing certain is known of the germination of the Orchidaceae."
-John Lindley (1799-1865)

Horticulturists have known since the 1840's that orchids live in close association with fungi, whose existence were not verified by isolation and culture for another 60 years. French biologist Noel Bernard was the first of those researchers who were to eventually culture out these organisms, and successfully demonstrated that natural germination occurred in the presence of these mycorrhizal fungi. At the time, demand for orchids was increasing rapidly. In an attempt to find routes by which orchids could be propagated, growers sought to germinate seed under all kinds of conditions; the greatest success was found when seed was sprinkled on the roots of a host plant. Still, producing seedlings was an uncommon event, and maintaining them was even more difficult. All the same, efforts continued to attempt to produce seedlings in this manner to try to meet the demand for live plants.

It wasn't until 1917 when Lewis Knudson, then professor of botany at Cornell University, modified aseptic culture techniques involving corn and legumes for use with orchid seeds. The work of Bernard, who had died in 1911, and Hans Burgeff, a German who had been working independent of Knudson and Bernard, was of great interest to Knudson, whose ideas and research to develop an asymbiotic route to germinating orchid seed were quite unpopular with European botanists of the time. While Bernard and Burgeff had thought that it was the presence of starch that supplanted the requirement of a symbiotic fungus, Knudson determined that it was the presence of sugars that caused orchid seeds to germinate *in vitro*. Experiments showed that, when the seed was provided with a variety of salts, a sugar (sucrose or table sugar, or glucose) and water, seedlings would develop and grow. Media lacking sugar would allow seed to germinate, but subsequent development of seedlings would fail. Experiments spanning nearly a decade indicated that the proper media would substitute the requirement for a symbiotic fungus, and eventually reliable techniques by which orchid seed could be germinated were developed.

Knudson's work was initially impeded by the shortage of live orchid seed; many growers were unwilling to release even small amounts of what was an extremely valuable commodity at the time. Eventually, Theodore Mead, a grower in Florida, supplied him with the seed he required, resulting in the techniques we use today. These techniques differ little from those described in Knudson's 1922 publication in the Botanical Gazette, "Nonsymbiotic Germination of Orchid Seeds."

He eventually succeeded in producing seedlings of *Cattleya mossiae*, sown January 14, 1919. Knudson's work was originally published in an "obscure Spanish journal" in 1921, and practically ignored until the follow up in 1922 in the Botanical Gazette (Schecter, 1970). His work was criticized by Dr. J. Ramsbottom, who was then involved in the commercial production of orchids via the symbiotic route, working for Charlesworth, Shuttleworth and Company, a British orchid nursery (Reinikka, 1972). Ramsbottom, a mycologist with the British Museum, believed that the techniques employed by Knudson could never be used to produce economically viable numbers of plants (Schecter, 1970), and voiced his opinions to that effect in *The Orchid Review* in 1922. Two of Bernard's affiliates, J. Constantin and J. Magrou joined the fray, going so far as to suggest that plants grown via asymbiotic culture were not capable of flowering.

Knudson's response was to flower a x *Laeliocattleya* hybrid asymbiotically in 1928, which pretty much ended the controversy (Schecter, 1970). Interestingly, much of the debate was due to differences between the two groups, based on one group exploring what was possible (i.e., growing orchids asymbiotically) versus those that were analyzing the natural function of the symbiotic relationship (Rasmussen, 1995).

It has been said that the term "breakthrough" is one that should be used sparingly, if at all perhaps once a century in a given field. For the Orchidaceae, the work of Knudson, and others, certainly falls into this category. For now, orchids could be reliably propagated in large numbers; hybrids, whose availability had been sporadic prior to these developments, came onto the scene in massive numbers. Even at this time, hobbyists were able to reproduce these results in their own home laboratories. An additional benefit was that seedlings *in vitro* grow faster than those in the wild, with one author estimating that monthly growth rates approximate annual growth rates in the wild (Warren and Miller, 1993).

There have been many contributions to the field since the 1920's, and several improvements, both to laboratory facilities and cell culture products and techniques. The first micropropagation involving cloning of orchids began when Gavino Rotor Jr. successfully propagated *Phalaenopsis* by stem node culture in 1949. Meristem propagation, which allowed for vegetative propagation (and, therefore, cloning) of orchids in large quantities arrived in 1960, the product of work performed by Georges M. Morel, in France, using techniques originally developed by American Earnest Ball. Arditti and Ernst (1992) attribute Rotor, Henry Thomale, Donald Wimber, Morel, and the French firm

Vacherot and Lecoufle with contributions that furthered the clonal propagation of orchids.

New products whose contributions to the orchid culture realm have not been fully exploited continuously appear on the market, and new techniques for dealing with stubborn problems are developed regularly. Despite more than 70 years of progress on the orchid seed germination front, we have done little more than scratch the surface, as a quick survey of the 600+ genera and thousands of species of orchids will reveal. Although we have made great strides in terms of propagating tropical epiphytic species, many species of terrestrial orchids remain reluctant to be brought into cultivation and have defied attempts to clone them; there are many frontiers yet to be explored in the Orchidaceae and many opportunities for study that remain wide open. Innovation is the key to the future with respect to orchid seed germination of these select members.

The explosion in biotechnology, in conjunction with improvements in chemistry and materials, has opened the gateway to future advances in the micropropagation of all plants. The development of the laminar flow hood, made possible by the HEPA[7] filter, has increased the size of workstations, increased comfort on the part of the worker, and reduced the chances of contamination in large-scale production facilities. We face many challenges with the orchids, but we must realize that challenges have been met before, and some of those presented to us today are ones that have already been overcome in other, similar disciplines. Not only do we have much to learn from new, original research, but we have much to learn from similar fields.

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[7] High Efficiency Particle Air filters, which produce essentially sterile air flow, and are critical to many applications in tissue culture.

Chapter III: The Basics

3.1 Orchid Seed Production

Producing orchid seed is not trivial. There are many factors that must be considered, some of which will be discussed here. Other factors, such as those involved in hybridizing, are beyond the scope of this text and are not discussed.

The first factor to consider is the parentage of the plants that will be used for the cross. When propagating species, it is best to select parents whose traits are superior: often, growers will flower tens or hundreds of members of a species, and choose only the most select clones for producing seed. When propagating for conservation, it is essential that the traits represented are characteristic of the species involved. It may even be important to produce crosses between plants that were collected from the same area.

Floral traits are most commonly used when choosing parents for a cross, but it is equally important to choose plants whose parents display desirable growth characteristics, such as disease resistance, ability to produce well-held flowering spikes, robust growth form, and so forth. It is unfortunate to see hybrids being produced among orchids that are weak, flower poorly, and hold these flowers so tenuously that staking and wiring are required to produce an acceptable display. Worse, growers that adhere to such strong spray regimes to defend against pests and diseases produce plants whose ability to survive in the absence of such chemical sustainence run the risk of driving orchids down the same route as roses, where fungicides and pesticides are essential life support mechanisms.

It is important not to produce orchids that are arbitrary hybrids, the products of whatever plants just happen to be in bloom; perhaps it would be a selfing (i.e., the plant crossed back onto itself), or perhaps it was outcrossed by hybridizing it with something else that happens to be in bloom. More often than not, these parents are complex hybrids whose parentage is unknown to the hybridizer.

Unfortunately, orchid hybridizing is a complex task; we may produce true gems, but more often than not, we produce garbage when arbitrarily producing crosses. This is certainly not meant to discourage the potential hybridizer; indeed, many fine breeding lines historically have been those produced by smaller growers and hobbyists, and more exciting lines will certainly be the product of those whose collections constitute a hobby and not a profession. But when making such crosses, one must accept the fact that there will be a great deal of time and energy invested in the production of these plants. The formation of the capsule often takes weeks or months, and sowing the seed itself may take hours of intensive preparation and work. The seedlings in flask must develop (requiring care along the way), and then be de-flasked, sown into compots[8], and raised to maturity.

By this point in time, you have invested several years to go from seed-to-bloom. If you are willing to invest this kind of time and money into such an effort, and are not dissuaded by the expense involved, then you must be a budding (no pun intended) hybridizer. Moreover, you may land yourself in deeper than you think, with hundreds or thousands of plantlets, begging for a home.

Therefore, emphasis should be placed on instruction and education before hybridizing; there is a good primer on genetics and inheritance in Rebecca Tyson Northen's book, *Home Orchid Growing*. Review of the literature and a good text on the subjects of genetics and inheritance will be of immense assistance. Hybridizing is not something that is to be taken lightly; many of the best breeders have been involved for years, and their education as to how to best produce hybrids is one that is hard-earned. To know, for example, that a particular shade of yellow is a dominant trait in a given parent, and will tend to carry through when crossed onto another plant, is something one must earn from experience–either by having performed the cross before, and seen the flowers that result in the progeny, or by having researched the records. None of these are easy tasks in any regard. Apprenticeship, arguably the best route, is reserved for the fortunate few that are on the inside track of an established breeding program.

Most hybridizers jealously guard their discoveries; after all, if one were to publish what they knew, it would be a simple matter for someone to pirate their ideas, and steal an entire breeding line. Studying crosses that interest you, and finding out more about their parents through published records, will help. Be forewarned: nobody ever said it would be easy. Moreover, a selfing will most likely not produce plants that resemble the parents; hybrid corn, after all, does not necessarily provide seed that can be sown next year to produce the same hybrid corn. It is necessary to study Mendelian[9] genetics to educate yourself as to the properties that may be imparted from the parents, as well as the manner in which they are imparted. This is beyond the scope of this text, but Northen's *Home Orchid Growing* will provide some good background, as will any introductory biology or genetics text.

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[8] Compots, or community pots, consist of pots with several seedlings, generally recently de-flasked plantlets.

[9] The principles of genetics as first recognized by Gregor Mendel (1822-1884), an Austrian monk who is generally regarded as the father of genetics.

Many of these will be simplifications; genetics is hardly straightforward or rarely proves to be as perfect as Gregor Mendel originally suggested. Today we are learning that the pollen donor plays a slightly different role in determining the ultimate genetic makeup of the progeny than that of the plant that hosts the capsule. For our purposes, right now, we may ignore these differences; it is important to know that they exist, but they are smaller than those influences imparted by strict Mendelian genetics and are complex.

The good news is that mother nature can fill your demand for seed by providing you with species whose lineage may go back tens or hundreds of generations. If we wish to produce seed for plants that will breed true, often provide good germination rates, and produce offspring from which we can predict many attributes (such as how appealing the flowers will be), all we need to do is find species with which we are content. With these, we may either trade pollinia between flowers on the same plant (called "selfing"), between plants produced from the same parents (called "sibbing," or "sib" as in "sibling cross"), or between complete strangers that are only members of the same species (called "outcrossing").

There are different advantages and disadvantages to each technique; if a specimen displays a given peculiarity (such as alba flowers), it may be desirable to "self" the cross so that the genetic code is kept as tight as possible, although inbreeding runs the risk of raising the incidence of undesirable traits. If one wishes to maintain the genetic code from a given colony of orchids, it may be best to sib the plant, or cross with other members gathered at the same collection site. Outcrossing may be desirable if we simply wish to maintain the species; one member may have excellent growth form, whereas the other members may have particularly well-held flowers. From these two, we could cross them, and hope that some of the progeny display both of these finer traits. With this we should bear in mind that selfing of crosses is undesirable and to be discouraged as it may result in unwanted traits manifesting themselves in the present or generations from now. If traits are to be conserved without selfing, it is best to find another specimen displaying similar features, producing either a sibling or outcross. Although this may not always be possible, attempts should be made.

With these factors in mind, it would be valuable to be able to determine the origin of a given plant or its parents for reference when it is bred later. It is interesting to note that in the cactus and succulent realm, the origin of a given plant is a valuable factor with respect to its identity. Cactus and succulent seed may have considerably less value when the exact location of where its parents came from is not known. Part of this is due to the restricted range of many cacti, which tends to produce colonies of plants that differ very slightly from one another; knowledge of the origin of these plants is valuable, both from the collector's standpoint, as well as that of the pure conservationist.

Both the family of orchids and the family of cacti are very young, and they share a surprising number of properties such as their great variability, large number of genera and species, and (with a considerable proportion of species) limited numbers existing in small populations. Indeed, it may be their relative youth that has produced such an abundance of species in the competitive environments in which they both may live. In this proliferation, not all species will persist; some are no doubt ephemeral, and their numbers may be dwindling as we watch (whether we participate in their decimation is moot in this discussion). What is vitally important is that we are able to document from which location and, if possible, which colony we have gleaned our genetic information; without it, we are doing very little in terms of preserving the species and its genetic heritage. Although we generally regard plants of the same species to be identical, the physical expression of genes may not be enough. Characteristics such as pathogen resistance, environmental requirements, and possibly such esoterica as flower coloration outside of the spectrum observed by humans (but still visible to pollinators) are conceivably different between different colonies of plants. Discussion of the complexities of conservation are beyond the scope of this book, but it is important to note that these issues should be addressed in any serious conservation attempt.

If so desired, limited *ex wild* protection of a particular colony of species may be afforded by assigning a collection number. Ideally, collection numbers consist of the initials of the collector, followed by a three- or four-digit number (e.g., "AJH-016"), and this number will follow the seed in a cradle-to-grave fashion such that it can be shown that a plant is descended from a specimen from a given collection. This number may be used for future reference by going to the collector and requesting additional information; it will be then up to the collector (or their survivors) to disseminate the notes that would lead researchers to the original collection. Obviously, detailed notes on the part of the collector are required. Global Positioning System (G.P.S.) coordinates will be of great value. Herbarium vouchers[10] should be taken when possible. Collection numbers also afford some amount of protection to a given colony; if greater protection is desired, then a general geographic location (within miles, usually) should be specified to prevent intrusion by collectors.

As with any breeding operation, careful notes should be taken. If one is to form capsules on a plant, they should be properly identified; use waterproof labels with indelible ink. Many growers use "Sharpie" pens, which

[10] A pressed plant deposited for future study that may later be used for identification purposes.

prove to have a relatively long life despite exposure to the elements. The cross should be labeled with the identification of both pod parent and the pollen donor, and the date the cross was made. It is important to make note of both when the cross was made, as well as when it was harvested; these capsule maturation times are of great importance to those that believe in green pod sowing.

Labels are always a problem, and even the best inks may fade after a period of time (Sharpie now makes a heavy-duty version of their markers, but it is not available everywhere). For particularly harsh environments or long durations, tags fashioned out of aluminum or brass tags, which are then inscribed with the data at hand, will be of value. Most hardware stores have brass tags, or even acrylic sheet which can be cut to size and then inscribed with an engraving tool or even a sharpened nail. A hole drilled through one end permits the use of a string or a wire tie to hold it in place.

Comments on Green Capsule Techniques

Some growers use "green pod" or "green capsules" for the propagation of orchids, also known as embryo culture. There are several advantages to green capsule techniques, including the fact that, unlike dry seed, green seed from an unopened capsule is sterile, and may be delivered to the growing media without undue chemical exposure. For this and other reasons, green seed may have a much higher germinability than its dried counterpart. In many cases, it may germinate much faster, decreasing production times. Some temperate species germinate at much higher rates from immature seed than from dried seed, but these results must be qualified by stating that this is variable between species. In any of these instances, green capsule techniques require that the grower harvest the capsule before it matures–specifically, before it develops to a point where it cracks open, and exposes the seed within to contaminants. It is removed from the mother plant with a sterile blade, and its exterior is chemically disinfected. After removal, it is cut open and its seed delivered to the media (described in Chapter IV). Despite the popularity of this technique, there are several problems and lingering concerns with green capsule seed production.

Some experimental data exists that indicate that viruses are probably not spread through dry seed. Yuen et al. (1979) report that Cymbidium mosaic virus (CyMV) was not transmitted from infected parent plants except for one seedling. This seedling was one of 123 that germinated from a 2-½ month old capsule; of 155 seedlings from 3-½ and 4-½ month old capsules, none were infected as demonstrated by bioassay techniques. There is the possibility that this one seedling was infected after removal from flask, or that it was infected when being removed from the capsule as the seeds from the former group were removed from immature capsules.

It is generally considered that virus particles are not capable of infecting orchid seeds; however, making such a broad statement is dangerous, because there are over 20 viruses that are known to infect orchids, and stating characteristically that none are capable of infecting progeny of any genera is entirely without evidence. Bertsch (1982) notes that "for all practical purposes, orchid seedlings (including those grown from green pods) are virus free until something wounds them." Although this seems speculative, it is not without evidence including the fact that if this were not the case, the incidence of viruses in collections today would be much higher, thanks to the prevalence of seed-grown plants in cultivation. Wild-collected plants are almost invariably lacking viral infection (Wisler et al., 1986).

It is also worth noting that although virus particles have been found in infected pollinia, there is "little chance" of infecting the pollen recipient "unless the receptor plant is actually wounded" (Wisler et al., 1986). Despite this, growers generally advise that pollen parents should be tested for viruses prior to using them for hybridization, particularly when valuable pod parents are involved.

Thus, while dry seed probably does not transmit viruses from parent to seedling, many growers consider the green capsule method to be messier in that it is not possible to completely separate the immature seed from the liquids of the mother plant. Accordingly, the procedures used to produce seedlings from the green capsule may introduce live viruses from the mother plant into the mother flask. Additional study is called for and, until such time, it is suggested that particularly valuable plants not be wholly entrusted to green pod techniques.

Pollination of Flowers

Orchid flowers are highly variable, and knowledge of the anatomy of the genera with which you will be working is important. Fitch (1980) details the pollination of *Cattleya* flowers, and Light (1990) has a good overview of pollination of flowers in general. Figure 3.1.1 is a cross-section of a representative orchid flower.

The ovary will hold the developing seeds, turning into a capsule as it approaches maturity. Its development begins with pollination, and it will swell soon after successful fertilization of the flower.

The column is tipped with an anther (sometimes referred to as an anther "cap,") where the pollinia are held. Behind the anther is the stigma, where the pollinia must be placed for pollination. Between the two is the rostellum

(Figure 3.1.2). Manual pollination begins with removing the anther, and extracting the pollinia. After removal, they are placed in the stigma.

Dissecting a large orchid flower is recommended if you are going to pollinate your own flowers; Figure 3.1.2 shows a ventral view of a typical orchid column, and the location of the anther, pollinia, stigma, and rostellum.

After familiarizing yourself with the flower at hand, the plant is prepared by moving it to a clean workspace; if working with small flowers (see below), the air must be still. Working over a large piece of white paper will assist in recovery of dropped pollinia or anthers.

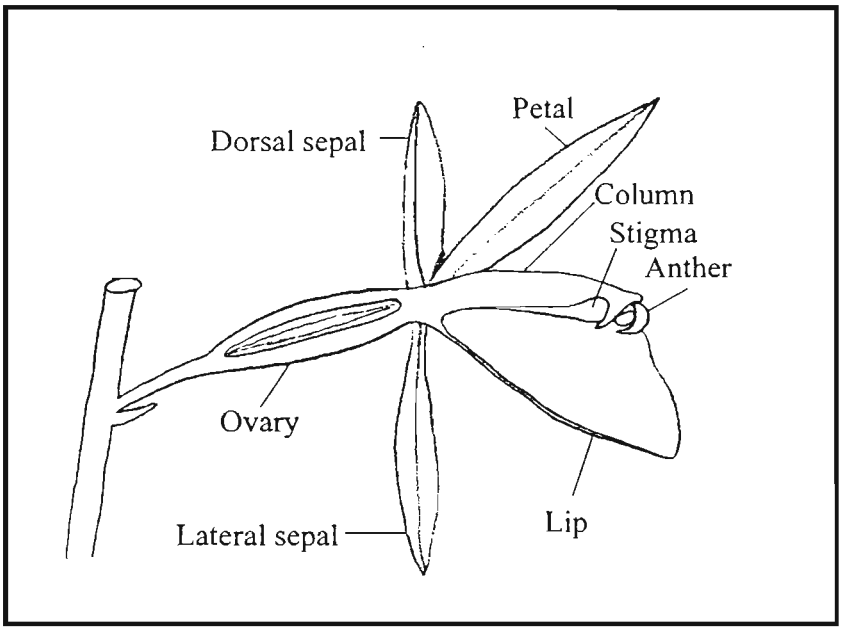

Figure 3.1.1: Longitudinal section of an orchid flower. (After Dressler 1993.)

Flowers should be mature and open (Figures 3.1.9 and 3.1.10). For ephemeral flowers, pollination should generally occur in the morning, after it is fully open. For longer-lasting flowers, they may have been open for several days or even a week or longer before beginning work. If scented, the strength of the odor may indicate the best time during which to pollinate. Old, "tired" flowers should not be used: the stigma should not be dry.

Select a "pod parent" to carry the capsule, and a "pollen parent" to donate the pollinia for fertilization. Some plants make better pod parents, and some make better pollen parents. Knowing the different abilities is a function of experience. Fitch (1980) suggests that if the difference in flower size between two plants is significant, the larger flower be used as a pollen donor. Pollen from a small-flowered species may be incapable of producing pollen tubes long enough to reach the ovaries of the host flower.

Your working tools should correspond to the size of the flower at hand. Toothpicks make the best all-around tool, except for the miniature flowers (see below). If necessary, whittle the toothpick to a sharp point to accommodate the smaller flowers.

The pod parent should be emasculated unless it is being selfed. Remove the anther cap (Figure 3.1.11) by flipping it up and away, and discard it.

The pollen parent should have the anther cap removed in the same manner, taking care to capture it as it comes off. Check to make sure the pollinia come with it, and do not remain on the flower (Figure 3.1.12). Gently tease one of the pollinia from the anther cap; the viscidium will be adhesive, and the pollinia generally adheres with good strength. Examine the pollinia carefully, using a magnifying lens if possible. Any signs of discoloration will indicate fungal infection.

After removal of the pollinia, the anther may be discarded. Holding the flower with your other hand, place the pollinia into the stigma of the pod parent, which will usually resemble a small liquid pool behind the small ridge called the *rostellum* (Figures 3.1.13 and 3.1.14). If desired, press gently to ensure the placement is sound.

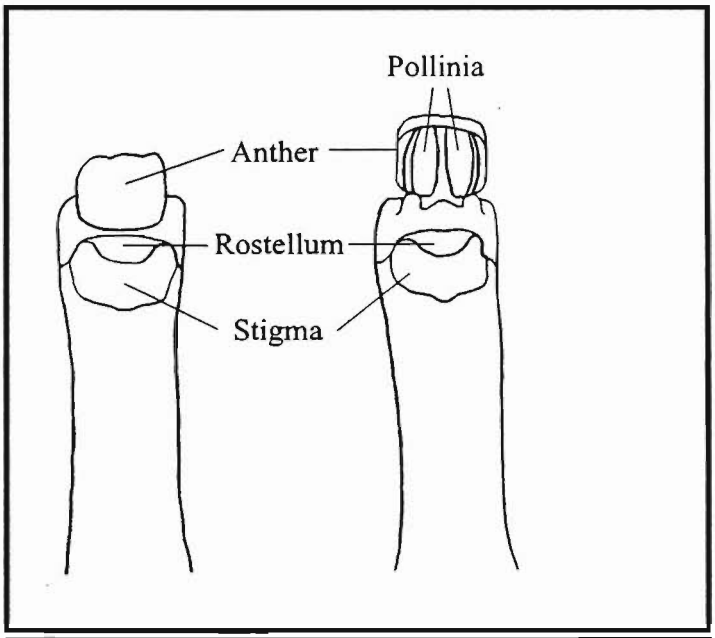

Figure 3.1.2: Ventral view of column, anther in place (figure left) and tipped away (figure right). (After Dressler 1993.)

Paphiopedilums, phragmipediums and other orchids with "pouched" flowers may produce their own problems. Wellenstein (1999) reports that a scalpel without a sharp tip may be used to remove the pouch prior to beginning work, flame sterilizing it between plants. It is also worth noting that these plants may have pollen that is poorly consolidated or otherwise very different from the pollinia of other orchids, and it may have to be smeared or otherwise delivered to the stigmatic surface with some degree of effort.

Additional pollinia may be wrapped in paper and stored in refrigeration (see Pollen Storage, below). Desiccant will be required if the ambient environment is humid.

Once the process is completed, use a small tag to identify the cross. With luck, once the petals fall the ovary will swell, initiating the formation of a capsule (Figure 3.1.8). The tag will serve to identify the cross that has been made, which will help identify it in the future as well as set it apart from other crosses if multiple capsules are hung on the same parent.

Since it is hard to discern which plants are better pod or capsule parents, a reverse cross may be made by placing the pollen from the first pod parent on the other plant. Some species are intrinsically poor pod or pollen parents, and many species are not self-fertile (will not form fertile seed when being selfed, often called "self-infertile"). It has been noted that if crosses are to be made between plants with flowers whose sizes differ greatly, pollinia from the larger flower should be placed on the smaller flower if only one cross is to be made. As the pollinia of the larger flower will form longer pollen tubes, these are more likely to reach the ovaries than with the reverse cross.

Production of Seed from Miniature Flowers

While the pollination of "major" flowers such as those of *Phalaenopsis*, *Cattleya*, and other large, readily-pollinated flowers, presents few problems to the experienced propagator, there is little doubt that miniature flowers, such as those of *Pleurothallis*, *Lepanthes*, and similar species, will present serious obstacles to even the most determined of growers. Indeed, with many orchid flowers measured best in single-digit millimeter dimensions, patience is but one of several factors: steady hands, high-quality magnification, understanding the mechanics of small flowers, and experience all lend themselves to successful pollination.

Despite the difficulties involved, several growers have succeeded in producing seed from such tiny flowers. In fact, intergenetic hybrids have been made between species whose flowers are best viewed under magnification. Behar (1993) suggests the use of handmade tools, including microspatulas constructed of fine wire that has been hammered and then cut at a very sharp angle so as to produce a very fine point. This tool can then be used to transfer pollinia when used in conjunction with a jeweler's glass for magnification.

Since the pollinia of orchids are often strongly adhesive (Figure 3.1.3), there may be less difficulty in removing them from the pollen donor: they can readily stick to implements used for pollination, provided they are used to entertain the flower in the correct manner. Behar notes that the difficulty occurs when attempting to place them on the stigma. Several attempts may have to be made, and success is never certain.

It is worth noting that removal of the pollinia from the anther may be difficult. For some plants with relatively large anthers (2-3mm in diameter), the pollinia may sometimes be removed through careful teasing of the parts with miniature tools such as a pair of sharpened wires or toothpicks. One trick is to drop the anther 10-15 cm (4-6 inches) onto a clean sheet of paper, carefully dislodging it from one tool with the tip of another tool. If the pollinia are ejected, they will be visible on the surface of the paper. Pick up the pollinia and place it in the stigmatic fluid. With miniature flowers, it is very important that the pollen be delivered to the stigma, and not to the rostellum, from which the anther is removed; it may appear to be a perfectly reasonable place in which to place pollen, when it is actually its origin.

Other recommendations made by Behar include the fact that although he sows dry seed from his efforts, the capsule is harvested green so that the seed is not prematurely released and lost. One hybrid between *Lepanthopsis* and *Lepanthes* was harvested at 60 days, having been made in April. The seed was flasked in August, and replated in November. Seedlings flowered *in vitro* starting in January 1993–less than two years after the cross was made. Behar goes on to state that losses are very high when seedlings are removed from flask, and he deliberately lets them grow on until they are mature, so they are more likely to survive the rigors of being de-flasked.

Pollination of *Vanilla*

In the wild, *Vanilla planifolia* is pollinated by *Melipona* bees (Fouché and Coumans, 1992). The number of flowers pollinated in the wild is very low (perhaps 1%), and probably much lower than this where the orchid has been moved but the pollinator is absent. Thus, for commercial production of vanilla flavoring, it is important that the flowers be hand pollinated.

Childers et al. (1959) note that hand pollination of the flowers requires the use of a small splint or stick, which

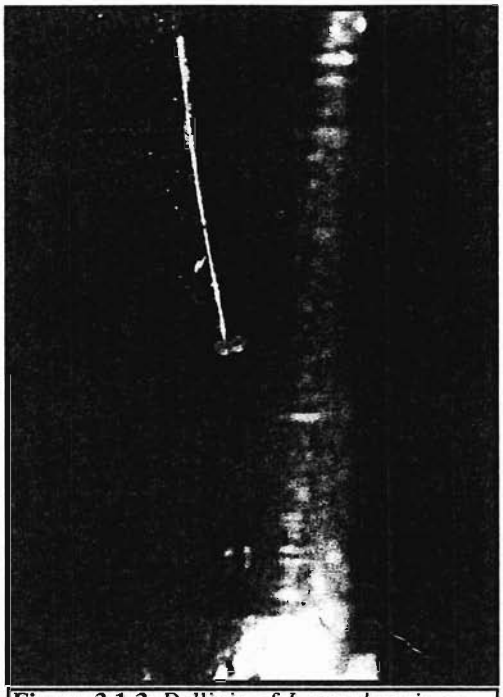

Figure 3.1.3: Pollinia of *Lepanthopsis* on microspatula. Photo by Behar.

Figure 3.1.4: Pollinated *Lepanthopsis* using sharpened wire. Photo by Behar.

is used to move the rostellum out of the way. When this is done, the anther is pressed directly into the stigma with a smearing motion to deliver the pollen. Historically, this maneuver is performed with a piece of stiff grass or a small, sharpened needle. A matchstick or toothpick will also work.

Fouché and Coumans (1992) present four different techniques which may be used to pollinate *Vanilla* flowers in the November, 1992 issue of the American Orchid Society's Bulletin, replete with diagrams and explanations. They also note that pollination of the short-lived flowers (one or two days) must be performed early in the day, preferably before noon.

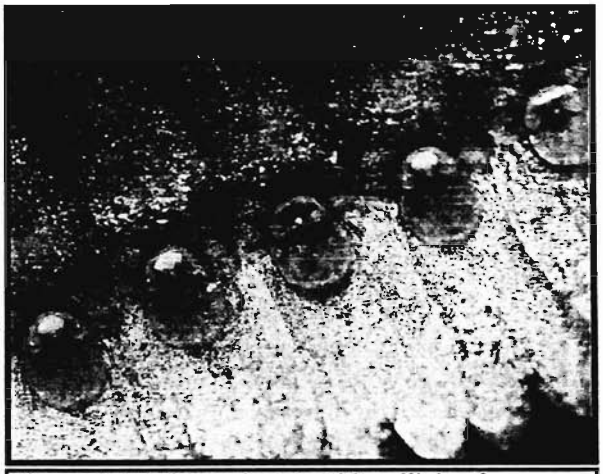

Figure 3.1.5: *Lepanthopsis* with pollinia of *Lepanthes*. Photo by Behar.

Pollen Storage

If so desired, some pollen may be stored for considerable periods of time. Pollen storage may be desirable for producing outcrosses between plants that are many miles apart, or that flower at different times of the year. Although there is little known about how long it may be stored, there are reports of pollen that is two or three years old being used successfully for breeding purposes (Wellenstein, 1999). Some pollen is ephemeral, and may last only days or weeks after removal from the flower, regardless of refrigeration.

It is important to use only clean, non-infected pollinia; it should not be discolored or mushy. Fournier (1997) indicates that "any shade of gray" is an indication it should be rejected. Once removed from the flower, the pollinia should be wrapped in paper; Fournier has had success with glossy magazine paper, and virtually any paper that "breathes" should work. Aluminum foil, and similar wrappers are not recommended, but Wellenstein (1999) reports that their *Paphiopedilum* pollinia are stored exclusively in waxed paper. Fournier (1997) deals with pollinia of *Paphiopedilum* in the following manner.

After the pollinia has been affixed to one end of the pollination tool (a toothpick), stick the unused end of the toothpick into a small block of Styrofoam. This will prevent the pollinia from coming into contact with anything that might damage it. The block can be stuck to the inside of a larger cardboard container, or into a test tube or other pro-

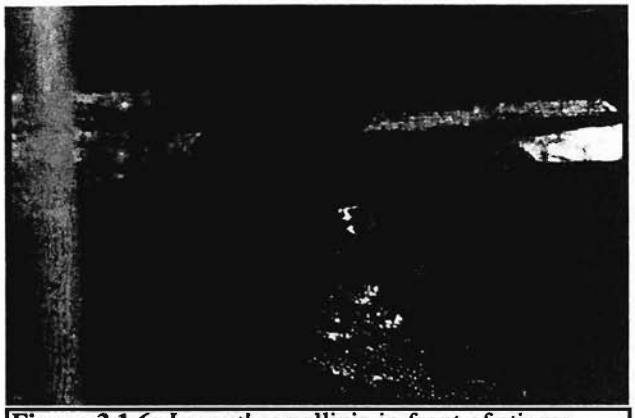

Figure 3.1.6: *Lepanthes* pollinia in front of stigma. Pencil in background for scale. Photo by Behar.

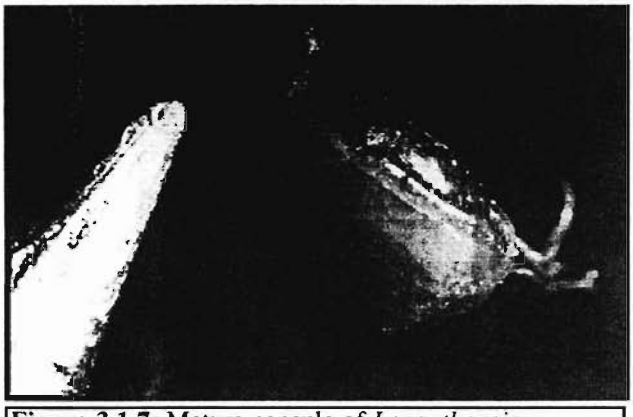

Figure 3.1.7: Mature capsule of *Lepanthopsis*. Photo by Behar.

Figure 3.1.8: Maturing capsules on *Epidendrum* species. Note dying flowers and swelling ovaries on second and third (from left). Photo by Karl Siegler.

tective cover for shipping.

Disagreement exists over whether desiccants need to be used; some will place a small amount of calcium chloride in the bottom of a test tube, followed by a wad of cotton and then the packet of pollen before capping it. Others will simply place them in a sealed container in a refrigerator. All the same, strong desiccation is probably not useful, and should be avoided.

Seaton and Pritchard (1990) suggest storing pollen and seed over a saturated solution of calcium chloride for three to four days, and then transferring them to individual vials which should be kept at 4° C. The purpose of the calcium chloride will be described in Section 3.3, Orchid Seed Storage. When used to control humidity in a closed space, it should produce a constant humidity of about 40% RH at 5° C; this is much higher than one would get with unsaturated silica gel, and therefore less likely to cause excess mortality from desiccation. It is not known what, if any, negative effects may result from allowing organisms to grow in this more humid environment, but they note that their work and the work of others indicate that silica gel causes loss of viability. Seaton and Pritchard (1990) go on to note that pollen "can be stored in the freezer at -20° C."

Pollen has also been stored by some growers in empty gelatin capsules, which may be purchased at the pharmacy. These in turn should be placed in a dry container, or a constant humidity chamber as described in Section 3.3.

Pollen Viability

It may be desirable to determine whether or not pollen is viable (more properly, germinable). Light and MacConaill (1996) describe the "hanging drop" technique in the North American Native Terrestrial Orchid Propagation and Production conference proceedings (NANTOC Proceedings, see Appendix III: Sources). This technique relies upon incubating pollen in a sucrose (table sugar) solution and searching for pollen tubes that extend from the germinating pollen.

In the hanging drop technique, a 1 molar stock solution of sucrose is produced by adding 34.2 gm of sucrose to a volumetric flask and adding deionized water until the 100 ml mark is reached. This stock solution is used to prepare a working solution for germination such that a 0.3 to 0.5 molar sucrose solution is used to germinate pollen.

Pollen to be tested is teased from the pollinia and placed on the inside lid of a petri dish, and labeled with a marker. Light and MacConaill use a small circle, within which the pollen is placed, next to the time, date, and particulars about the pollen itself. Add a small drop of the sucrose solution to the pollen; the authors note that it does not matter if the pollen floats. It is important not to add too much solution: you will need to invert the lid, and the liquid should not run. Once all the different samples are placed on the inside of the lid, a teaspoon or so of the solution is placed in the lower part of the dish; this will increase humidity inside of the dish so that the solution will not evaporate, leaving the pollen high and dry. When this is done, the lid is inverted and placed over the lower part and left for 36 hours at room temperature. The pollen should be examined for "finger-like" projections from the pollen grains under a low-power microscope. These are the developing pollen tubes.

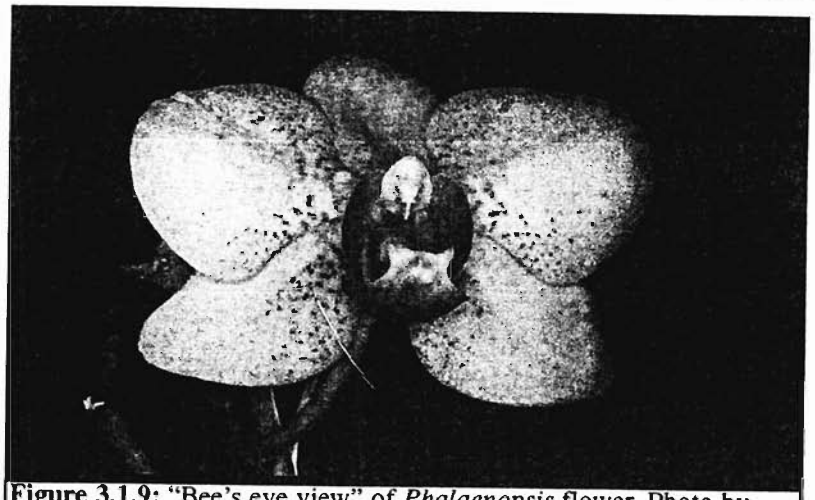

Figure 3.1.9: "Bee's eye view" of *Phalaenopsis* flower. Photo by Karl Siegler.

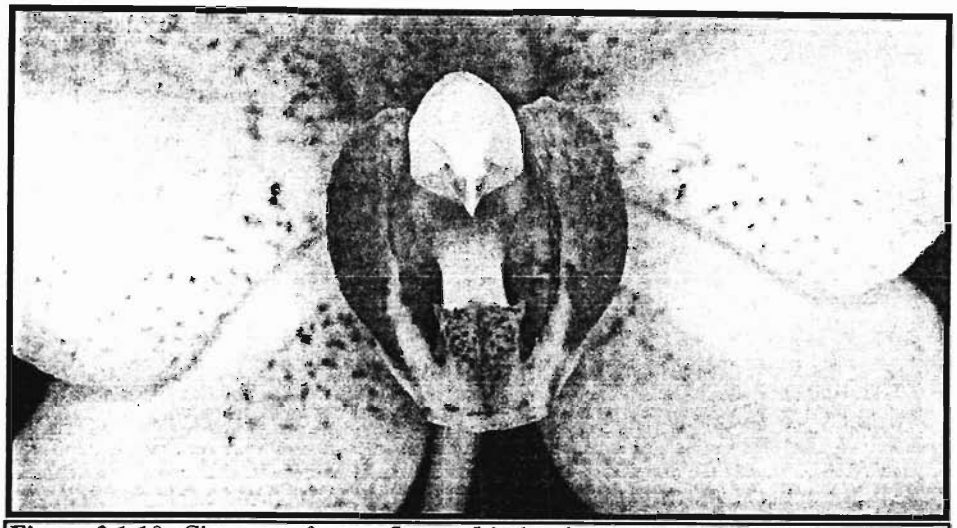

Figure 3.1.10: Close-up of same flower. Lip has been removed for clarity. Photo by Karl Siegler.

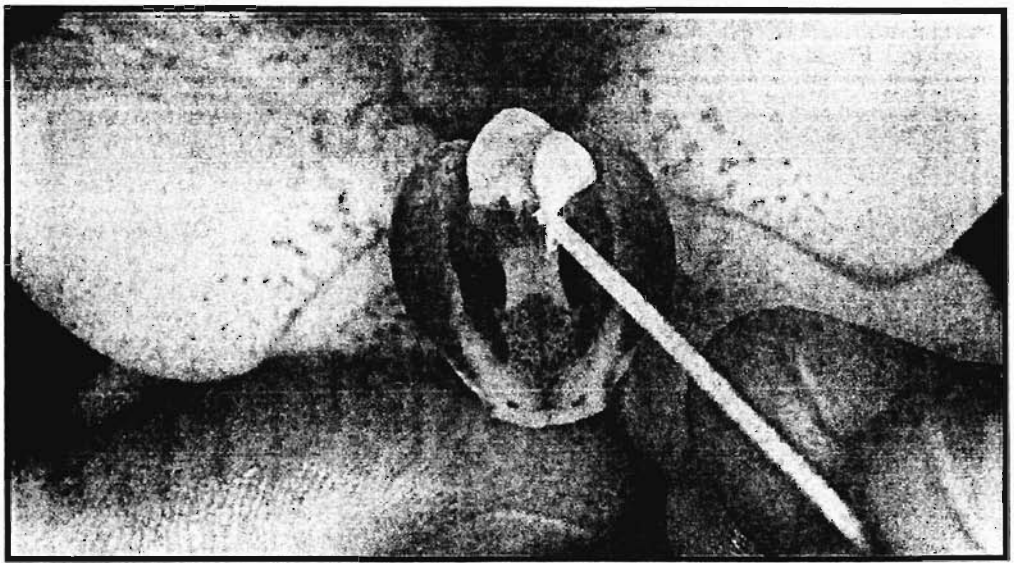

Figure 3.1.11: Removal of anther cap with toothpick. Photo by Karl Siegler.

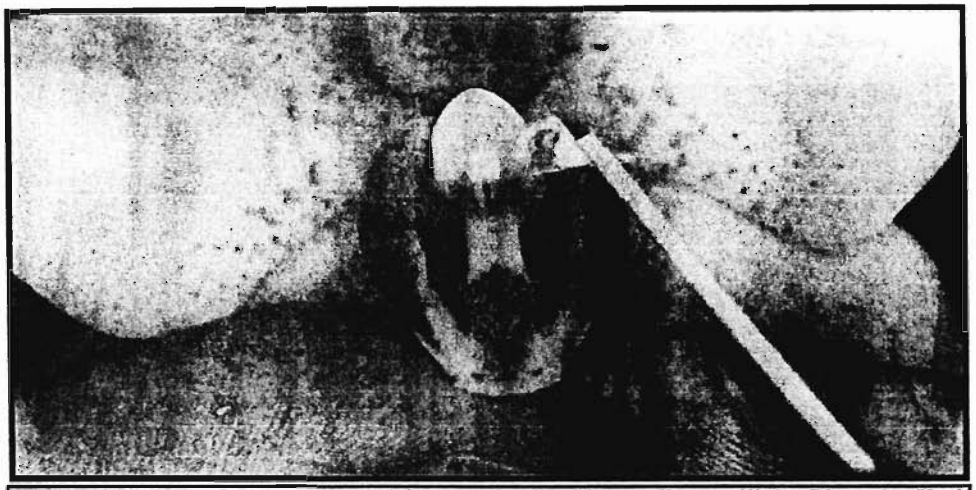

Figure 3.1.12: Anther cap, viewed from underside Note yellow pollinia. Photo by Karl Siegler.

Figure 3.1.13: Single pollinia (removal from anther cap not shown) being placed into stigmatic fluid. Photo by Karl Siegler.

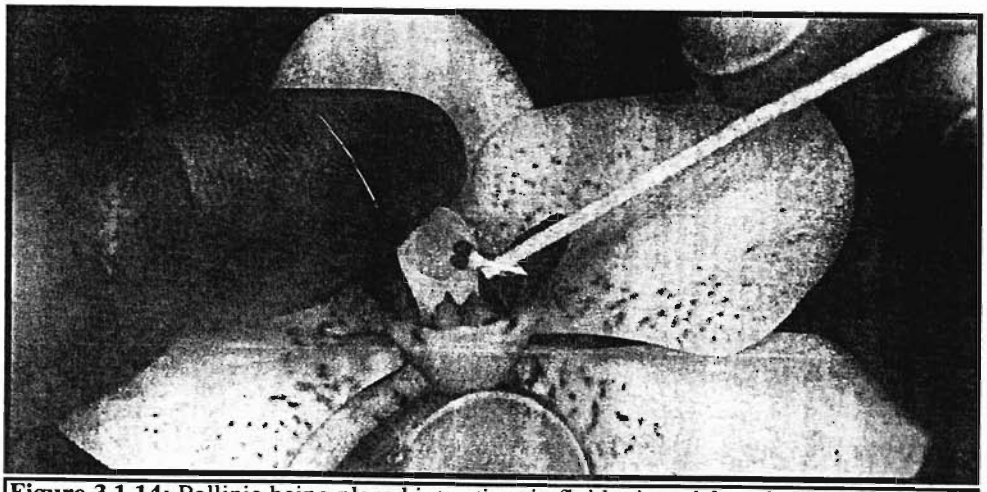

Figure 3.1.14: Pollinia being placed into stimatic fluid, viewed from below. Note large "pool" for stigma. Photo by Karl Siegler.

The relative number of pollen tubes that develop will serve as a guide as to whether or not pollen should be used. For the entire method, complete with photos, see the NANTOC Proceedings (see Appendix III, Sources).

Harvais (1980) notes that pollen of *Cypripedium reginae* was germinated using water ("fair" germination when squashed stigmas were mixed with distilled water), Harvais Culture Media V ("fair") and very good on Medium V + 0.1 ppm or 0.5 ppm IAA ethyl ester, which produced "pollen tube lengths as much as ten times the diameter of the grains after 6-8 hours at 20° C (60° F)."

TTC testing (see Section 6.8, Viability Testing and Seed Analysis) of pollen has been performed with other plant families, but I have found no references of this having been done with orchids.

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3.2 Orchid Seed Harvesting

The ripe orchid seed capsule[11] is a fickle thing. In the late stages of development, an orchid seed capsule may crack open (called *dehiscence*) and start to disperse seed without obvious signs. It is critical to keep a close eye on developing seed capsules, and to monitor them for the first signs of opening as a cracked capsule means the seed has been exposed to the atmosphere, and must be considered to be contaminated with fungi and bacteria. Indeed, capsules that are still green and appear far from maturity may crack open, and the seed may be lost to the whims of your air circulation system in very little time due to its extremely small size. Experience will guide the grower as to when a capsule may crack open; one of the indicators that maturity is imminent in some species is when the longitudinal lines of a capsule start to develop undulations or become convoluted. Some capsules may start to turn color (to pale green or even yellow), stop swelling or growing, or otherwise change characteristics. Tabular data, such as that provided by the Bakers (see About the Orchid Seedbank Project at the end of this text) may indicate expected times of maturity as capsule maturation time.

There are published tables for capsule maturation time, but these should be viewed in perspective: in the same way that babies develop differently despite similarities in age, there is no guarantee that an orchid seed capsule will ripen on schedule. Variations in climate, culture, and genetics may cause them to open outside of the time parameters suggested for a given plant. Capsule times are guidelines and are not meant to cover all possibilities. All the same, it is vitally important that seed be removed from the capsule in a manner such that, if it is not used immediately for

[11] A dry fruit that splits open at maturity, releasing seeds through slits. Sometimes incorrectly referred to as "pods" with orchids.

green capsule sowing, the seed is dried so that it will not rot.

Light (1990) suggests that measuring the diameter of the developing capsules at the midpoint along its length will serve to indicate maturity. When there is no additional increase in diameter, the seeds are probably mature and therefore capable of being grown from embryo culture. In fact, seeds are often capable of germinating when harvested a month, and possibly longer, before the capsule splits. Other indications noted by Light (1990) include the capsule turning downwards, the distal end of the capsule (to which the flower was attached) turning yellow, and the longitudinal grooves in the capsule becoming deeper. Also that color changes in the capsule may occur, particularly if the seed within is strongly colored.

Once a capsule has approached maturity, it may be separated from the plant (using a sharp, sterile razor blade or similar tool) and placed into a desiccator or in dry air so it will not rot. "Green" orchid seed contains a high concentration of water. Work done at the Orchid Seedbank Project on *Vanilla planifolia* showed that fresh seed dried over desiccant for 16 hours weighed an average of 28 micrograms per seed (326 seeds sampled). After drying for an additional 4 months over desiccant, the average weight was 21 micrograms per seed (571 seeds sampled). It is worth noting that these results may be peculiar to this species or genus, as this weight is very high; Rasmussen (1995) notes that *Galeola septentrionalis*, at 22 micrograms per seed, "may constitute a record within the family." *Vanilla* is in the same tribe as *Galeola* (Tribe Vanilleae), and Dressler (1993) notes that the seed structure and other properties are similar between the Galeolinae and Vanillinae sub-tribes. (As an aside, Dixon, Pate and Kuo (1990) note that the subterranean Australian orchid *Rhizanthella gardneri* average a whopping 75 micrograms per seed.)

An alternative to removing a capsule from a plant is to remove the plant from the growing area, place it in an area with little air movement, and then carefully bag the developing capsule with a smooth, porous paper cover so that the seed can be recovered. However, this technique runs the risk of having a capsule split entirely unnoticed, because the ongoing development cannot be observed. Stoutamire (pers. communication) goes one step further, suggesting that the capsule and its supporting stem may be removed and placed in a glass of water, much like a cut flower, as it approaches maturity. The capsule can be placed in an area where it is more conspicuous, and the climate more carefully controlled.

One other option is to place an inch or so of desiccant in the bottom of a Mason jar and use the lid and ring to form a tight seal, which will serve to keep the atmosphere within very dry. The capsule is removed from the plant as it approaches maturity, and wrapped in a single layer of thin paper, such as the tracing paper used to hold seed. This packet is then placed over the desiccant and checked daily for any changes. As soon as the capsule splits, you may move on to the next step. Another option is to cut open the capsule, spread it apart to increase exposure, wrap it in paper, and then place this in a desiccator (Figures 3.2.1 and 3.3.1).

Harvais (1980) notes that it is possible to use a small container, like a butter tub, to hold unripe capsules, and then carefully ventilate the contents with a stream of air to dry them. The lid of the container is perforated to accept two pieces of airline tubing. One is used to blow air in, the other to allow it back out. The exit tube should be fitted with fine mesh (Harvais suggests a small sample of pantyhose) to keep any seed from escaping. The inlet is connected to an aquarium air pump. The constant flow of air should keep excess moisture from accumulating, provided the system is not overloaded with wet, green plant material.

Once the capsule has been harvested, it can be placed on clean, smooth paper on a seed cutting board (see Section 3.4, Handling and Shipping of Orchid Seed) so that most of the seed can be recovered. One suggested technique is to use a small, clean, sharp blade such as a clean X-Acto knife blade, which can be inserted between the cracks that have formed in the sides of the capsule. Insert it and twist, being careful to keep the capsule over the paper on which you intend to recover the seed. It is also worth mentioning that this technique is an excellent way in which to perforate a finger or your hand with this blade; if possible, support the capsule on one side with the glass of the cutting board so that you will not injure yourself. Particularly recalcitrant capsules may be dealt with by clamping the blade in the jaws of a hemostat (available at electronics supply stores). Crack the capsule into as many pieces as seem necessary, and then using the back of the blade, gently remove seed that clings to the interior of the capsule. Eventually, you will reach a point where you are removing parts of the capsule itself, and it is recommended that you stop before you reach this point. Instead of removing fine, dust-like seed, you will be removing long, thin hairs; they look very similar to seed, but are of no importance to us. When dealing with species that produce long, hairlike seed (such as some dendrobiums and cypripediums), use your best judgement to determine when you have reached this point. Tapping the capsule with the handle of the knife may assist in bringing out as much seed as you can. Alternating tapping and very light scraping will yield the highest amount of clean seed possible.

It is important to remove as much of the chaff as possible, as this may contaminate your flasks. Using the point of the knife, remove any fragments of the capsule that may have fallen in with the seed; clean up the seed as much as is reasonably possible. Although not particularly harmful, orchid seed stores and ships better without the presence of this chaff. Moreover, contamination of the seed increases the likelihood of contamination of flasks–the external por-

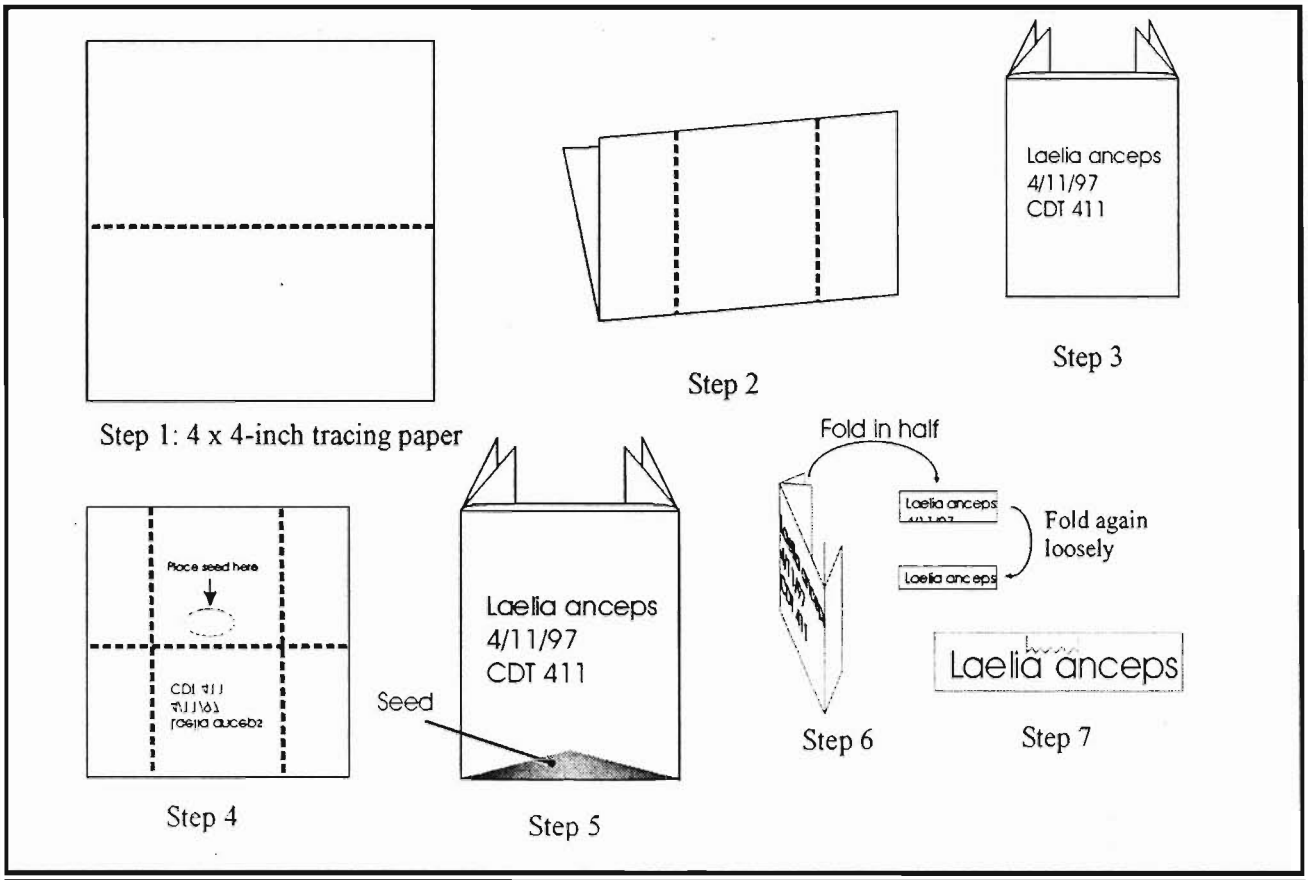

Figure 3.2.1: Packet folds.

tions of the capsule are more likely to harbor fungal spores and other sources of flask contamination than its contents. While removing seed from the capsule, examine it closely for any signs of discoloration; seed that has been spoiled by bacterial or fungal action may be slightly darker than unaffected seed. Discoloration is not necessarily a reason for disposal: sometimes the "discoloration" turns out to be the presence of embryos. Orchid seed ranges from white to tan in color, all the way up to the dark red coloration of *Angraecum* seed which may look identical to chili powder. Changes in coloration in different parts of a single capsule may be reason to suspect contamination, and segregating one color from another is generally recommended if this is the case.

If a capsule is allowed to get wet (such as a green capsule that cracks during shipping in a container unable to allow moisture to escape or be absorbed by desiccant), a lot of seed may be lost due to contamination. The rest of the seed should be treated aggressively with disinfectants and possibly a sugar pre-soak (see Section 5.1, Dealing With Contamination Problems) to deal with any spores that have almost certainly permeated the rest of the capsule.

Some orchids have seed that is mixed in with pulp; Dressler (1993) suggests that this is associated with *Vanilla, Palmorchis,* and *Cyrtosia,* and that some members of the genus *Neuwiedia* may "also have fleshy fruits." These species can make handling very difficult, particularly if this pulp is permitted to dry before the seeds have been removed. One suggested technique to remove the seeds (if green capsule sowing is not in order) is to slit open the capsule, and place it or parts of it in water; the seeds may be suspended by scraping, and then the larger pieces removed. The seed may be removed from the pulp by differences in density: when agitated, the seeds will often settle to the bottom of the flask, and can be removed with an eye dropper to a piece of filter paper or a coffee filter. Repeated washings may be necessary to keep seed from sticking to the filter when dry. Great quantities of seed may be lost unless you are particularly rigorous in your separation.

Seed may be stored in small paper packets folded from lightweight tracing paper, which is ideal for long-term storage of orchid seed. It is breathable, and translucent to allow visualizing the amount of seed held within the folds. Squares or rectangles of tracing paper are folded in half lengthwise (step 1, Figure 3.2.1). Make two more folds at about a quarter of the distance from each end (step 2). At this point, unfold and label the packet (step 3) and place the seed inside the packet (step 4) so that all seed will be caught inside the folds. Fold again (step 5) and lightly tap the packet to distribute the seed to the bottom of the packet. Roll the packet over once and lightly fold it, repeat and tape the packet together (steps 6 and 7). Do not crush the seed by making the last two folds heavy; the packet should be slightly springy when complete. See Figure 3.2.2 for another view of a folded packet.

An alternative fold was taught to me by Doug Martin of Biosource Orchid Lab. Starting with a square of tracing paper, fold across the corners (step 1, Figure 3.2.3). The edge that is formed is folded into thirds (step 2), and the seed is placed in the center. Fold over the crossed corners, and tuck the flap into the pocket formed by the corners (step 3). The resulting packet (step 4) may be taped, but it holds itself in place if folded carefully.

When folded correctly, little or no seed should escape a packet. A minimum of tape should be used to close any seed packet, as tape "eats" seed, from which it can never be recovered. It is critical to properly label all packets with the contents, complete with the name, collection data of the parent(s), if known, and the date of collection.

Figure 3.2.2: Finished seed storage packet from Figure 3.2.1.

Another option for storing seeds is suggested by Stoutamire (pers. communication), who uses small cellophane packets for storing seed. Manufactured by Drug Package Inc., the size used is a #5, and can be ordered by the thousand. He recommends placing the cellophane into a paper coin envelope, and writing all the applicable seed data on the envelope.

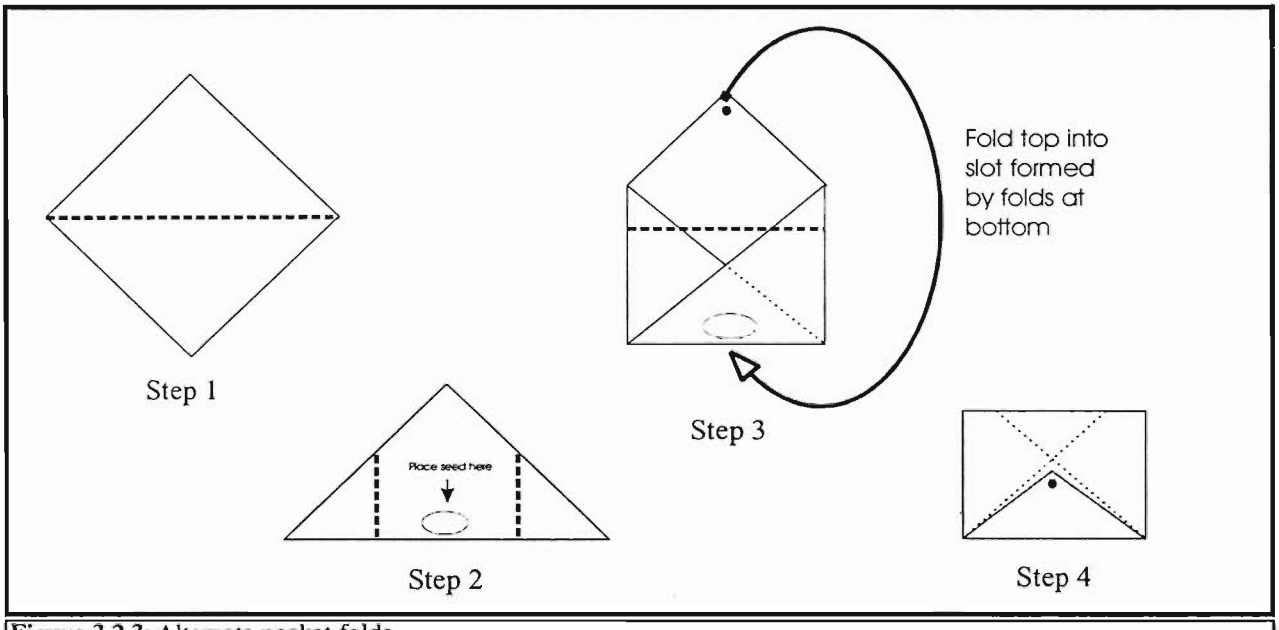

Figure 3.2.3: Alternate packet folds.

More than one clever grower has discovered that coffee filter cones are useful for storing relatively large quantities of seed. They are available at most grocery stores, and are pocket-shaped. They are cut down to the appropriate size, labeled and filled with seed. The end is then flapped over, folded and sealed. They provide good air circulation around the seed in conjunction with good recovery of stored material. They transmit enough light that one can visualize the remaining quantity of seed by holding it up to a light source. They tend to work best with larger seed.

The value of proper record-keeping cannot be emphasized enough. It is far better to provide more details than will be used than to be left wondering months or years from now. Record maintenance is best performed in a scientific lab notebook; if this is not available, most any notebook will do. Use a waterproof pen; pencil marks may fade with use. Write on only one side of the page; this will reduce the chances of data loss from the pen bleeding through, and will effectively halve the loss of data in the event of damage to your notebook. Orchids are very long-lived plants, and some may be essentially immortal. In any event, specimens exist that are known to be well over a century old by now. Greenhouse notebooks should be designed and treated to surpass the lifetime of the plants whose lives are recorded within them. Ideally, data should exist as two copies, kept in at least two locations. Data as hard copy (kept on paper) in two different formats may be used for cross-referencing. If information is kept as an electronic

copy as well as a hard copy, the electronic copy will permit rapid searching, easy backups, and can be used to provide paper copy later if required.

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3.3 Orchid Seed Storage

Orchid seed storage is a complex subject. Given that the Orchidaceae consists of several hundred naturally occurring genera, there is no simple answer to the question, "Under what parameters is it best to store orchid seed?" Indeed, it is unlikely we will ever be able to say there is one set of conditions that will satisfy the needs of all, or even most, orchid genera. Research in this area continues, but much of it is highly situational, and remains spotty. As with any biological product that undergoes decay as time progresses, the sooner the seed is used, the better. With some genera, such as *Disa*, there may be a rapid decrease in viability with age. Vogelpoel notes that there is the loss of viability "with great rapidity after a few months." (Vogelpoel, 1980) Others, such as many *Cattleya* species, can be stored for years and still maintain decent viability. Again, research in this area is tedious and time consuming. Much of this section will concern those who wish to research orchid seed storage, and will be above and beyond what most readers will need; the short summary below will serve the vast majority of growers. Those that require more detailed examination of the subject will find the rest of this section of value, possibly to assist with the creation of your own seed bank.

For those that do not wish to be bored with details, the following parameters for seed storage will suffice for most tropical epiphytic species: 4° C (39° F), 5% relative humidity (RH), or otherwise stored over fresh desiccant. Many growers do not even go this far, and once the seed is dried, it is stored in the crisper of the refrigerator (at about 4° C, or 39° F), which generally maintains a humidity around 20-30%. Stoutamire (pers. communication) reports some seed has been stored for as long as a quarter of a century under these conditions, while maintaining germinability. Admittedly not all species last this long, but it is uncertain as to why, or what causes otherwise healthy seed to fail to germinate after prolonged storage. Older seed may fail to germinate completely, or take prolonged periods of time to swell and germinate. This suggests that imbibition or other factors pertaining to moisture content of the seed has something to do with the problem, which has been studied by Pritchard (1993).

Another option is to store seed above a saturated solution of calcium chloride, for reasons that become apparent below. Species that seem to expire quickly in cool, dry storage (such as some members of the subtribe Stanhopeinae, including *Stanhopea* and *Coryanthes* species, and possibly the entire tribe Maxillarieae) may benefit from the increased humidity provided by the calcium chloride solution.

For those that were not deterred by the above statements, there has been much discussion about how to best prolong the life of orchid seed in storage. The general consensus is that reasonably lower temperatures are better, and humidity should be kept low. There are reports of orchid seeds being stored for more than twenty years and maintaining their germinability (Stoutamire, pers. communication, Arditti, pers. communication, Pritchard et al., Shoushtari et al.). Indeed, the seeds of many species of plants can be stored for hundreds or thousands of years provided the conditions are correct; for example, one species of Manchurian lotus produced seeds that still germinate readily despite having been buried in ancient lake sediments for more than a thousand years. Archeological digs periodically uncover seeds that still germinate despite their great age; many of these are from the arid southwest, where the dry conditions preserve organic material very well, and others have been frozen in arctic sediments or permafrost. Seaton and Pritchard (1990) note that the orchid species they have studied "can probably be classified as being so-called 'orthodox' seeds," which means that viability generally stays higher if they are stored cold and dry.

Orchid seed differs from that of other plants in that they lack an endosperm, and are generally very delicate structures. Orchid seed must be protected from storage at elevated temperatures, excess moisture and crushing. Moisture may not be a problem from the seed's perspective unless too much water enables fungal growth that consumes some seed and contaminates the rest. In fact, Rasmussen (1995) notes that many species of terrestrial orchids

do much better after prolonged soaking, and it is unclear what role, if any, this could play in enhancing the germinability of tropical orchid seed.

Seeds of the tropical species do not seem to be harmed by freezing. Storage at cryogenic temperatures evidently causes no harm. However, short-term cryogenic storage seems to offer few advantages over low temperature storage, and the complexities of storing seed for long periods of time in liquid nitrogen (LN_2) or with dry ice requires expensive refrigerators that may thaw out when power is lost or Dewars that rely on periodic resupply of the refrigerant. Shoushtari (1994) has reviewed several articles discussing exposure to freezing and cryogenic storage of seeds. One report from 1943 where *Cattleya* and x *Laeliocattleya* seeds were lyophilized by freezing in coconut water at -78° C, and then stored at -5° to -10° C for two hours while under vacuum indicates they "germinated well." Another researcher reports that *Cattleya*, *Dendrobium*, and x *Laeliocattleya* seed stored at -79° C for 12 to 15 months "germinated well," and yet another reports that when seeds of "several terrestrial and epiphytic orchids" were exposed to liquid nitrogen (-196 C) for 15 minutes, they too "germinated well." Pritchard (1984) reports that several species of terrestrial and epiphytic orchids were unharmed (i.e., differences in germination and embryo growth were not significant) after their storage vials were plunged directly into liquid nitrogen and held there for five minutes. The germinability and growth of one terrestrial species, *Orchis morio*, was enhanced by repeated freeze/thaw cycles in this manner. Recent work performed at the Orchid Seedbank Project has shown that species of *Laelia*, *Coryanthes*, *Stanhopea*, and *Peristeria* are capable of surviving 30 day immersions in liquid nitrogen with no adverse impact on germination (Hicks, unpublished data).

Still, cryogenic storage is not a panacea. Bowling and Thompson (1972) reported that collections of orchid seed held over calcium chloride at -10° C were found to be dead after 8-10 years of storage (Pritchard, 1986). Data from experiments lasting longer than this are few and limited in scope; Koopowitz and Thornhill (no date given) have shown that seed of *Encyclia vitellina* stored at -40° to -24° C presents upwards of 95% viability despite storage for 10 years under adverse conditions including power outages and other disruptions, and recent results indicate that ongoing experiments from this lot of seed continues to yield good results (Koopowitz, pers. communication).

Although its value as a conservation tool remains dubious, there is clearly much work that needs to be done with respect to the cryogenic storage of orchid seed. Gene banks that store major crop seeds, whose commercial value are much greater than that of the orchids, are in dire straits as the result of diminishing germinability of the stored material in conjunction with the lack of funds to continue such operations. Clearly, periodic renewal of the stock is required, which entails thawing out the seed, growing plants which then set seed, and then storing the fresh seed. With orchids, this would take years and a lab instead of single seasons and a garden for most food crops. Such long-term storage anticipates future availability of resources by which the efforts will not have gone to waste; this is a great burden, with little more than speculation to support it. If food crops cannot be supported in such a manner, how can we expect to support a much less lucrative and much larger group of plants?

Still, the value of intermediate-term storage in cryogenic suspension is significant; prolonging the life of seed in storage is an important issue, and the technology and tools are readily available, but the process can be expensive. Worse, there is no evidence that cryogenic storage necessarily prolongs the life of orchid seed. Although research in cryogenic storage of seeds has shown that lower temperature invariably extends viability, Pritchard (1986) indicates this is evidently not true of the orchid seed of species tested.

For those interested in pursuing cryogenic storage, Dr. Loren Wiesner, of the National Seed Storage Lab in Ft. Collins, Colorado, advises that they do not have any members of the Orchidaceae in their frozen nor cryogenic collections. Their protocol for liquid nitrogen storage consists of placing the vials into the vapor of liquid nitrogen; it is worth noting that there are no vials currently manufactured that are designed to withstand immersion in liquid nitrogen. It is not the low temperature alone that does this: liquid nitrogen may enter the tubes, which then expands when the tubes are removed, causing the cylinders to explode. Given that the tubes may be stored for weeks, months or years, even the tiniest leak may cause the tube to rupture when eventually removed. Thus, tubes must be stored in the vapor phase until a more acceptable system is found. The tubes may be shrink-wrapped to exclude liquid nitrogen and prevent rupture, but this is expensive and tedious.

An alternative to tubes are straws commonly used for preservation of semen and other biological fluids (Figure 3.3.1). When seed is stored in these tubes, kept in place with cotton plugs at both ends, immersion in liquid nitrogen will not result in container rupture upon removal. Moreover, at least one provider (IMV) produces a device capable of heat-sealing these tubes for immersion in liquid nitrogen. Their SYMS cryopreservation system is designed to maximize storage integrity and efficiency. Innovations in the cryobiological field have yielded many products that may be of use to seed banking in liquid nitrogen, including straws that are small and pack

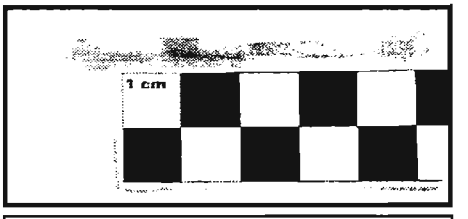

Figure 3.3.1: Approximately 13,000 seeds of *Epidendrum parkinsonianum* are stored in this small tube for cryogenic storage.

efficiently and coded for identification. Many forms are available, from bar-codes that can be read with a laser scanner to conventional alpha-digital printing. CryoBio Systems (of which IMV is a division) markets the largest variety of these products (see Appendix III, Sources).

The Orchid Seedbank Project has begun extremely limited experiments in cryogenic storage of orchid seed, and is gearing up for short-, intermediate-, and long-term experiments in this poorly researched field. Results will be slow to develop, but given the importance of determining factors in cryogenic orchid seed storage, this type of research must go on despite the difficulties to surmount.

There are reports of seed having successfully been stored at room temperature for prolonged periods of time with no undue effects. Zytaruk (pers. communication) reports having stored seed of a primary *Lycaste* hybrid on a shelf under ambient room conditions for almost two years. When flasked, it was later determined that germinability was almost 100%. Sadly, this is not typical of orchid seed, and many seeds become non-viable after several weeks or months under room temperature and humidity.

Humidity

Moisture control in orchid seed storage is an important factor; if stored in the presence of moisture, the seed may be eaten alive by fungi. Reduced temperatures in conjunction with arid conditions will adequately control such undesirable activity, but a balance must be struck between removing enough moisture to inhibit the growth of undesirable organisms, and providing enough moisture to optimize storage durations. Desiccants are most commonly employed in the reduction of ambient water for such purposes, and several different types are available. In order to adequately explain the best parameters for orchid seed storage, a few concepts including hydrates, humidity and constant humidity solutions, desiccants, and some specific compounds must be introduced and discussed.

Hydrates

Hydrates are compounds that have a high natural affinity for water, and when this water is removed (typically by heating to a sufficiently high temperature to cause the water to turn to vapor form, then removing the vapor) they become anhydrous. Such compounds are usually expressed as, "calcium chloride, anhydrous," or "$CaCl_2$, anhyd." In this form, they will chemically combine with any ambient moisture, reducing the humidity in a sealed vessel. There are other compounds, called drying agents, such as phosphorous pentoxide (P_2O_5) that chemically react with water. These compounds are much more efficient, but they are dangerous and difficult to work with. In fact, they may be too efficient and remove virtually all available water in a container; to the best of my knowledge, the effects of drying agents have not been studied with respect to orchid seed storage. Accordingly, ultra-low water storage should be undertaken only as research, not as routine, until we know the long-term effects on orchid seed.

Relative Humidity

"Relative humidity is perhaps the most familiar humidity parameter used—misused, confused and abused. Most of the abuse and confusion arise from a misunderstanding or ignoring of a simple principle. Relative humidity is a ratio, to obtain which two quantities must be known; if relative humidity is used as a humidity parameter then for completeness a second piece of information must be known, and usually the best to give is the air temperature."

- H.L. Penman, Humidity

Relative humidity (RH) is the concentration of water in vapor form, expressed as a percentage of the total carrying capacity of the air. For example, if a given sample of air holds 75% of the water vapor it possibly could under those conditions (pressure and temperature), we say that the relative humidity of that air sample is 75%. The amount of water that air can hold decreases with a corresponding drop in temperature; dew forms on the lawn early in the morning for this reason. Air is a solution, and the carrying capacity of the solute (water) decreases as temperature drops. The "dewpoint" is the temperature to which a sample of air must be reduced in order to completely saturate that sample of air; in other words, to raise the relative humidity to 100%. The relative humidity at which we store orchid seed is low (5% at 4° C). In order to achieve this level, we must use fresh desiccants in conjunction with vessels that can be sealed tightly.

Several literature sources state that they store seed below "5% humidity," but how they arrive at this figure is

unclear. In many cases, commonly available desiccants will achieve equilibrium (i.e., a concentration of water vapor that will not vary significantly, and therefore a specific relative humidity with confinement) at a relatively high figure–usually higher than 5% RH. As the desiccant absorbs increasing amounts of water, the amount of water vapor, and therefore relative humidity, increases until saturation is achieved. Once a desiccant achieves saturation, it either ceases to work, or it maintains a constant humidity atmosphere (see Table 3.1 below). Thus, while new and fresh desiccant may produce a very low humidity in an enclosure, its efficiency decreases as it absorbs water. Absorption of water will lead to progressively higher humidity levels as desiccants achieve satisfaction through combination with water, potentially producing humidity levels outside the desired range.

Measuring Humidity

Humidity is difficult to gauge under most conditions; most dial hygrometers are inaccurate even when calibrated. Most common hygrometers rely upon a polymer film, which changes length dependent upon humidity; older models relied upon human hairs. They are inaccurate, and identical models will often produce very different measurements in a side-by-side comparison. The "comfort" zone of 30-70% is difficult enough to gauge, but accurately measuring the amount of water in a sample at around 10% humidity is almost impossible without resorting to complex assaying techniques. Sling psychrometers, which function based upon the differences in temperature between a dry thermometer and a thermometer whose bulb is surrounded by a wetted piece of cloth, are much more accurate, but again have a narrow range within which they work best. Further, most cannot be operated in tight quarters, such as those in a small, sealed vessel for seed storage as they must be swung in the air for a time to establish a temperature difference between the two bulbs; several models rely upon mercury thermometers due to the excellent properties of mercury, and should be used with great caution, as it is not practical to shield the bulbs from impact. If you should break the bulb when using the psychrometer as it is designed to be used it is possible to spray a room with mercury, whose vapors are toxic. There are also models that use a small fan and blow air over the wet bulb, rather than cause the assembly to be swung around, but this type of psychrometer is more expensive.

Without expensive analytical equipment, the area below 10% RH becomes a range where guessing is as good a technique as any. As a result, packing your sample over clean, fresh, dry desiccant or constant humidity solution as described below are the best ways to assure that as your seed, pollen, and capsules dry they are stored under the best possible conditions.

Constant Humidity Solutions

It is worth noting that there are several chemical mixtures called constant humidity solutions whose intrinsic properties will allow them to form overhead atmospheres with a very specific humidity when contained. Producing them is fairly simple; they are either liquids, such as sulfuric acid (H_2SO_4) at a set concentration, or a slurry consisting of distilled water plus enough of the given solid that a saturated solution with undissolved solid exists. Set into a confined space, these constant humidity solutions will maintain a fixed concentration of water in the headspace of the vessel within which they are confined. As a result, we now have a technique for knowing with a fairly high degree of accuracy what the humidity will be. These solutions may be very important for future research, given the dubious techniques by which humidity has been determined in previous long-term orchid seed storage studies.

Table 3.1: Constant Humidity Solutions

Compound	Temperature Range (°C)	% RH
H_2SO_4 (70% wt. Solution)	5	4
H_2SO_4 (69.4% wt. Solution)	25	5.0
$NaOH \cdot H_2O$	15-60	6
$LiBr \cdot 2H_2O$	10-30	6
H_2SO_4 (65% wt. Solution)	5	8
$H_3PO_4 \cdot ½ H_2O$	24	9
Lithium chloride, LiCl	5	11.3
Potassium acetate	25	23
Magnesium chloride	25	33
Calcium chloride, $CaCl_2 \cdot 6H_2O$	5	39.8
Potassium carbonate	25	43
Magnesium nitrate	25	52
Calcium nitrate	25	54
Sodium bromide	25	57
Ammonium nitrate	25	60
Lithium acetate	25	70
Sodium chloride	25	75
Sodium carbonate	25	89

Before using constant humidity solutions, users should educate themselves about the risks involved; sulfu-

ric acid, for example, is extremely caustic in the concentrations given in Table 3.1. Sodium hydroxide (caustic soda or lye) can generate great heat and spatter when combined with water. Eye protection and patience is called for when manufacturing or using such solutions. Containers with such solutions must be clearly labeled as to their contents. Moreover, it is important to consider that some of these chemicals are caustic, or toxic. As a result, they should not be stored in a refrigerator used for food, nor where they can be accessed by children or others that are not aware of the hazards presented.

The following section contains information that will be of little value except for researchers; the material is presented for those that wish to perform research in this field, both in rehydration of orchid seed as well as seed storage.

Constant humidity solutions are discussed in the CRC Handbook of Chemistry and Physics, ASTM Standard E 104 and Helleiner (1980); for these compounds, we are able to calculate with some precision the relative humidity we would expect to receive when we store materials over these desiccants. Although not necessarily addressing the temperature range of interest (i.e., 4° C), the relative humidity produced by these saturated solutions will not differ by more than a few percentage points when the temperature drops (usually increasing slightly with a decrease in temperature). The margin of error will be within 1 percentage point of the given value for relative humidity in most cases, and those solutions specified at 25° C (77° F) may be useful for calibrating hygrometers, as per Helleiner (1980).

Knowing that solutions of sulfuric acid (H_2SO_4) at specified concentrations will produce an overhead atmosphere with a specific, known concentration of water vapor when contained is important; these precise values would be useful in order to standardize research in this field. There is no information as to the potential damage to orchid seed that long-term storage might result from exposure to vapors from this type of acid. The vapor pressure of a given liquid will indicate how much of it will escape as vapor. Sulfuric acid has a very low vapor pressure (much less than 1mm Hg), particularly at such low temperatures; all the same, questions remain about prolonged storage in contact with these vapors. It is conceivable that sulfuric acid could be stored with the seed if the vessel of acid were covered with a thin filter that was impregnated with a mild alkali such as sodium bicarbonate (baking soda).

Another possibility is phosphoric acid (H_3PO_4); this acid should serve as an excellent desiccant, and have an even lower vapor pressure than that of sulfuric acid. Further research is called for with respect to storage of seed in the presence of both of these acids, as well as with other compounds that reduce humidity to low values.

One issue with the acids is that they will eventually lose strength as increasing volumes of water are absorbed from the atmosphere which they "protect," unlike slurries (saturated solutions). Monitoring liquid solutions (such as sulfuric acid) through the use of a hydrometer is suggested. Constant humidity solutions employing saturated solutions with a slurry are easily "regenerated" by the addition of more solute as the water accumulated from the air dissolves more solid.

Probably the most commonly prescribed desiccant used for orchid seed and pollen storage is calcium chloride, or $CaCl_2$. For small quantities of seed, pollen, or other items that must be kept very dry, it is often employed by placing a small quantity of anhydrous calcium chloride in the bottom of a test tube, followed by some cotton. Seed or pollen, wrapped carefully in paper, may then be placed on top of this.

It is worth noting that, although calcium chloride will serve as a desiccating agent, as seen from the following table, it is not a very good one when saturated with water, particularly at low temperatures. It is further worth noting that there has been success with storing pollinia over calcium chloride for prolonged periods of time (several months, in many cases), but this is generally the dry compound, and not a slurry (as with constant humidity solutions).

Table 3.2 shows the level of humidity that will be achieved at the given temperature once calcium chloride is saturated with moisture.

Table 3.2: Calcium Chloride and Constant Humidity Solutions

Temperature (oC)	% RH
5	39.8
10	38
18.5	35
20	32.3
24.5	31

There are other desiccants that are commercially available. Some of these are commercial preparations designed for use as commercial desiccants, such as Drierite, which is anhydrous calcium sulfate. The material can be purchased with or without a cobaltous chloride indicator; the indicator is blue when the material is dry and below capacity, and gradually turns pink as regeneration or disposal is required.

Silica gel, commonly used as a desiccating agent, has markedly poor efficiency below 40% RH. As a drying agent, it can be used to maintain dryness in enclosed environments, where desiccant efficiency is highest, and then only when fresh. It has a very high capacity for moisture at higher humidity levels, and therefore may be useful for pre-treatment when drying out capsules or other large, moisture-laden articles.

Some clays possess a great affinity for water once they are heated to drive off ambient water and they tend to be more efficient at low humidity than silica gels.

Most efficient of all are molecular sieves. As their name implies, they sort available molecules by size, and water happens to be a "convenient" size due to its molecular structure. They also have a higher capacity for water than either silica gels or clays. It is not clear whether they represent a distinct advantage over other desiccants because it is not known if their high efficiency may damage seed by removing too much moisture. It appears likely that excessively dry conditions may cause higher seed mortality, so the use of molecular sieves is discouraged for long-term storage until further study has been performed. Molecular sieves are difficult to regenerate, unlike other desiccants. While molecular sieves must be regenerated at very high temperatures, assisted by being put under vacuum, most desiccants need simply be placed in an oven at a little more than 100° C, provided they are capable of being regenerated (check with the manufacturer).

It is also worth noting that several well-known individuals who propagate orchids from seed do nothing more than dry it, then place it in envelopes in the refrigerator. No desiccants are used, and depending upon ambient conditions outside of the refrigerator, the humidity level inside may be adequate for proper storage. They do not report any significant loss of viability as a result, but this may be because no control was used by which their results may be judged: very little comparative research has been performed in this field.

An important factor in seed storage is to allow storage containers to reach room temperature before opening them; by taking this extra step, we prevent condensation on the individual packets, and therefore extend the life of the seed as well as the life of the desiccants involved. Much in the same way that droplets of moisture accumulate on the outside of a glass with an iced beverage on a hot and humid summer day, water will be drawn from the air onto the cold surface of seeds and their packaging when removed from refrigeration, potentially spoiling seed.

At a sufficiently low humidity, even a cold object (say, a seed storage container) kept at a given temperature (say, 4° C) will not be able to draw moisture from the air. The dewpoint of a given body of air is the temperature to which the air must be dropped in order for condensation to form (i.e., that the relative humidity of such air is brought to more than 100%). Still, there is a small amount of deposition that occurs at a temperature slightly higher than this, caused by micronucleation of water droplets. Table 3.3, below, will give the dewpoint and, therefore, the minimum temperature to which a seed storage container should be brought before the dewpoint is met. In other words, if you know the temperature and humidity of the room in which you are working, and your seeds are stored at 4° C (about 39° Fahrenheit), the following table will indicate whether or not gross amounts of condensation may form, which will prematurely saturate your desiccants.

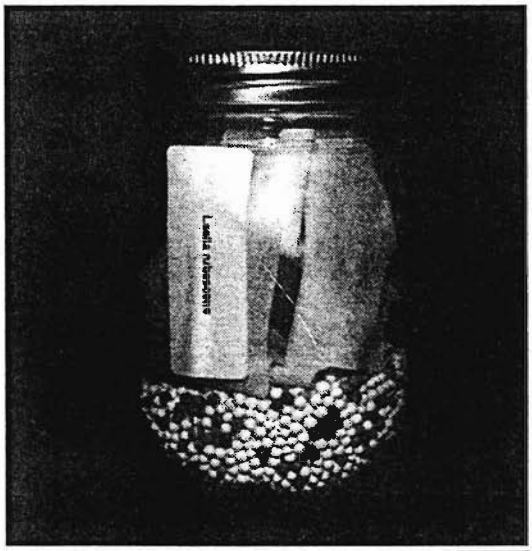

Figure 3.3.1: Mason-jar desiccator for fresh capsules. Note bead-type desiccant in bottom.

Table 3.3: Temperature, Humidity and Dewpoint

Humidity of room air, %RH

Temperature, degrees Fahrenheit	5	10	20	30	50	70	80	90
55	-14	0	15	24	37	46	50	53
60	-10	4	20	28	41	50	54	57
65	-5	8	23	33	46	55	59	62
70	-3	12	27	37	51	60	63	67
75	1	15	31	42	55	65	68	72
80	3	19	36	45	59	70	74	77
85	7	23	39	50	65	74	78	82
90	11	26	44	55	69	79	83	87

Data taken from: Relative Humidity and Dew Point Table, U.S. Department of Commerce Weather Bureau, TA No. 454-0-1D, 10-63.

How to use this table:

Find the approximate temperature of the room in which you will be working, in the left-hand column. Go across, using the number at the top of each column for the relative humidity in your workspace. The number at the intersection will be the MINIMUM temperature which your seeds should have reached before you begin work to prevent excess condensation.

Thus, if your workspace is at 70° Fahrenheit, and your relative humidity is 50%, your seeds should be at 51° Fahrenheit or more before proceeding. A rule of thumb is to avoid opening seed storage containers until any condensation that may have appeared on the outside has disappeared. Only the largest operators may find this section of importance; hobbyists and those with relatively small collections of seed should simply be made aware of these factors.

Longevity

Orchid seeds have demonstrated the ability to survive for long periods of time in storage. Shoushtari et al. (1994) note that seed of various genera that had been stored for as long as 20 years still germinated when stored at 4° C (39° F) over calcium chloride desiccant. Still, not all of the seeds kept in storage were viable after such a duration and all had initially displayed viability when put into storage. For example, of the 11 species of *Cattleya*, composed of 16 different collections, stored for between 12 and 19 years, only 5 of these collections coming from 4 species eventually germinated. Pritchard and Seaton (1993) did exhaustive research showing several collections of seed that demonstrate germinability despite years of storage under a variety of conditions (typically dry refrigeration). Unfortunately, examples of seed that perish after days or weeks of storage despite refrigeration and reduced moisture content are also presented.

To date, the properties that determine the ability of seed to retain germinability after prolonged storage times are not known.

Short-lived Seed

There are several groups of orchids whose seeds are short-lived. It is unclear as to why they keep so poorly in storage, and there is debate as to whether these seeds expire at a higher rate once stored as dry seed, or if they are already dead once they are in dry storage.

Anecdotal evidence is that disa seed is very short-lived; again, the reasons are unclear. It has been suggested by Cesar Zapata (pers. communication) that the seed tends to germinate while in the capsule, and then perishes when the

seed is harvested and desiccated. It is possible to determine potential viability of *Disa* seed by soaking it in distilled water for 24 hours, and then observing it under a microscope. Viable disa seed will swell when immersed in water for this long, providing an excellent indicator that it is still of use. Indeed, seed of *Disa* species is quite capable of being germinated either *in vitro* or on wet *Sphagnum* moss, and it is difficult to judge which method is best. Zapata reports success with *Disa uniflora* seed that has been soaked in distilled water, at least until it starts to swell, before sowing it on Sphagnum, and claims that success rates are much higher using this technique. (**Note:** Caution is called for with the use of *Sphagnum* moss as it is known to carry fungal spores that may cause sporotrichosis, which may result in infection of the skin or lung. Wear gloves and a protective respiratory mask if dust or aerosols may form. Wash with soap and water when done.)

Vogelpoel (1980) recommends germinating *Disa* seed on "sterilised" [sic] (boiled) *Sphagnum* moss, or "clean, preferably sterilised [sic], imported peat moss." A 2 cm thick layer of *Sphagnum* is laid over coarse river sand, sitting in a rain gutter to help increase drainage which in turn helps reduce the growth of algae. It is not to sit in stagnant water, nor should it ever dry out. Protocorms may develop within three to four weeks. Vogelpoel goes on to note that germination via this route is "excellent," and preferred over aseptic techniques when growing this species.

It has been reported by Thornhill and Koopowitz (1992) that *Disa uniflora* could be stored for long periods of time under cryogenic conditions (-70 C) with minimal loss of viability, but these are extrapolations from experimental results over a much shorter time frame. Moreover, *Disa uniflora* differs from most orchid species in that it is a "feet-wet" terrestrial orchid with very large seeds and is native to South Africa. Differences between the cryogenic storage of epiphytic orchid seeds whose properties may or may not be very different from *Disa* may be considerable.

Stoutamire (1990) notes that viability of *Disa* seed "decreases rapidly within a year, but some seeds retain the ability to start germination for at least 10 years," and that plantlets that develop from old seed do so slowly, and generally die.

Several growers have reported that members of the subtribe Stanhopeinae are very short-lived in storage. Böhm (pers. communication) suggests that seed of members of *Stanhopea* and *Coryanthes* rapidly lose their germinability, but several collections I have tested have demonstrated good germinability after more than a year in dry refrigeration at 4° C (Hicks, unpublished data).

Rehydration

Several growers have noted that orchid seeds that are old tend to take longer to swell and germinate. This may be related to problems surrounding rehydration; damage may result as a product of protracted storage under cold and dry conditions. It is uncertain as to the nature of this damage, and how to prevent or reverse it. It has been suggested (Hicks, unpublished data) that constant humidity solutions are a potential answer; thus, if seed has been stored at low humidity for a long period of time, its germinability may be enhanced by exposing it to slightly higher humidity for a period of time prior to attempting to sow it. This hypothesis has not been fully explored, nor have parameters such as the length of rehydration or humidity levels.

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3.4 Handling and Shipping of Orchid Seed

Due to its small size and light weight, orchid seed can be difficult to manipulate. Worse than losing seed is cross-contaminating it with the seed of other species; it is vital to prevent seed of one species or hybrid from being contaminated with that of another. When working with orchid seed out in the open, it is essential to minimize air movement, and to work on a clean, smooth surface. One such surface is a seed cutting board, which is highly suggested for anyone who works with a large volume of seed.

A cutting board (Figure 3.4.1) can be designed and built inexpensively from a piece of 6mm (¼") thick glass; the dimensions are not important, but 30 cm x 60 cm (1' x 2') is about the minimum. Pay the extra dollar or so to have the corners rounded, and the edges ground so they are not sharp, and less prone to chip. After you bring it home, carefully clean one side of the glass, and wipe it down with acetone or a similar solvent if you so desire. (Caution! Acetone and other organic solvents are volatile, toxic and inflammable. Use only with sufficient ventilation and with due caution. Wear gloves to prevent dermal absorption.) Lay it clean side up on a sheet of newspaper, and spray it with a thin layer of black spray paint. When it has dried, carefully pick it up and hold it up to the light; look for any thin spots that you may need to repaint, but even a patchy job will produce a black slab once the contact paper is added (see below). Add an extra coat if you want, and let it dry for several hours. Protect your paint job with a layer of adhesive contact paper of the sort used to cover shelves, available at a hardware store; trim the excess with a razor blade. Flip it back over, and you have a (literally) glass-smooth, high-contrast surface on which you can work with your seed.

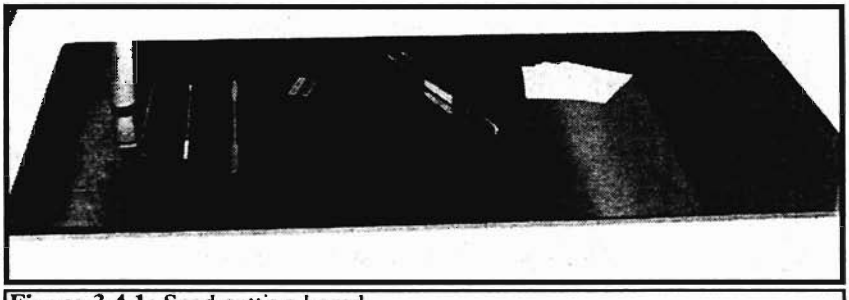

Figure 3.4.1: Seed cutting board

When working with seed, the cutting board provides a clean surface upon which to work; when switching between different species, the cutting board provides for a readily cleaned surface, preventing contamination between different collections of seeds. One must be certain to inspect and wipe down the glass, if necessary, to remove any stray seed. A lint-free cloth works well, but adhesive tape can be used to remove small amounts of seed. The smooth glass will allow for quick recovery of the seed if spilled. The use of a small light on the side of the board away from the user, kept at a shallow angle to reflect light and reveal remaining seed, is suggested to make sure the board is clean between different lots of seed.

When shipping orchid seed, it is important to protect the seed from crush damage. Recent work by Stoutamire (1992) suggests that, despite the tiny size of orchid seed, if left unprotected in, say, an unpadded envelope, orchid seed will be damaged, and much of the potential viability will be lost. Padded mailers, such as those with bubble wrap liners, are recommended.

Even more protection is afforded through the use of rigid mailers or boxes, such as those used to mail video

and audio media like cassette tapes, video tapes, and compact discs. These containers are relatively inexpensive, and virtually assure that no excess pressure will be applied to the seed. Other alternatives include the use of metal or plastic containers, or inserting seed in cardboard cutouts. To create these packages, a hole large enough to accept the finished seed packet is cut in one or two thicknesses of corrugated cardboard. A cutout may be used with an envelope, provided the trip is not expected to be a long one. If the seed packets are small enough, plastic film canisters make good shipping containers. If you can find them locally, metal film canisters about 4 inches in diameter, used to hold small reels of 35mm film, are excellent; check a local film lab or photographer that uses film supplied in bulk to see if they will save them for you.

Not acceptable are flat mailers used for computer floppy discs or photographs; although designed to protect valuable magnetic media or pictures, they will not suffice to protect orchid seed from crush damage.

Sometimes, when shipping particularly large volumes of seed, or particularly valuable seed, it would be advisable to add a small quantity of desiccant with the shipment, especially if it is going to or being sent from a humid region, or contains components that may not be entirely desiccated when packed. Small desiccant packets are available from commercial sources, and they are also packed in with pharmaceuticals; ask your pharmacist to save disposable desiccant packets in an airtight container for future use. Enclose the seed along with the desiccants into a small, airtight plastic bag so that they may remain dry for the entire trip. Remember that desiccants have a limited function, and their ability to absorb water is not infinite; without an indicator such as cobaltous chloride, it is not possible to visually determine when a desiccant is saturated.

Static Control

The science of controlling static electricity has come a long way in the past few years, much of which is due to the demand for effective static control in the electronics industry. Some of these tools may be used in conjunction with orchid seed processing.

Due to the small size and the tendency for seeds to acquire an electrostatic charge, control of static electricity may become necessary under extreme circumstances. Static electricity is primarily a problem in dry, arid climates, and with the Orchid Seedbank Project operating in the middle of a desert, it is no surprise that we occasionally have to combat this problem. Orchid seed clings to papers, sticks to glass, and is generally very difficult to handle when there is excess static buildup. The best way to control static electricity is to boost the humidity–rarely an appropriate solution when it comes to orchid seed. Devices such as grounding straps, although effective at reducing the static hazard of the operator, does little to reduce the static charge on a piece of paper on which orchid seed is stored. Working on a surface covered in aluminum foil, which is in turn grounded, may help the problem, but is very hard on the eyes after a short period of time. A larger slab of gauge metal may serve to cover a portion of a workspace, grounded with a clamp or a screw. Another option is to wipe the cutting board down with an anti-static laundry softener dryer sheet. There are commercially available cleaning solutions that are used to control static on working surfaces, and are available through specialty companies.

There are small brushes and emitters which use radioactive polonium to disperse the accumulated charge from a surface; emitting alpha particles (a helium nucleus without electrons), it absorbs electrons when it contacts the working surface, discharging the static. Alpha particles are essentially harmless to humans unless they strike living cells, and given that alpha particles are readily stopped by a sheet of paper, a few inches of air, or the dead skin cells that cover most of your body, they require ingestion or entry through an open wound to be harmful to you under the described working conditions. Unlike the human body, however, orchid seeds are relatively naked, and are theoretically subject to damage by alpha particles; therefore, radioactive sources should not be used to eliminate static with seeds.

Zerostat anti-static tools, which use a piezoelectric action to create either positive or negative charges, are available, but expensive and have a limited lifetime; nevertheless, they seem safe for use with orchid seeds. Sigma chemical (see Appendix III, Sources) sells at least one model.

Ionizing air sources, or ionizers, come in two types: those with blowers, and those without. They are designed to work by protecting electronic repair or assembly stations with a source of charged air that neutralizes static charges. The units with blowers are less effective because they will probably have to be placed far enough from the working space that the air flow does not disrupt the seed. Those without blowers are much better because they often have points through which the ionizing charge is emitted and they must be equipped with safety features to prevent shocking the operator in the event of accidental contact. They are very effective and useful where there is an extreme static problem.

It is worth mentioning at this point that provided the worker uses very smooth paper, there are very few problems with handling orchid seed with respect to static electricity. The greatest problems occur when plastic surfaces

(such as polycarbonate) are present, or when dealing with very large seed (such as the *Epidendrum secundum* type, which may reach 4-6 mm in length). Smaller seed is very easy to work with, even under extreme conditions.

Laws

Unfortunately, orchid seed is not removed from laws. Under the Convention on Trade in Endangered Species (CITES), all orchids are classified under Appendix II. In the United States, the members of *Paphiopedilum*, *Selenipedium*, *Phragmipedium*, and *Mexipedium*, as well as a handful of single species, are placed under Appendix I. Seed of Appendix I material may not be sent between countries without permits, but it may be freely sent to other individuals within the same country. The permit application process and laws are different for each nation; inside the United States, one must go through the Office of Management Authority, under the US Fish and Wildlife Service.

Office of Management Authority
4401 North Fairfax Drive
Room 430
Arlington, VA 22203
(703) 358-2104, x 5437

All other species (Appendix II species) are exempt from CITES. Despite this exemption, plant health and quarantine regulations may require phytosanitary permits in order to be imported. The US does not require phytosanitary permits to import orchid seed, but other nations may have their own restrictions. If this is the case, it is up to those who are shipping seed to send the seed through one of the designated ports of entry along with the appropriate paperwork and a fee ($23 for most cases at the time of this writing). If not accompanied by the proper paperwork, customs may refuse entry, and confiscated packages are generally incinerated if they are not returned.

Check with the US Department of Agriculture for permitting instructions and your nearest Animal and Plant Health Inspection Service (APHIS) port of entry inspection station. Check with the US Department of Agriculture (USDA) to find out where your port of entry is. Remember that orchids are only rarely handled by some ports of entry, so finding an expert may be difficult.

Some nations that take particularly stringent measures to ensure that no noxious plant pests are imported may further require special permits on the part of the recipient, along with a fee that can be very expensive for small quantities of seed.

At the time of this writing (1999), Hawaii does not require a phytosanitary permit on orchid seed from the continental United States.

Once placed in flask, orchids are exempted from CITES regulation regardless of their listing (i.e., Appendix I or Appendix II). Most, but not all, nations regard flasked material as exempt of their regulations; again, it is best to check.

Due to the esoteric nature of orchid seed with respect to customs, it is not uncommon for properly routed and permitted material to be delayed. Identify packages as perishable so that they do not spend weeks sitting in customs.

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3.5 Laboratory Basics

"We're at that point where biology leaves off and chemistry begins."
"I thought we were at the point where chemistry leaves off and biology begins."
Two scientists in a cartoon, "Today's Chemist at Work," March 1998

For most small home flasking facilities, the room in the house that most resembles a lab will be the kitchen. It is here that we can cause the most damage, particularly in terms of strained relationships when your spouse comes home to find the remains of unsuccessful experiments in the sink. The best way around this problem is to choose another location, if space permits. Important attributes that should be kept in mind when considering simple lab facilities include:

- Cleanliness. An environment as free of stray dust and excess traffic as is reasonably possible is essential
- A stainless-steel or ceramic sink with hot and cold water
- A reliable power supply, preferably with GFI power interruption circuits
- A comfortable, desk-level workplace, and a comfortable chair with back support
- Non-porous surfaces that are readily sterilized with bleach or other solutions
- A heater or other power source sufficient to drive an autoclave or pressure cooker

Note that we will use the terms autoclave and pressure cooker interchangeably, unless otherwise noted.

Labs should be constructed with safety in mind; with the possibility of spills around electrical power, a GFI (Ground Fault Interrupter) is invaluable for cutting off power when voltage might accidentally leak to ground, presenting a safety hazard. There is a fire hazard any time alcohol burners or other open flame is used, and a fire extinguisher suited for use on A, B, and C fires should be available in any laboratory, as well as a smoke detector for when you leave the lab for "just a minute." Although the home lab is not subject to regulation by OSHA safety laws, common sense and respect for the chemicals and hazards that a lab presents should provide the reader with the dangers at hand. Although perhaps no greater than cooking dinner over a gas stove, few of us are as comfortable in the lab as the kitchen. As a result, greater safety measures should be taken.

Chemical Safety

When we work with potentially dangerous chemicals around the house, it is important to have available and use protective safety equipment. Strong acids and bases can irritate the mucous membranes with their vapors, liquids can splash on the skin and into the eyes, and dusts and powders of otherwise innocuous compounds can irritate the skin and eyes.

It is important to know the hazards inherent in the chemicals that we are exposed to, both in the home and in the home lab. Labels are not for the edification of the manufacturer; they are there for the consumer, so that appropriate safety measures may be taken. One relatively recent development is the MSDS, or Materials Safety Data Sheet. As provided for by law for virtually every product containing chemical compounds for commercial or industrial use, an MSDS should be made available for the consumer of any chemical sent to a home lab, as well as complex mixtures, such as flasking media. Know how to read these sheets, and understand the hazards that this information describes. Moreover, know how to minimize hazards indicated within the MSDS.

Eye protection: Use safety glasses, safety goggles, face shields, or even larger protective shields to defend the eyes against injury. Eyeglasses, unless constructed of impact-resistant plastic, may provide only partial protection against injury, particularly from splashes that may strike the face sideways or run into the eyes. With the availability of comfortable and inexpensive eye protection, the reader should be certain to defend one's eyesight appropriately. Whenever working with chemicals, it is important to remember not to touch or rub the mouth or eyes.

Skin protection: Use of gloves is highly recommended. Be certain to use gloves that protect against hazards such as those that you will incur. For example, PVC gloves will dissolve in acetone. Although not particularly relevant at this stage (most of the simple compounds we are dealing with here will not penetrate gloves to any significant degree), some solvents will migrate through latex and PVC gloves; it is important to use heavier gloves that have known resistance to these chemicals. Contact the manufacturer if you are uncertain: Use of an apron, particularly when dealing with corrosive or toxic liquids, is suggested.

Respiratory protection: When working with contained vessels, such as a glove box, we will find that a high concentration of chlorine gas or alcohol vapors may have accumulated when we open it. It is important to ventilate under these conditions because chlorine gas can be very damaging and ethanol (ethyl alcohol) is flammable. If you find yourself becoming hypersensitive to chlorine gas due to frequent exposure, there are masks and filters available to remove such damaging vapors. Similarly, some reagent alcohols, primarily ethyl alcohol, contain concentrations of methyl alcohol or other compounds to denature them (prevent human consumption). Methyl alcohol, also known as "wood alcohol," is very poisonous, and is widely known for causing blindness. It is important to ventilate as protection against any chemical fumes, particularly if they are flammable or poisonous.

Heat protection: Although perhaps common sense, just as the same as when we are cooking, care should be taken not to injure yourself when preparing hot media. Wear heavy gloves or oven mitts when moving hot flasks, and wear shoes that offer protection in the event that hot, liquid media is splashed or spilled. An apron is also suggested.

Toxic compounds: There are a number of compounds whose properties are such that they are not recommended for use in the home lab. Some growers wish to use colchicine on their plants; this should be done only after careful study of the properties of this carcinogenic chemical that penetrates through the human skin. Similarly, before working with different chemical compounds, you should know how to read, and understand, an MSDS, and be capable of dealing with the hazards as appropriate.

Commonly available orchid media and their components do not present significant health risks when treated the same as other non-food chemicals. They should be treated with respect, for formulations vary; it is within your right to request an MSDS sheet for these formulae so that you can judge for yourself the risks at hand.

It would be worthwhile to familiarize yourself with a Materials Safety Data Sheet; they often cite strong warnings for relatively common compounds and, indeed, many of these are quite dangerous (drain cleaner, shoe polish, the gasoline you put in your car, etc.). All the same, we must read and understand the MSDS in context; the reader should be acquainted with these before becoming unduly concerned from these seemingly exotic compounds when in many cases they present no more of a health risk than chemicals we use on an everyday basis.

Lab Glassware

Laboratory glassware is an important element in chemistry, and its proper use and maintenance is a priority if we are to produce our own media. Many of our techniques rely upon repeating the work of others, and these procedures often call for the use of stock solutions, dilutions and other procedures that require precise volumetric work. Clean, accurate glassware will allow the user to produce work of the highest standard.

Glassware comes in many different forms: we will be interested in using Pyrex, Kimax, or other heat-resistant glassware when heat is involved, but for other applications, it is not important. Indeed, certain vessels (such as volumetric flasks[12]) must not be heated, or we run the risk of ruining their accuracy. Pyrex is also safer than soda lime or common glass when broken. Although it will still form sharp edges, it is less prone to breaking into long, extremely sharp shards. If uncertain, soda glass is greenish on edge when chipped; Pyrex is gray.

Laboratory glassware is expensive; it should be treated with care, and cleaned with appropriate detergents after use, or with acids (chromic acid in particular) if they become so dirty that detergents alone will not clean them. Care should be taken with chromic acid, or other aggressive cleaning agents. One should also be aware that some cleaning agents actually dissolve tiny amounts of glass each time they are used, and therefore change the specific volume of glassware. This will not be significant unless glassware is left in these solutions for a long period of time, or used repeatedly, and is generally not important unless dealing with volumetric glassware.

One other factor worth noting is that several states have passed legislation to the effect that certain types of glassware are illegal, or must be permitted (this is to say, the owner must have a permit to possess it). In the drug paranoia that has swept this nation, Texas, for example, requires permits for the possession of Erlenmeyer flasks (Texas State Health and Safety Code section 481.080). The enforcers of such laws, one would hope, are capable of discriminating between vessels in use for plant cell culture, and those used in methamphetamine manufacture. Fortunately, Mason jars are cheaper, easier to find, and make better flasks anyway.

Pipets

Pipets are used to accurately deliver volumes of fluid, generally from 1 to 10 ml or so. They are not to be used like soda straws, i.e., the liquid within is to be drawn up using a small bulb designed for such purposes, and not by mouth. The fluid within should be brought up to slightly higher than the calibration mark, and then air introduced slowly to drop the level of the fluid to the mark with the pipet held vertically. The bottom of the meniscus[13] of the liquid should be at this mark precisely. Hold this level either with the bulb or by placing your index finder over the end. The tip is then wiped clean of any remaining droplets with an absorbent tissue, and placed into the next vessel for delivery. Release the liquid by removing your finger or bulb, and allow all the liquid to trickle out.

If the pipet is designed "to deliver" (marked with a "TD" on the barrel), then allow the tip of the pipet to touch glass–gently touch the tip to the inside of the vessel while draining. Maintain contact and hold it vertical for several seconds after flow has ceased, and then remove the pipet. Some tiny amount of liquid will remain inside the tip of the pipet; the glassware is calibrated with this in mind.

If the pipet is designed as a "blow out," then a bulb is to be used to eject any remaining liquid. Rarely seen are "TC," to "to calibration" or "to contain" pipets; filling these is the same as the other types, but once the liquid has been delivered, the barrel of the pipet is to be washed with solvent (water, for our purposes) so that no traces of the original solution remains.

Another safe method is using so-called pipettors (piston-stroke pipets) that are now standard in virtually every professional laboratory. These pipettors are available for all required volumes from 0.1 ul to 5 ml. Some are preset to one single volume (for example, 0.1 ml, 1 ml, 2 ml, 5 ml, etc.) These appliances are operated using disposable tips.

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[12] Flasks calibrated to contain a very precise quantity of liquid, often used in analytical chemistry.

[13] The curvature experienced by liquids in glass, caused by adhesion between the liquid and the walls. With water, the meniscus is always measured from the extreme bottom of the curvature (see figure 3.5.1).

Although these pipettors may be considered to be very expensive at first, they may prove to be more economical than their glass counterparts in the long run. Indeed, glass pipets easily break and precision pipets are inherently expensive. Furthermore, they must be washed after each use, requiring a pipet washer, detergent, and time. Some must be autoclaved before re-use. The disposable tips for the pipettors are fairly inexpensive and can either be ordered as non-sterile or sterile. The lower part of the pipettor (which is in contact with the tip) can also be sterilized.

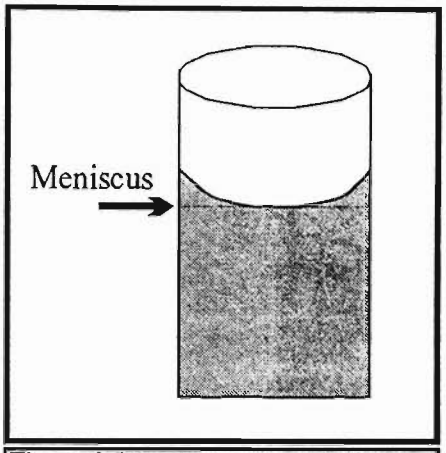


Figure 3.5.1: The meniscus indicates the point at which the volume is read.

Volumetric Flasks

Volumetric flasks are used to determine the volume of liquid very accurately, generally from 50 to 1000 ml, but much smaller and much larger ones are available. They are expensive, and should be treated with great care. One liter versions can be used to hold the different constituents of a media as delivered from different stock solutions, if the volume is critical. If it is not critical, or the media is "messy" (i.e., has activated charcoal or would otherwise be very difficult to clean), then something else should be used, such as a wide-mouth beaker or a Fleaker (a tall, narrow-mouthed heat-resistant flask with many lab purposes manufactured by Corning).

As with pipets, the volume of the liquid should be brought up so that the meniscus (Figure 3.5.1) is in contact with the calibration mark. While adding solvent (water for our purposes), a squeeze bottle may be used to rinse water down through the neck, taking all remaining droplets of liquid or crystals of media or other components into the bottom of the flask to assure recovery of all components. After filling to the calibration mark, the flask should be capped tightly and inverted as many times as is necessary to assure the contents are homogeneous.

Filter Flasks and Büchner Funnels

These two go together in that the funnel is mounted in a stopper such that it can be placed in the mouth of the filter flask. The filter flask then is connected to an aspirator or other source of negative pressure. The Büchner funnel then supports the filter media as liquid is run through, and caught in the filter flask.

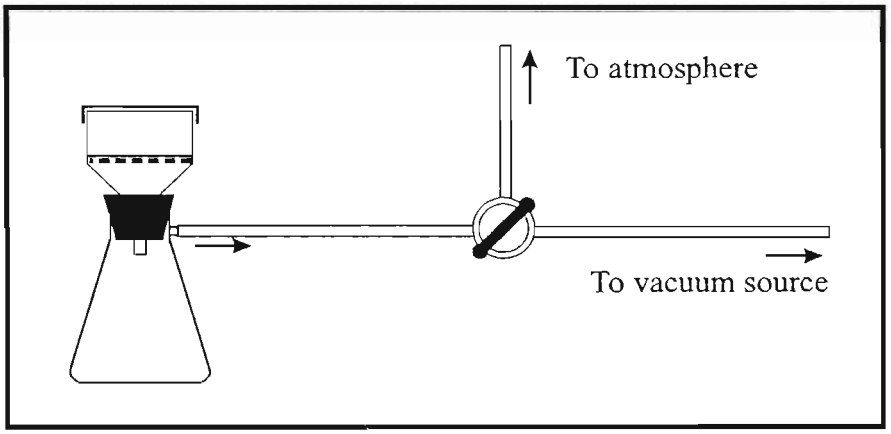


Figure 3.5.2: A vacuum flask with valve to prevent backflow.

Always be careful when operating flasks under negative pressure; although filter flasks are designed to resist implosion, and are heavy-walled, one should not attempt to draw them to high vacuum. Some users wrap them in tape specifically designed to retain glass fragments in the event of failure. Many flasks manufactured today are coated in a special layer of plastic, which will serve the same purpose. Drawing a vacuum sufficient to implode a vessel is difficult, but the results are unpleasant and dangerous. Only glassware designed for such purposes should be used as such, and should be in good condition, without chips, cracks, or other signs of weakness.

Since very few households have a vacuum pump for lab use, you will probably have to invest in a small fixture that operates upon the venturi principle. It connects to the end of your faucet, and draws a vacuum roughly proportional to the velocity of the water flowing through it. These are commonly sold to help drain water beds, and may be modified with adapters for lab use. You will also want to put a "T" in line with a valve, so that any vacuum may be broken without "burping" water through the system (Figure 3.5.2).

Erlenmeyer Flasks

Erlenmeyer flasks are most commonly known for holding media and seedlings; they serve other purposes in the lab, such as storage of stock solutions and other miscellaneous jobs. Once used as flasking vessels, they have

been replaced with everything from canning jars to empty spaghetti sauce bottles.

Pasteur Pipets

Pasteur pipets are most useful for the "quick-and-dirty" flasking techniques involving simple disinfection and washing routines. This is to say, they are inexpensive pipets that have no calibration marks on them. They are cheap (pennies apiece when purchased by the hundred), disposable, and very useful in the labs. Small rubber bulbs are made specifically for use with Pasteur pipets, and should be purchased as semi-disposable items themselves.

Purchasing Glassware

Glassware is expensive, and it can also be difficult to find. The reader is encouraged to find glassware via surplus sources, or though other inexpensive means. Accurate, calibrated volumetric glassware is important for research purposes, but the home grower will find it expensive and difficult to find. Moreover, without a complete array of expensive analytical instruments, it will be useless. For those that are willing to expend the money for an analytical balance, heat/stir plates, thermometers or thermocouples, desiccators, and other apparatus, volumetric equipment should be purchased new, or through reputable sources. Hobbyists and even small production labs will be able to get by with non-graduated glassware, or glassware with etchings that indicate only approximate volumes (within 5%).

Another substitute is to use plastic; Nalgene bottles can be used for stock solutions, and are much harder to break.

Other Lab Equipment

Stir Plates

One of the most useful lab tools is a stirring hotplate. The beaker or flask which requires stirring is placed on top of the stir plate, and a coated magnet is dropped in. The rate at which the magnet spins is controlled on the plate. If the stir plate also heats, this allows for rapid heating and homogenization of media without requiring stirring or agitation. Although generally expensive ($400 or more when new), these tools are valuable time-savers that quickly pay for themselves.

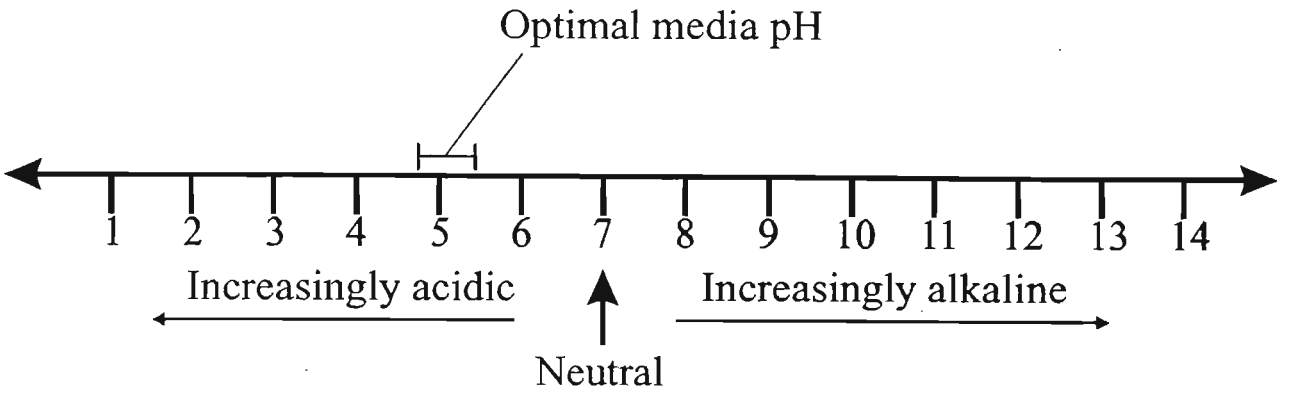
Inoculating Loops

Primarily used for bacteriology, inoculating loops are generally used for manipulating bacterial cultures. For orchid propagation, they may be useful for manipulating seed once it has been disinfected, or used for removing seed from disinfectant or filter paper and transferring to media. Loops come in plastic and wire (generally platinum or nichrome). Plastic loops are generally sterile upon receipt, and must be chemically sterilized to be re-used. Wire loops are much more expensive, and may be thermally sterilized by flaming or autoclaving.

Loops are just that: a tiny loop of plastic or wire at the end of a handle, and they may be made out of music wire. Unfortunately, stainless steel wire is difficult to find, and therefore, it is generally best to purchase high-quality loops for transferring seed.

pH

The pH of a solution is a measurement of how acidic or basic it is. Acids have a low pH, and alkalis (bases) have a high pH. The pH of a solution is a measurement of the hydrogen ion concentration; pH (which stands for potential hydrogen) is an open-ended scale whose endpoints are at 0 and 14, but concentrated, powerful acids and alkalis can be outside of this range. The scale for pH is logarithmic, so the difference between, for example, a solution with a pH of 3 and one with a pH of 4 is that the former one (pH 3) has 10 times the concentration of hydrogen ions. The difference between pH 3 and a pH of 5 is a factor of 100.



The simplified formula for pH is:

$pH = -\log [H^+]$
(Equation 3.1)

The "operational definition" of pH allows us to adjust for differences in temperature. Since pH varies relatively little within the range of temperatures with which we will be working (15 to 25° C), we will ignore this calculation in favor of the more practical aspect: direct measurement.

Quite simply, if one knows the concentration of hydrogen ions (the H^+ in Equation 3.1) we can calculate the pH by taking the log of this number, and then taking the inverse (i.e., turning a negative number into a positive one) to get the pH. Calculating the pH via this route is reserved for technical applications such as buffers or set solutions, and dealing with the pH by direct measurement is generally the most convenient route. A pH of 7.0 is neutral, a pH above 7.0 is alkaline, and a pH measurement below 7.0 is acidic. The further from 7.0, the stronger the acid or the base. Thus, if we have a solution that has a pH of 5.0, but we wish for it to be 5.6, we will need to add small quantities of an alkaline solution in order to raise the level to where we want it to be.

Pure, distilled water with no gases dissolved in it is pH 7.0 by definition. All the same, a simple chemical reaction occurs when carbon dioxide (CO_2) which is present in part per million concentrations in the air around us, dissolves in water.

$CO_2 + H_2O \Leftrightarrow H_2CO_3$
(Equation 3.2)

Equation 3.2 demonstrates how carbon dioxide dissolves in water to produce carbonic acid. Carbonic acid then weakly dissociates and produces a hydrogen ion (also known as a proton):

$H_2CO_3 \Leftrightarrow H^+ + HCO_3^-$
(Equation 3.3)

The protons produced (the H^+ in Equation 3.3) in this reaction make fluids acidic. Thus, carbon dioxide in solution is a weak acid, producing a pH slightly less than 7.0, where we would expect to see distilled water.

That having been said, pH is an unreliable measurement at best. The pH of a solution is fickle in part because of ion interactions and the scale's temperature dependence. For example, carbon dioxide dissolves much more readily in cold water than warm; if you heat a saturated solution of carbon dioxide, its pH will rise (all other things being equal) as the carbon dioxide comes out of solution. Solutions that are buffered may be difficult to measure under certain circumstances, and the pH of ultrapure water (in which there are exceedingly few ions) is very difficult to measure. You may find it easier to use the experimental value for water exposed in air (around pH 5.6), as discussed above when it combines with carbon dioxide, than to bother trying to measure it.

There are a number of pH meters available on the market; most are very simple to use, and prices have come down to the point where they are affordable. Better pH meters will cost more, in general, and can cost hundreds of dollars. Simple ones will be $30-$40 and are still fairly accurate. It is worth noting that a pH meter is only as good as

its calibration. A pH meter must be calibrated regularly against one or more buffered standard solutions whose respective pHs are already known. Most require a two-point calibration; two convenient buffers that bracket the best range for pH measurement are pH 4.01, and pH 7.00, both of which are readily available commercially, and are accepted calibration points for most meters. Bear in mind that pH meters and probes require proper treatment. Instructions provided by the manufacturer should be followed carefully.

Another viable route to follow is to use pH papers, which are much less expensive and more than reliable enough to use for orchid seed germination. Best of all, they are shelf-stable and do not require calibration as do pH meters. The papers cover different pH ranges, so select a pH paper whose range includes the desired pH of your media. However, be aware that their resolution is generally lower over a broad range than for those that center around a very narrow range. One would not wish to use papers that read from, say, 2 to 12 (with a resolution of 1.0 pH units) when one desires a pH of 5.2; instead, one would be much better off selecting a paper that ranges from, say, 4.8 to 6.0, with a resolution of 0.2 units.

These test papers are available from several commercial suppliers (see Appendix III, Sources); they are inexpensive and last for years when stored properly. They are perfect for the home flasker, and present several advantages over pH meters, such as their relative inexpensiveness, ability to test a relatively large range with ease, no need for calibration solutions, and certain brands possess the ability to measure the pH of turbid solutions by being washed off after having been used without changing the results. Moreover, there is no maintenance, such as that required by many pH meters, which may periodically require new electrodes or other components to function properly.

Buffers

Buffers are important to biologists and chemists alike. Buffers or buffer solutions will resist pH change when chemical reactions occur that would otherwise change the hydrogen ion (proton) concentration. As discussed above, the pH of a solution is dependent upon the concentration of hydrogen ions. If we were to design an acid-base system that relied upon the intrinsic chemical properties of certain compounds to absorb or release hydrogen ions to maintain a stable concentration of protons, we could overcome specific problems with respect to maintaining constant pH.

The pH of a solution is particularly important in biological applications, but for "chemical" reasons, reaction rates often depend upon the availability and therefore, when in solution, the concentration of ions. Further, many organisms rely heavily upon an external environment that is constant, and the availability of different nutrients may change with differences in pH. Inside living organisms, the difference in pH may radically alter particular reactions; a difference of ten in proton concentration can cause a given reaction rate to change by one to five orders of magnitude (10 to 100,000 times).

For obvious reasons, pH inconsistency becomes relatively important with orchid media. The plant is dependent upon this media for its nutrients, and will be unduly harmed if the pH is outside a range which the developing plants prefer. Knowledge of the equilibrium constants of the acid-base pair which we will use to prepare a specific buffer will allow the user to determine the pH of the final solution in advance. The concentration of each component is critical, and tabular values for commonly used buffer solutions are available in sources such as the CRC Handbook of Chemistry and Physics.

A buffer commonly used with orchid media is MES, generally referred to as "MES, free acid." Its useful pH range is generally given as 5.5 to 6.7, and is a constituent in several orchid media. However, its use is not without controversy because some growers have decided that it has an undesirable phytotoxic effect. Others claim it has no effect or that the benefits outweigh its detractions. That it maintains a slightly acidic pH is one of its best traits, helping combat the change in pH that may be experienced as seedlings mature *in vitro*.

The following phosphate buffer may be used to maintain pH and is given by Arditti (1982) for maintaining a suitable pH with Knudson C media.

A 0.1 molar solution of potassium phosphate (KH_2PO_4) is created (13 grams of the compound in a liter of solution). A 0.1 molar solution of dipotassium phosphate (K_2HPO_4) is created (17.4 grams per liter). Create a stock solution by mixing 975 ml of the first solution and 25 ml of the second solution together. Test the pH, and adjust to 5.1 to 5.4 if required. To each liter of culture medium, 18 ml of this stock solution is added.

Adjusting pH

Adjusting the pH of a solution may be necessary. Premixed media for orchids may or may not already be prepared to deliver a satisfactory pH. Nevertheless, these media will give instructions to mix into a set volume of distilled water in order to work correctly. These media rely upon the use of buffers, which in turn rely upon intrinsic chemical properties to deliver a pH that, although not exact every single time, will be well within a usable range when mixed and cooked as the manufacturer suggests.

Otherwise, if the requirements are for a media with a pH other than that which we have found during testing, one will need to adjust the pH of the solution prior to autoclaving. Two pH adjusters that are commonly available in the home are sodium bicarbonate (baking soda) and acetic acid (vinegar). Sodium bicarbonate is a weak base, and can be used either as a powder or a solution to raise the pH of a solution gradually. Acetic acid is a fairly strong acid but in a 5% solution, such as that found in household vinegar, it is less aggressive. With either preparation, one should only add tiny amounts of acid or base in order to achieve the desired pH. It is very simple to overshoot the desired pH, and then you will have to titrate with the other solution. Although not detrimental in the small amounts we discuss here, baking soda and vinegar both add something to the media that is unnecessary. After adding anything to adjust the pH, the media must be thoroughly mixed prior to measuring the pH again. The best technique for mixing is to perform adjustment while the media is being stirred by a magnetic stir plate.

Other, more powerful solutions can be made with commercially available laboratory reagents. Possibly the best agent to lower pH is phosphoric acid, which has the advantage of introducing phosphorous into the mix which plants can then use. However, this acid does not otherwise present a significant advantage over other acids. Hydrochloric acid is satisfactory, as most seedlings are not affected by the chloride (Arditti, 1967), and nitric and sulfuric acids will also prove satisfactory.

For raising pH, one can use potassium hydroxide, which will serve to supply potassium. Arditti and Ernst (1993) suggest that ammonium hydroxide and sodium hydroxide may also be used for increasing pH.

Strong acids and bases should be treated with great care, as they are aggressive reagents; diluting them before adding them to different media may be desirable to prevent radical swings in the pH. Before adding to media, acids and bases should be diluted by a factor of around 20 to prevent over-adjusting.

To dilute, all acids and bases should be added to water, not the other way around. This is to say, if water is added to either of the pure compounds, large volumes of heat may be generated, and spattering and spraying of hot solutions of strong chemicals may result. Only small amounts of acid or base should be added, with constant stirring to dissipate heat. Eye and skin protection is strongly recommended. Sulfuric acid is most dangerous, and should be added in very small quantities only; it may spatter and erupt violently if care is not taken. Keeping the solution cold in an icewater bath, using Pyrex or other heat-resistant glass, may be necessary.

Suitable pH for Germination of Orchid Seed

There are several valid parameters for the "proper" pH to use when germinating orchid seed, and they may vary from species to species. Martin (pers. communication, 1998) recommends pH 4.8 to 5.2; Torres (pers. communication) advocates pH 5.4 to 5.7; Arditti (1982) notes that culture media should be between pH 4.8 and 5.5. Some species may enjoy or even require a particularly low pH, but few grow in the range above this. In fact, some species of *Paphiopedilum* that prefer alkaline limestone or dolomite soils will enjoy a higher pH, possibly higher than 5.8 or so. Some native American and European terrestrials are not at all choosy about pH, provided it is moderately acidic. Others have suggested (Knudson, 1951) that pH is important only for germination, and that there are few problems with replating to a media with a pH that might be unsuited to germination. The pH of the media used for growth may have to be optimized, so this does not mean that pH is irrelevant to optimal growth on replate media as "survival" may be very different than "thriving."

A pH too low or too high may cause deficiencies, as some compounds are only available to plants within a certain span of pH. Arditti and Ernst (1993) note that phosphorous, for example, is available in useful concentrations in soil only between pH 4.5 to about 8.1. They recommend that if no specific figure is given, that media be adjusted to pH 4.8 to 6.0.

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3.6 Media, Sterilization, and Bacteriology 101

Bacteria and fungi are ubiquitous, as are their spores. Fighting hard enough to maintain a sterile work space is an uphill battle, but one that we must be willing to fight in order to produce quality flasks with a reasonable contamination rate.

There are many ways in which we may kill bacteria, fungi, and their spores. All techniques have their advantages and disadvantages. We will discuss those that are suitable for use in the home lab, as well as some that may be useful for the larger lab.

Chemical Sterilization

Some of the easiest techniques by which surfaces and tools may be sterilized rely upon the use of chemicals. In the same way that seeds may be disinfected with a bleach solution, tools and non-porous working surfaces may be sterilized by wiping them down with a 10% bleach solution. Commercially available bleach is a 5.25% solution of sodium hypochlorite, which is formed by bubbling gaseous chlorine through a solution of sodium hydroxide, or lye. It is very caustic due to the sodium hydroxide, and will cause metals such as aluminum to be severely pitted if they are left in it long enough. Wiping the surfaces down and waiting 15 minutes or more will sterilize a non-porous surface. This solution will produce a lot of chlorine gas, which may be strongly irritating or dangerous in a confined space. Proper ventilation is called for when opening a glove box full of bleach vapors, for example, as the interior atmosphere may have quite a concentration of chlorine gas built up. Chlorine-based disinfectants, which include bleach, calcium hypochlorite, and Virkon S (below) are the most effective for our purposes.

Surface sterilization may be achieved with alcohol as well; 70% rubbing alcohol (isopropyl) or ethanol (ethyl alcohol) will sterilize surfaces in a manner similar to that of the aforementioned bleach solution. It is worth noting that the vapors from both of these chemicals are flammable, as are the liquid form. As a result, it is not suggested that large areas be sterilized with these or other alcohols (isopropyl and methyl may also be used). Caution should be exercised with methanol, isopropyl, and denatured ethanol, as these are toxic.

There are other chemicals that are widely used in microbiology for aseptic work; these are proprietary, and tend to have properties that make them less hazardous than those chemicals mentioned above. Biological supply companies will serve as the best source for these disinfectants. Alcohol can be used in place of chlorinated disinfectants, but is much less effective, probably due to its inability to deactivate spores. Lysol is another commercial preparation that can be used for sterilizing surfaces; it consists of ethyl alcohol and a small amount of a quaternary ammonium disinfectant ("quat").

Ethylene oxide (EtO) is a gas sterilizing agent sometimes used in hospitals. It is satisfactory for use on tools and surfaces that cannot be disinfected thermally or with a liquid, but is not practical for home use.

Virkon S, mentioned earlier in this text, may be suitable for disinfecting large surfaces while performing flasking. I have not personally tested it extensively, and hesitate to mention its use for these purposes, but I mention it for those who wish to experiment. A 1% weight solution is effective for surface disinfection, and may be used for disinfecting seed. Heavily contaminated seed may be treated with a 2% solution.

Thermal Sterilization

Thermal disinfection can be used for many different aspects in seed germination. Prime amongst these uses is sterilizing media.

Autoclaves and pressure cookers function in the same manner; this is to say, they both rely upon boiling water to produce pressure, which in turn elevates the boiling point of the water. Boiling water at sea level (100° C) is insufficient to kill all spores; in order to do so, the temperature must be elevated to at least 110° C for a period of time. Most pressure cookers and autoclaves build up one atmosphere (14 or 15 psi) of pressure, which is sufficient to boil water at about 121° C. If sustained for 10-15 minutes, these conditions virtually assure sterility of the contents. Although automatic autoclaves present certain advantages over home canning pressure cookers, there is little or no advantage in terms of function: either will sterilize the media effectively. Unless otherwise specified below, autoclaving and pressure cooking may be used interchangeably, provided each physically fits your needs (i.e., is large enough to hold the size and type of vessels in use).

It is worth noting that thermal disinfection has several levels. Pasteurization raises the temperature of a product so that many of the organisms are destroyed. Total sterility is not achieved, but product life, such as that of milk, may be prolonged because most pathogens are destroyed. Boiling goes a step further, raising the temperature to about 100° C, depending upon the water purity and atmospheric pressure. As with Pasteurization, boiling will not destroy

all the organisms. In fact, many bacteria and fungi have resistant growth phases which can lay dormant for long periods of time, and can survive brief exposure to such temperatures. Spores of some species cannot be destroyed in a reasonable period of time without autoclaving. Autoclaving is the next step of thermal disinfection, and is absolute when performed correctly. Fungi, bacteria, their spores, and viruses are all destroyed by the autoclaving process.

When autoclaving larger volumes of media (more than 50-100 ml at a time), it must be at or near a boil when placed in an autoclave. One should not expect the contents of a liter of cold solution to reach the temperatures necessary to kill any potential contaminants simply by placing it in an autoclave for 15 minutes. The best method is to heat the media, and when one is certain it is homogeneous and satisfied that all components are in solution, pour it into flasks at 100-150 ml per flask, and then place the sealed flasks into the autoclave for thermal disinfection. This way, with such a small quantity of media per flask, sterilization is assured.

It is important to note that tightly sealed vessels may break or explode during or after autoclaving. For this reason, it is important to either leave lids on only loosely, or vent caps using cotton or other plugs to allow for pressure equalization. Do not cool pressure cookers rapidly: using water to cool a pressure cooker or autoclave is dangerous and may result in injury. Once removed from heat, pressure cookers should be allowed to cool on their own.

One other technique mentioned by Bergman (1996) for thermal sterilization of flasks involves Tyndallization, and may be used when neither an autoclave nor a pressure cooker is available. Tyndallization requires steaming 3 or 4 times, once every 24 hours. Thus, flasks should be steamed until their contents reach the boiling point; this temperature is maintained for a few minutes, returned to room temperature and boiled again 24 hours later, and repeated as necessary. Stoutemyer and Cooke (1989) note that Tyndallization in a conventional oven at a temperature of 180° F repeated three times also works.

Wire loops and similar objects that weigh very little, and are very heat-stable, may be sterilized either by "flaming," or by dipping in alcohol and then passing it through a flame to let the alcohol burn. Another technique uses a loop placed into the flame of an alcohol or gas burner with the tip of the tool pointing down at about the 7 o'clock position until it turns a dull red color, and then is removed. When cool, the loop can then be used to pick up seeds or seedlings. Flaming the necks of bottles is also acceptable for reducing contamination by destroying any stray spores that may have settled in an opportune location.

One other solution for thermal disinfection consists of commercial heaters, used either for sterilizing tools for microbiological work, or modified for such use. The Bacti-Cinerator, used to quickly incinerate bacterial remnants on tools, is commonly used to sterilize transfer loops and other small tools. Some other growers have modified lead melting pots for their purposes by filling them with sand, and inserting them into the hot sand to sterilize them. Another useful tool for this purpose is the glass bead sterilizer. It goes without saying that these are not without their hazards, and should be treated with respect. They are not only incredibly hot, but produce a fire and explosion hazard when used in conjunction with flammable liquids.

Filtration Sterilization

For liquids or compounds in solution that decompose at elevated temperatures, filtration may be used for sterilization. By passing liquids through filters that physically remove all particles larger than a given size, the resulting solution will be rendered sterile by physically removing organisms and spores. Although previous generations of filters relied upon expensive and complicated setups, some modern filters consist of little more than prepackaged, sterile filter discs in a special housing, along with a syringe for forcing the liquid through the filter. These devices are fairly inexpensive, and quite capable of producing sterile solution components upon demand. The primary disadvantage is that they generally have limited capacity. Employing them to sterilize an entire batch of media would not be possible for most formulations, as many types of media possess colloidal components (such as finely powdered charcoal) that would quickly fill the filtration surface, rendering it useless. Many companies require purchasing filters in units of 50 or more; be certain to purchase filters that are pre-sterilized, as sterilizing them in the home is not practical.

The smallest bacteria known are approximately 0.3 microns in size; a 0.22 micron filter is considered sufficient to sterilize liquid components, but would optimally be labeled for such purposes.

Denaturation

The use of solvents to prepare stock solutions will render them sterile when used in the proper concentrations. Solutions of 70 to 95% ethanol may be used without other sterilization (thermal, filtration, etc.), and Arditti (1982) notes that up to 5 ml of 70% ethanol in a liter of media has not produced harmful effects. Methanol should not be used for this purpose.

Ultraviolet Light Sterilization

Some short-wave ultraviolet (UV) light sources are available that may be used to sterilize surfaces when not in use. These devices should be equipped with interlocks such that direct exposure to UV light, particularly to the eyes, is not possible. These lamps can be expensive, but may assist in maintaining the sterility of a workspace. Some correctly argue that UV radiation is dangerous, and it must be stressed that exposure to humans must be minimized. There are also questions about their effectiveness, as they do not produce absolutely sterile workspaces.

Radiation Sterilization

Ultrahigh dosages of radiation are capable of sterilizing a variety of tools and media; although home use is obviously not an option, many components are available as prepackaged irradiated objects that are completely sterile. Many things that would otherwise degrade under thermal and chemical disinfection are otherwise disinfected through high doses of radiation, all of which leave the components non-radioactive (in case you were wondering). However, some components will also degrade under such high doses of radiation, and therefore is not a universal solution.

Microwaves

It is worth pointing out that microwave ovens, such as those used in the home, possess no intrinsic sterilizing capacity. This is to say, a contaminated object placed in a microwave does not have its microscopic denizens annihilated simply by being exposed to microwaves. It is possible to kill large numbers of bacteria and other microorganisms through prolonged microwave exposure when the microwaves heat an object, but this is no different from putting it on the stove to boil. It is the heat that destroys them, not the microwaves themselves. The net upshot is that although you have killed the vast majority of the actively growing organisms, you will not destroy all of the dormant spores; recall that it only takes a single spore to contaminate a flask. Microwave ovens are not absolute, but the infection rate may be low enough to satisfy many hobbyists.

Although it is possible to produce a small number of flasks if you are willing to accept an increased contamination rate in this manner, it is suggested that you are risking valuable time and effort on your part in your flasking operation by circumventing one of the easiest links in the chain–autoclaving. Further, since many growers will use canning jars, it is unsafe to put metal parts (such as rings and lids) into a microwave.

Still, there are techniques that have been developed by which media may be prepared using a microwave, and some report success with these techniques. Given the availability of microwave ovens over autoclaves, it may be desirable to use these techniques for preparation using instructions specific to these techniques. Tyndallization, as described above, may be used with microwaves, or the microwave may be used in conjunction with anticontaminants (see Section 6.1, Media Modifications).

Arditti and Ernst (1993) note that microwaves have been used for sterilizing media, and notes that there are several safety requirements, including:

- As with conventional heat sources, microwave heating of tightly sealed vessels presents an explosion hazard.
- Use of any metal, including caps or aluminum foil, presents a significant safety hazard in the microwave.
- Cotton, either as stoppers or vent plugs, must not come in contact with the media as they are a contamination hazard.
- Complex additives, such as oatmeal, contain contaminants that may not be deactivated by the microwave oven, and cause contamination later.

One technique discussed for sterilizing media in a microwave oven consists of heating media in the oven until it boils. The flask is then to be removed, agitated, and cooked again until it boils once more. After this point, most of the organisms will have been killed, and if left for several days prior to use to see if anything grows, it may be considered sterile if growth is absent.

Introductory Microbiology

Bacteria, fungi and their spores are everywhere. They are suspended in the air on particles of dust, and they are present on most every surface in your home. They survive in water despite the chlorine in the water supply. They are in our food and in our drink. Fortunately, most are in small enough quantities that they do not make us ill unless we

ingest large numbers of them or their toxic products, or we are susceptible to a particular organism and get sick when it infects us.

Unfortunately for us, nutrient agar does not require tens or hundreds of organisms to infect a flask; it takes just one, and reclaiming an infected flask is difficult, time-consuming, and frustrating. The best way to solve the problem of contamination is to employ ruthless disinfection techniques, and refrain from taking shortcuts. Experience, patience, and practice will go far in terms of becoming adept at adhering to aseptic technique, and prevent one from becoming disappointed when flasks become contaminated.

For our purposes, nutrient agar is little more than a jelly with specific concentration of nutrients, sugars and salts added to provide the germinating orchid seed with the sustenance it requires. Unfortunately, it will also happily support the growth and survival of many, many other organisms, all of which are undesirable for our purposes. After all, nutrient agar is used for culturing many different organisms in the laboratory setting. These techniques are used in many different ways by microbiologists, and it is possible to grow virtually all bacteria and fungi on these media, or media with special modifications. Raising plants on nutrient agar media is a relatively specialized field, but one that has great economic importance. There is a substantial body of research behind it; unfortunately, much of this work is proprietary and therefore not available to the home flasker. Also, most of these techniques would be beyond what the neophyte would be able to perform in any event.

Some of the organisms that we may accidentally culture are pathogenic, most are not. All the same, caution is called for when dealing with infected flasks; some people autoclave their accidental creations before disposal, others will chemically disinfect their contaminated flasks with bleach or other solutions, before discarding the media. It is important to take caution to destroy infected media, because even relatively harmless organisms may produce unpleasant reactions in sensitive individuals when exposure to large numbers of organisms occurs.

There are a number of media that do not rely upon the use of a gelling agent (typically agar, but new components have come on the scene that supposedly do not have phytotoxic effects like those of agar–more on this in Section 6.7, Alternative Gelling Agents). These are liquid media, and require mechanical agitation to suspend developing plantlets and provide them with nutrients and gases. Their use is beyond the scope of this text. It is worth noting at this point that "agar" and "agar-agar" are equivalent, and are often used interchangeably.

References:

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Arditti, J. and Ernst, R. 1993. Micropropagation of orchids. John Wiley & Sons, Inc., New York, Chichester, Brisbane, Toronto and Singapore.

Bergman, J. F. 1996. Seed sowing the easy way. *The Orchid Review* 104: 23-25.

Stoutemyer, V. and Cooke, R. 1989. Orchid flasking simplified. *American Orchid Society Bulletin* 58: 572-578.

3.7 Preparation of the Media

Selection of the proper medium for germinating a given species (or genus) of orchid seed is an important factor. Members of many "common" genera, such as *Cattleya*, *Dendrobium*, *Phalaenopsis*, and so forth, may germinate and grow quite happily when placed upon media whose nutrients are obtained from a relatively high concentration of salts. Still, there are many genera that present problems when used with such media, such as pleurothallids and masdevallias, which are reported to be salt-sensitive (Light, pers. communication). Guidelines for germination when using different media are best acquired through the manufacturer. Rare, unusual or poorly-known species should be sown on multiple media formulations to maximize the likelihood of germination success.

When preparing media for flasking, it is important to determine if a given medium is complete, or requires further preparation before final sterilization. Some media are supplied with all the necessary ingredients, except for the agar or other gelling agent, and will not gel when cooled. Some media require (or strongly suggest) the addition of banana puree, pineapple juice, coconut milk, or other components. Further, you will need to chose between "mother flask" and "replate" media. Mother flask is used when you are sowing seed, and replate media is designed for when you transplant protocorms[14]. Replating maintains protocorms in flask to develop into seedlings so they will be able to survive when de-flasked. Replate media may contain compounds that help with plant growth, but may inhibit germination, and are thus poorly suited for mother flask. This does not mean it cannot be used as such, and personal expe-

[14] Small, undeveloped masses of plant tissue that develop soon after germination.

rience has shown that seeds that do germinate on replate media will generally do just fine. There are exceptions, of course, and one such example is *Gongora armeniaca*, which in one experiment germinated well on Phytamax P-6668, but almost not at all on P-1056 (Hicks, unpublished data). The difference between the two is that the latter has 30 gm of banana powder (50/50 banana and maltodextrin) per liter. Banana has been reported as inhibiting germination by some growers, but it is a primary component in some replate media as it often contributes to accelerated root and shoot growth. However, Maunder (pers. communication) notes that the absence of banana often results in poor germination with the genus *Masdevallia*.

One other important component is the water that will be used. Tap water is a complex brew, displaying an incredible level of variation between locations, as well as seasonal and even hourly changes. Many people produce their flasks with tap water, or even rain water; provided the quality is good, and there is no problem with this. However, bear in mind that buffers may act very differently when they are placed in solutions that have high concentrations of dissolved solids. If your water is poor, and certainly if you are performing flasking as a serious proposition and require repeatable results, you will want to use distilled water. If you prefer, high quality, reverse osmosis water (often sold as "purified water") is also satisfactory, provided it is run through an activated charcoal filter before being pushed through a high-rejection reverse osmosis (RO) unit. Either should be available at a grocery store for under a dollar per gallon, or about $.25 per liter of media.

For larger labs, a still may be required for producing distilled water. Scientific Glass Company manufactures the least expensive commercially available still, at under $700 (see Appendix III, Sources). Alternatively, RO units are relatively inexpensive, and may be purchased through local water purification sources, or hobbyist sources such as horticultural speciality or aquarium supply. RO units can produce water that is better than distilled when used in conjunction with deionization columns. Such high purity water is only a consideration with larger production labs, or with research facilities. With either of these situations, water quality must be carefully controlled, and consultation with a specialist is recommended.

The availability of commercially prepared media allows for growers to purchase a mix that is carefully formulated and manufactured to prevent nutrient deficiencies; the different constituents are added in concentrations such that they can successfully sustain a relatively broad variety of orchid genera with no signs or symptoms of poor nutrition. Those professionals inside the realm of *in vitro* plant culture are conscious that the plants in their care are absolutely dependent upon a media that provides every single chemical and element required for their existence, sometimes for months or even years at a time. Proper nutrition for seedlings can be more like a shotgun than a rifle, in that we provide a diet with what we hope is slightly more than the plant will need, without the concentrations approaching a level that will inhibit growth. Some plants are pickier than others, but it is still possible to use a media whose parameters suit the needs of a broad variety of plants.

The typical preparation of a liter of media begins with finding a Pyrex flask, preferably with graduations on the side, that will hold the contents of the flasking job at hand. Using a balance to weigh out the media (unless it has come pre-weighed), add an appropriate quantity of media you have selected to the flask. Add distilled water, agar or other gelling agent, and any media supplements (such as banana, pineapple, etc.) sufficient to make up to 1000 ml of solution (if one is making a liter of media), and homogenize, being careful to break up all lumps.

The amount of agar (or other gelling agent) is a matter of experience and personal preference. However, a good starting point is 10-12 grams of agar per liter for mother flask, and 7-10 grams per liter for replate. More agar, and therefore harder media, for mother flask may be desirable to make it easier to skim protocorms off the media. Softer media, with as little as 7 to 7.5 grams per liter, in replate will help ease replate by making it easy to seat the protocorms. It is also worth noting that agar is available in a broad range of purities. Agar of a lower grade will have more impurities, requiring more for the same strength of gel as a higher quality agar. Although high purity agar is not required for orchid seed germination, staying with a consistent source is important because when changing sources, you may have to reformulate to achieve the same gel strength.

At this point, if the solution is not buffered, you will need to measure and, if required, adjust the pH of the solution. Once satisfied with the pH, heat the flask to dissolve all of the components. It is important that the solution is well-mixed and heated to dissolve all the agar: a mixed batch of flasks that have well-consolidated media along with flasks that gel poorly or not at all are the product of insufficient heating or mixing of the flask to make certain the agar has dissolved. The use of a magnetic stir plate is recommended to homogenize the media and heated stir plates that will quickly heat the media while stirring are commercially available. If the media is not stirred, components that settle to the bottom will burn, ruining the mix. Fill the flask to the 1 liter mark with distilled water and mix thoroughly. Then you may dispense into individual flasks, as discussed in Section 3.9, Flasks. This should be done briskly, and agitating the media between pours is recommended in order to keep the mix homogeneous.

It is worth noting at this point that not all media needs to be mixed exactly as the instructions specify; indeed, many growers use commercial media such as Sigma Phytamax formulations at between ½ and ¾ label strength.

When used with the appropriate amount of agar, these mixes (some of which are considered to be too strong by many growers) become very useful as mother flask and replate media. Thus, if Phytamax P-1056 is to be used at about 57.3 gm per liter, it can be mixed at, say, 40 gm per liter, perhaps with a little more pineapple or other accessory sugars added to make up for what is lost in dilution. Most media that come prepackaged with a set amount of agar are designed to be used at the label concentration, and will not gel correctly if mixed at less than label strength unless additional agar is added to make up for what is lost in dilution.

The amount of nutrient mix per flask varies from grower to grower and one will generally add enough of the media to maximize surface area. If using square-bottomed polycarbonate flasks designed for plant tissue culture, it is a simple matter to dispense enough media to a depth that will satisfy the grower by simply looking at it. With Mason jars and Erlenmeyer flasks, the grower may wish to experiment a little in order to determine the appropriate volume of liquid. For wide-mouth Mason jars, 100 to 150 ml of liquid seems to work well; this is equivalent to 3-⅓ to 5 fluid ounces. Some growers prefer to use only the bottoms of Mason jars, which means that about 100 ml will be adequate. Mother flasks do not require a thick layer of media if the seedlings are quick to develop, but replate flasks will require a thicker layer of media to accommodate a healthy root system. About an inch of depth is a good starting place, but orchid seeds may germinate on far less than this–even bare glass at the neck and the sides that is wetted with the liquid that accumulates on the surface of the agar.

After dispensing the media, put the lids or caps on loosely, and cover them with one or two layers of aluminum foil if using cotton plugs to keep condensation from getting them wet while autoclaving. The foil should extend a reasonable distance beyond whatever cap they are protecting (Mason jar lids, etc.). Some types of plastic may be used, and held in place with wire. However, their ability to withstand autoclaving must be tested first. Place them in the autoclave and sterilize them.

Once the sterilization regimen is complete and the pressure has dropped to the point where the autoclave can be opened, remove the flasks and, if using Mason jars, tighten down the rings on each jar. Set them on their side, using a shim if necessary, to let the media cool at an angle; the agar should not slosh forward far enough that it touches the inside of the lid or stopper! It is important not to disturb the flasks during this process, and allow them to cool without undue disruption. One way to produce consistent slants in Mason jars is to stand the box they came in on its side, but tipped back slightly, and allow the jars to cool inside the individual dividers at an angle, producing a "heel" of media that maximizes surface area. If using other types of bottles, they should be allowed to cool in whatever position works best, obviously.

If so desired, flasks may be placed into the flasking box or flow hood immediately after their removal from the autoclave while their exteriors are still very clean and uncontaminated; indeed, the entire pressure cooker can be moved into your flasking area if so desired, so that its sterile interior is not disturbed. If your flasking box has a glass bottom, place the flasks on a towel to insulate them and keep the glass from cracking from the temperature difference.

As soon as the agar has solidified, the flasks will be ready to sow. Do not be alarmed if the agar does not gel immediately; agar media may approach room temperature before it solidifies, and it may take some time for it to become solid. If your agar does not solidify, or does so only in some flasks, you did not sufficiently homogenize and heat the mix before pouring into individual flasks, or you did not add enough agar or other gelling agent. If none of these problems pan out, check the pH of your media, particularly if you have added anything or are using diluted media. The buffers may have been unable to deal with the chemical differences, and the pH could be outside of parameters. One other possibility is that if you did not allow the media to cook when preparing it, the agar will not have dissolved. Stirring and heating the mix to the boiling point will be enough to allow all the available agar to dissolve.

For our purposes, it is useful to know the exact contents of a flask before it is sown, particularly if you use a variety of media. Label the flask once it is cool enough. One identification scheme uses the date manufactured: number them sequentially, i.e., March 10, 1994 A, March 10, 1994 B, and so on, along with the type of media. The lab notebook for that date should then detail the contents of that lot, particularly if they were variations on a commercial media. It is also suggested that the flasks be marked "Mother" or "Replate" for quick identification ("M" or "R" on the lids will generally suffice).

Some pen markings are removed by disinfecting solutions; for this reason, test your pens against alcohols and hypochlorite solutions. It is also possible to lightly inscribe the lot number and type of media on an aluminum foil cap, using a ballpoint pen and a light touch. This is not permanent, and care should be taken to provide a more permanent form of identification once the risk of chemical obliteration has passed.

An article by Light and MacConail (1990) also describes selection and preparation of media in the September 1990 American Orchid Society Bulletin.

Types of Media

It is worth noting that there are many variations on media; there are the commercially available media such as Sigma's Phytamax, G&B, Murashige and Skoog (M&S), Gallup and Stibling, and others. There are many variations on each of these, and it is even possible to mix your own at home (more on this and other fertilizer-based media below).

Phytamax is a good starting place; P-6668 or PhytoTechnology's P-668 is good for seed germination for beginners. It is reliable, works with a good spectrum of genera, and is inexpensive. The difference between this media and P-1056 (to be used for replate) is that the latter has 30 gm of banana powder (50% maltodextrin) added to each liter of mix. Seeds will germinate and develop slowly on most germination media, but grow rapidly on replate media. There are many different formulations, but most replate media have high concentrations of sugars, and compounds like banana, that help growth but may sometimes inhibit germination. Some replate media are too "hot," with too many sugars, salts, and other compounds that burn sensitive species. Occasionally, plants that dislike a media but still manage to grow will fail to develop healthy root systems, or have roots that grow up and out of the media to avoid touching it.

Specialized media, such as those used to multiply the number of protocorms, are also available. Used when germination is low or few seeds are available, protocorms may be transferred to protocorm multiplication media to produce more seedlings. Note that this will result in less genetic diversity among the resulting plantlets than if they were all produced from seed alone.

Fertilizer-Based Media

Media for asymbiotic seed germination are composed of salts and sugars. The salts are composed of a cation[15] (calcium, magnesium, ammonium, etc.) and an anion[16] (chloride, nitrate, sulfate, etc.). The salts used for seed germination overlap with those found in balanced fertilizers such that they can emulate the components normally used. Thus, with a little experimentation and knowledge, seed germination media may be formulated from fertilizer, sugar, and extracts such as banana and pineapple.

Although such "improvised" media may or may not yield the same results as commercial formulations, they are capable of growing seedlings within months. MacDonald (pers. communication) has produced healthy seedlings that have grown quickly using the formulations given below.

The importance of these media for use in developing countries where commercial formulations are not readily available is obvious. Behar (1998) notes that it is important for improvised media to be developed in order to meet the needs of labs closer to the native habitats of most orchids. As the needs for conservation and dissemination of our dwindling natural stands increase, we find ourselves tested to develop techniques and media whose properties facilitate the flasking process for those not fortunate enough to enjoy the facilities commonly available in plant propagation labs. In addition, some reagents are not commonly available in some countries as they are under government control for fear they will be used as explosives. The following is recommended not only for the home grower and hobbyist, but for those growers who work with limited lab equipment and must function with only rudimentary tools and chemicals.

Improvised Media

MacDonald suggests the following as a simple, workable media that can be made and used in the home.

Place ¾ ounce of agar agar from a Chinese food store in a large stainless steel stew pot with 6 cups of water; set heat to "high." Take 4 bananas which have been frozen and then thawed, which reduces them to mush and produces a much smoother homogenate, add two cups of water, ½ teaspoon of Peter's 20-20-20 fertilizer with minors, and ½ teaspoon of "SUPERthrive" vitamin additive. Place this second mix in a blender, and liquefy. Add the banana mix to the agar mix, which should be hot by now. Stir the mix constantly to keep it from burning; check to make sure all the agar has dissolved. At this point, add 2 tablespoons of powdered activated charcoal; MacDonald makes his own in a blender, using fish tank activated charcoal, until fine powder is produced.

From this mixture, add about ⅓ cup into each of 24 pint Mason jars, and cap them with the lids, which have been modified in the following manner: after having drilled the lids with a ⅛" diameter hole, plug the hole with cotton, fabric, or some other type of vent. MacDonald pierces the lid with a #2 Phillips screwdriver, paints the exposed metal with clear nail polish to help prevent rust from forming, and plugs the hole tightly with a small square of fabric.

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[15] An ion with a positive charge.

[16] An ion with a negative charge.

Bottles are cooked at 15 pounds for 15 minutes for the first batch, which should still be hot from preparation. The second and subsequent batches are cooked at 15 pounds for 20 minutes, to compensate for the difference in temperature. He strongly suggests that the pressure cooker not be cooled directly with cold water, as it is possible to boil-over the flasks, which is very messy.

McDonald has also had success improving root growth by using vitamin tablets. As noted below in section 6.1 Media Modifications under "Vitamins," B vitamins have proven to be useful in stimulating growth of orchids *in vitro*. A vitamin B_1 or B-50 tablet is dissolved in 120 ml (4 ounces) of water. From between ½ and 10 ml of this liquid is added per liter of mix, but he reports that there is no significant gain after more than ½ ml of this solution is added. Although commercial preparations vary, B-50 tablets consist of a variety of B vitamins, each at 50 mg per tablet, with the possible exception of B_{12}, folic acid, and biotin. For operating in less-than-optimal lab conditions, the use of such a B vitamin table may be a practical source of B vitamins.

Yanagawa et al. (1995) used a modified Kyoto solution for their experiments involving anticontaminants in media (see Section 6.1, Media Modifications):

Hyponex fertilizer (6.5/6/19)..... 3 grams
Sucrose (table sugar)..... 20 grams
Agar..... 8 grams

The components are mixed and water added to produce 1 liter of solution. The pH is adjusted to 5.8 using either hydrochloric acid or potassium hydroxide. Although the authors used hypochlorites as anticontaminants, this solution could also be autoclaved, producing an acceptable media.

Arditti (1982) lists the following medium utilizing Hyponex fertilizer:

Hyponex.....3 grams
Sucrose.....20, 30, or 35 grams
Peptone or tryptone.....2 grams
Activated charcoal..... 2 grams
Agar.....8, 10, 12, or 15 grams

The first three components are added to distilled water for a total volume of 800 ml, and the pH is adjusted to between 5.0 and 5.4, depending upon the species involved. The volume is then brought up to 1000 ml, and heated to boiling while agar is added. Charcoal is then stirred in, and the homogenized media poured into culture vessels, and autoclaved. *Calanthe* and *Cymbidium* species were successfully germinated on this media.

Stoutemeyer and Cooke (1989) cite the following media for seed germination, developed by "orchid growers in Hawaii," but give no further details as to the specific origin:

Two level teaspoons (or "two grams," but this latter figure seems too low) of water-soluble plant fertilizer plus 20-30 grams (5-7 level teaspoons) of table sugar, plus 1 to 1-½% agar (about 12 level teaspoons) per liter of water. For replate, a 100 gram ("one smaller than average") banana is added, and agar is reduced to 0.8% (about 8 level teaspoons).

pH adjustment will be required with these types of media as noted above.

Fournier (web URL) manufactures his own media using 6 grams of the fertilizers listed below plus 38 to 43 grams (1-⅓ to 1-½ ounces) of Chinese food-store agar per 4 liters (1 gallon) of mix. Sugar, "super ripe" bananas and other components are added as required. Fertilizer used includes G&B 20-10-20 for *Cattleya* species and hardy orchids, Peters 12-36-14 for ghost orchids (leafless species), and 10-60-10 "Bloom Plus" by Schultz for *Phalaenopsis*. Replate of *Cattleya* and hardy orchids involves using Miracle-Gro for roses 18-24-16.

Kyte and Kleyn (1996) note that Bridgen (1986) had developed the following formulation whose components are readily available:

Table sugar..... 30 cc (⅛ cup)
Tap water..... 237 cc (1 cup)
Nutrient solution from stock solution containing 1.25 cc (¼ tsp)
all-purpose 10-10-10 fertilizer in 4 liters (1 gallon) of water... 118 cc (½ cup)
Inositol tablet (250 mg)..... ¼ tablet
Vitamin tablet with thiamine..... ¼ tablet
Agar flakes.....30 cc (2 tablespoons)

The components are combined in a flask, boiled while stirring, dispensed into culture vessels, and autoclaved.

Research at the Orchid Seedbank Project has shown that baby food jars can be used for production of protocorms in the following manner using readily available components.

The glass culture vessels used will depend upon what you have. Baby food jars have been used with success, but other jars with lids that may be secured tightly are fine. The lids are perforated with a sharp object, and the hole plugged tightly with cotton.

A piece of absorbent paper is cut to the size of the jar's bottom diameter. Whatman #4 filter paper was used, but any tough, absorbent paper will work. This paper is placed in the bottom of the jar.

The following are added to a 1 liter beaker:
Commercial sugar (sucrose)..... 2 level teaspoons
Miracle Grow fertilizer, 15-30-15..... ½ level teaspoon
Approximately 150 ml of bananas from baby food (no sugar added), or pineapple juice

Distilled water (tap water or rain water may be substituted) is added to the 1 liter mark. Make certain all components are dissolved or suspended by stirring. Pour a small quantity of this liquid into the bottom of the bottle. Only enough liquid to wet the paper and leave a small quantity of liquid standing under the filter paper should remain: if there is any more, the seeds will be washed off the surface whenever it is moved. The flasks are then autoclaved or pressure-cooked; if this is not possible, Tyndallization as described in Section 3.6, Media, Sterilization, and Bacteriology 101, may be used.

Seeds are then sown in these flasks using techniques described later (Chapter 4). Once protocorms develop, they may be replated onto this media with agar added for support, or the media may be absorbed into a matrix like glass wool, perlite, or other compounds. When used with a piece of blotting paper, paper towel, or filter paper as the substrate for germination of seeds (not seedling culture), a liter of media will fill from 50 to 100 baby food bottles, and is very efficient for testing small batches of seed.

There are many modifications that may be required for successful germination on this media. The banana may be replaced with pineapple if the former proves to inhibit germination. More or less sugar or fertilizer may prove to be helpful. Replate media may contain agar or an other gelling agent, as well as McDonald's vitamin B solution, as described above. Furthermore, it is imperative that research continues to find the most suitable formulations that may be generated and used in the field.

Formulations for Expedient Orchid Seed Germination

Unfortunately, this is not a how-to section so much as an appeal to researchers. Behar (1998) suggests that simplified formulations and techniques must be developed in order to facilitate the seed propagation of orchids. Techniques used by commercial growers are poorly suited to applications in countries where chemicals, products, and techniques are difficult or impossible to acquire. Certain aspects of flasking require labware that is not commonly available in less developed countries or deep in the jungle. As a result, it is in the best interests of conservation that techniques be specifically developed to facilitate these efforts.

Behar (1998) notes that Bayer's "Bayfolan Forte" can be used in conjunction with a "commercial plant growth regulator" called Kr-ESE to germinate orchid seed. The mixture was put in the bottom of small culture vessels with a small quantity of lava rock (although perlite or another similar substrate could be used instead). A piece of absorbent paper is placed on top of the substrate to keep it evenly moist, and suitable for orchid seed germination.

Flasks were sterilized by preparing them in a conventional oven. The thermal disinfection of flasks, lids, substrate and filter paper was performed at 350° F for 30 minutes. The solution was boiled for 15 minutes and poured into the cooled flasks, bearing in mind that the paper must be "wet, but not submerged."

Sowing was performed with a 0.5% active chlorine solution, prepared from a stock solution of calcium hypochlorite. Five grams of calcium hypochlorite added to 300 ml water, followed by filtration, were used to produce a 1.0% active chlorine solution. Then a 50:50 dilution of this was used to sterilize the seeds. This solution was also sprayed in the interior of the flasks just prior to closing them.

Replate is performed with similar solutions using a technique involving flamed forceps. The chlorine solution used for disinfecting the interior of the flask was 0.25%.

Unfortunately, Bayfolan Forte is not available in the United States, and I have been unable to locate Kr-ESE. Accordingly, reproducing Behar's work has not been possible, but similar formulations have been used, some of which are noted in this text. Behar had good success, but notes that his techniques have only been used with *Lepanthes* in some very limited experiments, and that other orchid genera should be tested. All results are preliminary, and merit further research.

As such, modifications of McDonald's techniques shown elsewhere in this book may be useful as a baseline for developing new formulas. Behar notes that fruit juices may be used to replace sugars, and many commercial

media formulations include banana as a major component already. Pineapple juice, honey, and cane sugar are other carbon sources for seedlings. Nitrogen sources could include commercial fertilizers, which would also serve to supply the other major and minor salts and elements.

In any event, all components for newly proposed media should be commercially available, particularly in nations that are less developed than the industrialized nations. Mixtures that require the use of analytical balances and precise measurements are not desirable. Requirements for accurate measurements should be exceptions, and not the rule, when considering formulations.

Similar choices should be made when considering substrates. Perlite, lava rock, and other inorganic materials that survive the heat of dry sterilization are good, but "wet" sterilization via room-pressure steam should also be considered. Substrates such as gels are not desirable, but if some other compounds are found to be suitable, these too would be useful. Other potential substrates include cotton wool, glass wool, paper, and vermiculite. For the development of rootless protocorms, nothing more than a few layers of filter paper may suffice, and it may be possible that paper towels or even cotton cloth may be substituted. Once development has progressed to protocorm stage, it may be possible that seedlings could be transferred to thicker media to accommodate root growth.

Another important issue is water quality. Since distilled water is not available everywhere, techniques using rainwater are important. Emphasis should be placed on hydrogen peroxide disinfection, either singly or with "sugar hatching" for when hypochlorites are not available. Techniques using bottled bleach, which is arguably easier to acquire than calcium hypochlorite, might be emphasized. The use of electrolysis to generate hypochlorite solutions for water treatment in remote areas has been discussed, and can possibly be adapted for orchid propagation. The production of small, simple flasking cases to lower infection rate would be desirable.

In any event, there are large gaps in our knowledge, and in order to facilitate flasking in remote areas, we should work to aid production of orchids via seed under difficult field conditions. Some factors have been mentioned here, but the actual research belongs in the hands of those that are willing to experiment and pass along the accumulated knowledge. Assisting in these processes will help developing nations produce large quantities of orchids indigenous to their respective countries, thereby preserving their natural heritage and possibly supporting the commercial production of plants for growers elsewhere. Such techniques may reduce collection pressure locally and emphasize conservation of native species. These efforts should be facilitated in any way possible.

Stock Solutions

For those that use large quantities of media, the use of stock solutions will provide a way of quickly and inexpensively producing your own media. Although more intensive than the use of prepared media, stock solutions increase flexibility in the creation of your own media. Larger labs that require media by the tens and hundreds of liters may find this to be the most efficient. The reader is advised to research papers on the subject and read Arditti's *Orchid Biology: Reviews and Perspectives II*, Kyte and Kleyn's *Plants from Test Tubes*, or Thompson's *Orchids from Seed*. Stock solutions for several specialty formulations, particularly those for propagation of cold-tolerant terrestrials, are given in the literature.

Arditti (1982) defines a stock solution as "a concentrate prepared for repeated and convenient use." As mentioned above, the primary advantage of stock solutions is convenience. If we were to create a liter each of several solutions, each with a different media constituent, at a concentration that was, say, 100 times that of the media, we could then remove 10 ml ($1/100^{th}$ of a liter) of each solution and mix them to create a liter of media at the desired concentration. Then components for which stock solutions are not possible (sugar, agar, etc.) are added, and the mix is homogenized, poured, and sterilized.

Another advantage is that for those components that appear in much smaller concentrations in the media, a 1000x stock solution is made, and 1 ml of this solution is then added per liter of media. This eliminates the requirement for repetitive weighing of components, not to mention the balance required to weigh such tiny quantities.

Let's say that you are going to make a media that requires 250 mg of potassium chloride per liter of media. You have a stock solution of 25 grams per liter; call this concentration C1. You want to find out what volume of stock solution is required to make a liter of media with concentration C2, or 250 mg per liter of potassium chloride.

Remember: all units must be the same. Convert milliliters to liters or vice-versa, and work in grams rather than kilograms or milligrams. Then cross-multiply to find the volume, V.

$$C1 = \frac{C2}{V}$$

Thus, if C1 is 25 grams per liter, and you know C2 is 0.250 grams per liter, we have:

$$25 \text{ grams/liter} = \frac{0.250 \text{ grams/liter}}{X \text{ liters}}$$

Cross-multiply, and find that you get (X liters) (25 grams/liter) = 0.250 grams/liter

Divide both sides by 25 grams/liter, and we get 0.01 liters. Therefore, 10 ml of stock solution of this component must be added to make 1 liter of mix. We can use a 10 ml volumetric pipette to add this quantity quickly and accurately, and repeat with all the other components.

Stock solutions are very handy when one only has limited access to an analytical balance. Few people have access to an analytical balance that can weigh out, say, 0.01 mg of potassium iodine, much less do it 10-15 times for a set of flasks. It may be easier to weigh out, say, 10 mg of potassium iodine for 1 liter of stock solution, and then use 1 ml of the stock solution for media preparation. For some media, several different microelements (those used in relatively low concentrations when formulating media) may be combined into one stock solution, and then 1 ml of this may be used for media preparation.

Stock Solution Preparation

Stock solutions must be prepared with attention to detail. Components must be measured accurately, and preparations must be refrigerated or frozen when stored, and checked for bacterial contamination regularly; stock solutions containing nitrogen are readily contaminated by microbial growth, and must be frozen or mixed immediately prior to use (Arditti, 1982). Label each stock solution with all pertinent data. Certain components are incompatible, and may form insoluble precipitates if mixed together. Kyte and Kleyn (1996) suggest the following for Murashige and Skoog (M&S) salts formulation. Note: For orchids, ammonium nitrate and potassium nitrate should be ¼ strength of true M&S salts, and this correction has already been done here.

Each stock solution is prepared by carefully weighing the components, and then adding them to a 1 liter volumetric flask. Sufficient distilled water is added to dissolve the components. Additional distilled water is added to the point where the volumetric mark is met (see glassware section in Section 3.5 Laboratory Basics). The flask is then capped, and inverted or agitated to mix the contents completely. The contents may then be poured into a 1 liter bottle for storage. Stock solutions must be kept refrigerated and discarded if any signs of bacterial contamination occur; this will typically appear as cloudiness, but do not confuse this with precipitated material that can be re-suspended.

If the work being performed is not critical, a 1 liter flask may be used in place of a volumetric flask, but graduations on these cylinders is often off by 10% or even more.

Nitrate stock:

	Potassium nitrate (KNO_3)	47.5 grams
	Ammonium nitrate (NH_4NO_3)	41.25 grams

Sulfate stock:

	Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	37.0 grams
	Manganese sulfate ($MnSO_4 \cdot H_2O$)	1.69 grams
	Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)	0.86 grams
††	Cupric sulfate ($CuSO_4 \cdot 5H_2O$)	0.0025 grams

Halide stock:

	Calcium chloride ($CaCl_2 \cdot 2H_2O$)	44.0 grams
	Potassium iodide (KI) (often omitted)	0.083 grams
††	Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	0.0025 grams

Phosphate stock:

	Potassium phosphate (KH_2PO_4)	17.0 grams
	Boric acid (H_3BO_3)	0.62 grams
	Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0.025 grams

Iron stock:

	Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	2.78 grams
	EDTA disodium salt (Na_2EDTA)	3.73 grams

Iron stock may require heating for components to completely dissolve, and should be stored in the dark.

Inositol/thiamine stock:

Inositol..... 10.0 grams
Thiamine hydrochloride.....0.04 grams

Nicotinic acid/pyridoxine stock:

Nicotinic acid..... 0.1 gram
Pyridoxine hydrochloride..... 0.1 gram

††Stocks using cobalt chloride and cupric sulfate stock solution may be created by adding 25 mg of each compound to 100 ml of water. Ten ml of the cobalt chloride solution is added to the halide stock, and ten ml of the cupric sulfate solution is added to the sulfate stock, each providing the requisite 2.5 mg per liter of these stocks.

Although the authors note that both the inositol/thiamine and nicotinic acid/pyridoxine stocks should be stored in the refrigerator, as mentioned above, it is best to refrigerate all stock solutions to slow the growth of contamination. Arditti (1982) notes that 70% ethanol should be used for stock solutions of vitamins and hormones, changing the pH with acid or base if required to dissolve components, and goes on to note that stock solutions of inositol, sugar or agar should not be made (Kyte and Kleyn probably intend for the inositol/thiamine solution to be used soon after its preparation, but this is not specified).

To produce a liter of M&S media using these stock solutions, 10 ml of each solution (nitrate, sulfate, halide, phosphate, iron, inositol/thiamine, cobalt chloride, and cupric sulfate) are added to a 1 liter flask, and water added to the 1 liter mark ("made up to 1 liter"). Then the pH should be adjusted, agar added, and finally the media sterilized.

These are only a few examples of stock solutions. As mentioned above, there are many more, some of which are designed explicitly for specific genera or even species. As a result, growers are encouraged to explore stock solutions if performing research in a specific field, or discover a need for large quantities of media.

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Yanagawa, T., Nagai, M., Ogino, T., and Maeguchi, R. 1995. Application of disinfectants to orchid seeds, plantlets and media as a means to prevent *in vitro* contamination. *Lindleyana* 10(1): 33-36.

3.8 Aseptic Sowing Area

As we have discussed above, most media for the germination of orchid seed will happily sustain cultures of many organisms which, although exciting, will ultimately lead to nothing but frustration for growers. The single most important aspect to maintaining an aseptic environment is restricting air circulation. By doing so, we remove potential sources of contamination such as dust particles that can be blown in. Many germs and spores are not independent articles; instead, they hitch a ride on a mote of dust. Thus, if we eliminate the dust, we can prevent contamination of our flasks. Do not be frustrated if your first attempts result in contamination because good results are the product of technique and experience and do not always come immediately.

The simplest form of protection from contamination of our flasks is a simple sheet of glass. If we perform our work under a clean sheet of glass in a room with a low concentration of dust, we can achieve results almost as good as those with a glove box or laminar flow hood (see below). The reason is quite simple: in still air, dust drifts downward, and therefore falls into open flasks or tubes. Eliminating drafts and minimizing contamination from above will reduce the chances of stray infectious agents contaminating flasks. As with any technique used for flasking, minimiz-

ing the amount of time for which flasks are open while using the glass sheet technique will be important. We can further lessen the chances of contamination by working on a hard, non-porous surface such as Formica or another glass sheet, which is then covered with a paper towel soaked in a disinfection solution. There are many such solutions commonly available for work in bacteriology, but chlorine solution such as that used to disinfect the seeds are superior. We can also lessen the chances for contamination while using this technique by keeping flasks horizontal, so their openings are not facing upward. A sheet of aluminum foil, sterilized by autoclave or dry heat, may be substituted for the working surface.

A step up from the glass sheet is a glove box. Glove boxes range from aquariums tipped on their side to complex steel and glass structures, replete with ports for gloves, ultraviolet sterilizing lamps, air locks, and other features. The glove box serves to produce an entirely enclosed area that can be sterilized, thereby reducing the risk of contamination even further.

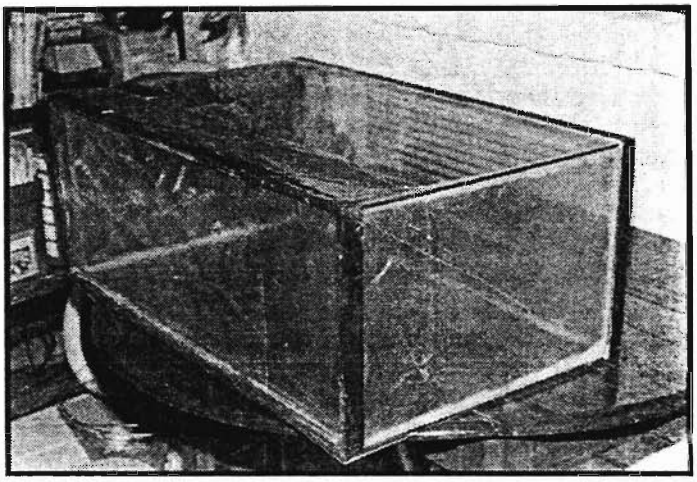

Figure 3.8.1: An aquarium may be used as a sterile workspace for flasking and replate work.

An aquarium makes an excellent glove box. When tipped on its side, the open end may be covered with a thin sheet of plastic to reduce air circulation (Figure 3.8.1), with slits for your hands or equipment to be passed through to the inside. Sold under the name "Tough-Glas," it can be found in hardware and discount stores, sometimes sold as vinyl sheet for covering kitchen tables. The use of a small shim to tip the front edge back just a bit to keep from spilling bleach solution all over the kitchen is suggested (something about the diameter of a pencil in most cases). A small towel soaked in your disinfection solution may be kept in the back and used for wiping down surfaces to eliminate contamination. Similarly, a little ingenuity and labor will permit do-it-yourself home flasking devotees to produce their own customized cabinet. These may range from cardboard boxes lined with aluminum foil and covered with plastic, to glass cases with silicone rubber seals. More details on these cases are given in Section 6.3, on making your own glove box.

To determine the effectiveness of your sowing area, Light (1990) recommends preparing 6 flasks of media, and leaving each open for progressively longer periods of time: 10, 20, 40, 60, and 120 seconds, and leaving the sixth as a control. Incubate each flask in a warm area for at least 3 days, and check for evidence of contamination in the form of fungal or bacterial growths. In the event that contamination occurs, review your sterilization techniques, make adjustments as necessary, and begin again.

Laminar flow hoods (Figures 3.8.2 and 3.8.3) are decidedly the best option when it comes to having a large, sterile work space. The laminar flow hood possesses a High Efficiency Particle Air (HEPA) filter that removes virtually all of the particulates suspended in air. Recall that, since most bacteria and fungi that might infect your flasks are traveling on these dust particles, removing the dust will render your air flow virtually sterile. As with other work areas, your tools, bottles, etc. will need to be sterilized either prior to their introduction to the flow hood, or chemically or thermally sterilized once in the work area. For flasks and some other components, a bucket of disinfectant may be used as a dip, in which the entire item is immersed, or partially immersed with sensitive areas (such as the vent) sprayed or wiped separately. A spray bottle filled with disinfectant also works well.

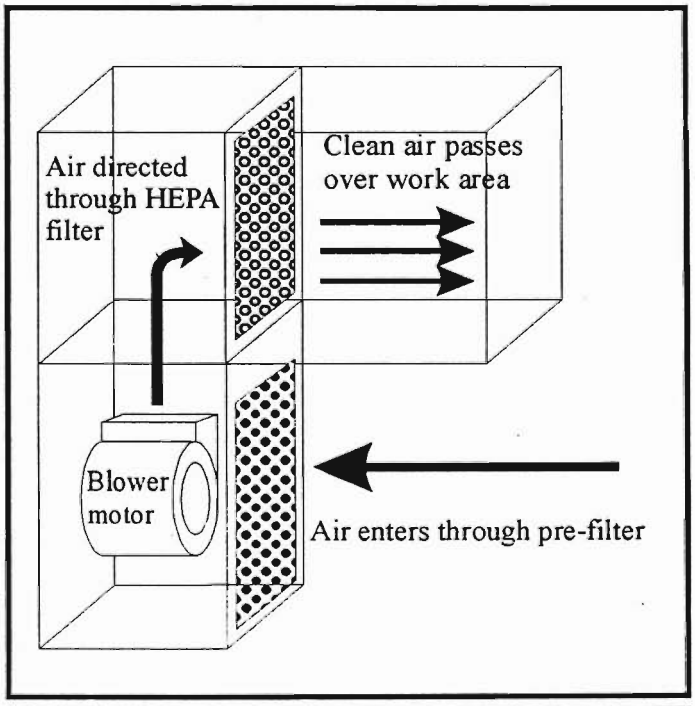

Figure 3.8.2: A horizontal flow laminar flow hood.

There are several different types of flow hoods. However, the two main types are vertical flow and horizontal flow. With vertical flow models, the air is

driven downward by the blower, usually onto a flat working surface. Provided that there are three walls and an open front for the operator, these units are useful for aseptic work. Horizontal flow units are more popular for these techniques, and as the name implies the air is pushed towards the operator from the rear in a horizontal direction. With either unit, air flow should exceed 100 linear feet per minute, and all surfaces exposed to the air should be capable of being chemically sterilized.

It is worth noting that laminar flow hoods are commonly used in microbiology labs as well as in ultra-clean rooms, such as those used to fabricate electronics devices. They have a fixed useful lifetime, at which point they are commonly surplused, and may be purchased for the home lab, often for a fraction of their original cost. Most units are glorified air filters, in which a blower serves as the only moving part. They also have a large, expensive filter component, some of which may be proprietary and therefore unavailable once the original unit is out of manufacture. Still, this should not discourage one from considering the acquisition of a used unit, provided one is willing to invest a little time and research into how to rebuild or maintain a laminar flow hood, which often costs thousands of dollars when purchased new.

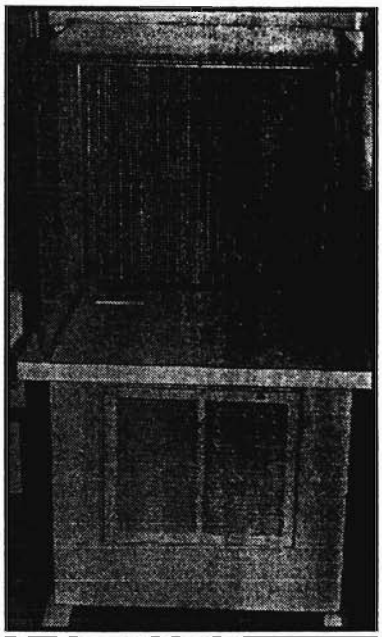

Figure 3.8.3: A small HEPA workstation.

It has been suggested by several growers that it is possible to make your own miniature flow hood by using commercially available HEPA air filtration units that are designed for domestic use. If one uses one of these filter units to vent into a box so that the air flows towards the user, the air stream may be sufficiently clean to perform the aseptic work required for flasking and micropropagation. Availability of these units has increased in recent years such that they are now available in discount stores in the United States, as well as the filter unit alone. A little ingenuity may go a long way, but these units should not be confused with commercial-quality flow hoods: the filter elements are tighter and heavier in domestic-grade units such that they have an abbreviated life span, as well as a higher pressure drop across the filter. Considering the price difference, these hoods may not be objectionable to the home grower. At least one domestic model boasts a 99.97% filtration rate for particles 0.3 micron and larger for a price under $100.

One other modification involves using a HEPA filter in conjunction with a glove box, forming a turbulent (versus laminar) flow hood. Instead of having a large working space requiring large filters and fans to maintain the 100 linear feet per minute generally recommended for laminar flow hoods, attaching a HEPA filter to the back of a glove box to force purified air towards the worker serves to maintain a clean workspace as the air exits the glove/hand ports only. Although a far cry from a full workstation, a turbulent flow station permits enhanced work quality and worker comfort over a glove box.

A short article by Roberts (1986) in the September 1986 issue of the *American Orchid Society Bulletin* shows how to create a small, clean-air workstation using a 12" x 12" HEPA filter for purification.

Appendix IV contains a set of directions to assist in the construction of a HEPA filter suitable for aseptic plant propagation, written by G.W. Forester and D.W. Burger.

When using a HEPA filter, it is best to test its efficacy in filtration before using it for production. After sterilizing the interior of the workspace, turn on the fan for at least ten minutes before beginning work. Then open one or more petri dishes or flasks with nutrient agar, and let them be exposed to the flow of air for one to five minutes. Close them again, and allow them to incubate for several days. If there is no sign of bacterial or fungal growth, the workstation is ready for use. If contamination is present, run the filter for progressively longer periods of time, and clean and disinfect all working surfaces again.

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Light, M. H. S. 1990. Doing your part for conservation (part 3 of 3): Doing it! Sterile techniques, flasking, the works. *American Orchid Society Bulletin* 59: 1014-1022.

Roberts, A. 1986. Making a clean air station. *American Orchid Society Bulletin* 55: 924-925.

3.9 Flasks

It is important to select a flasking vessel that is appropriate to the task at hand. The novice using only simple components that are immediately available will want to use pint or quart canning jars, which have several appealing features. Most importantly they are inexpensive and can be purchased at almost any supermarket. Secondly, they have very wide mouths, making them easy to work with for replating, de-flasking, and cleaning. Further, when rested on their side at a slight angle, very little media goes to waste, unlike with expensive Erlenmeyer flasks that waste a great

deal of media when rested on their side. Also, canning jars are made to be autoclaved, as are their lids and rings.

Many growers will use labware such as Erlenmeyer flasks. Others use glass bottles used for drinks or other household products, but aside from wasting media, they also make it challenging to reclaim seedlings. Seedling removal may necessitate smashing the flask, and replating can become quite difficult if it is required. Some growers have noted that such bottles should be washed with hydrochloric acid prior to use, to reduce leachable compounds. Others have suggested that this is not necessary (Torres, pers. communication). In either event, an acid bath will clean the surface of the glass very well. Hydrochloric acid should be treated with caution, wearing eye and skin protection as well as a rubber gown to prevent injury to the skin and damage to clothing.

Today, there are a number of clear, autoclavable plastic plant cell culture vessels that are commercially available. Some are square-bottomed, offering maximum use of your seedling growth areas.

Different culture tubes have different venting requirements; some are simply plugged with cotton, and others have their own special membranes that provide for gaseous exchange. Canning jars are easily prepared for flasking by perforating the lid with a sharp object like an arch punch, or ice pick or nail to form a hole approximately 4 mm in diameter which is then stuffed with cotton. The purpose of the cotton plug is primarily to allow for pressure equalization, especially when cooling down, but also helps with gas exchange in the sown and replate flasks. After being autoclaved, the lid may be covered in plastic wrap if one is not covering them with aluminum foil during autoclaving; this helps keep down the chances of contamination.

Agar "slants" can be used to germinate small quantities of seed or grow small quantities of seedlings (Figure 3.9.1). These small (2.5 cm or 1 inch diameter) glass tubes are autoclaved with a small amount of media in the bottom, and set to cool at a 45-degree angle to maximize surface area. They are plugged with cotton, and capped with a polypropylene or similar lid. These setups allow small volumes of seed to be germinated, or a small number of seedlings to be cultured. Baby food jars are another option, and special, translucent lids are commercially available that will permit using these inexpensive vessels for plant culture. These tubes and lids are sold by several different companies, including Sigma and PhytoTech Labs (see Appendix III, Sources). There are several reasons that one may wish to go with slants or baby food jars, including:

- Testing seed to see if it is viable before sowing larger numbers of flasks
- Testing seed to see if it is heavily contaminated
- Seed is rare, or only small volumes are available
- Testing the same species of plant on several different media on a smaller scale, preventing waste
- Germinating on nutrient agar media in an aseptic environment, so cultures are relatively free of international shipping restrictions
- Making smaller flasks for trading/sharing

Figure 3.9.1: Sterile culture tubes, as agar slants.

Because of their small size, and the fact that they are stuck in the bottom of a test tube, slants are much more stable in transit than are larger flasks that tend to become jumbled, sometimes resulting in high mortality rates. Slants are very efficient, but are more time-intensive to produce, and are obviously not very effective when one needs to produce large numbers of plants. Further, once in flask, seedlings are relatively free from international restrictions governing transport of species internationally. Still, it is important to check regulations with all parties concerned, as sometimes permits such as phytosanitary certificates are required. Mason jars will be commonly used in home flasking. They are inexpensive, available at most grocery stores, and of uniform size and shape such that they pack conveniently in a pressure cooker. It is worth noting that almost any glass jar with a suitable lid will do, but given the low cost of Mason jars (about $.50 each locally), it is difficult to rationalize using anything else unless Mason jars are locally unavailable. Check at garage sales, as used canning jars may be available in large quantities- sometimes by the tens or hundreds.

Carefully note the variety of Mason jar you are purchasing. There are "regular" jars with flat sides that increase surface area, and jelly jars that are round and not quite as good. There are also a variety of sizes. For larger operations, you may wish to use quart jars. Pint jars are very good for mother flask if you don't have much seed, and if you like to give away small flasks as gifts, half-pint jars are occasionally available and very convenient to work with. "Wide-mouth" jars are desirable, but as Mason jars all have very large openings, the difference between these and regular jars is not an overwhelming factor.

Baby food jars are appealing because they take up less space, and hold fewer seedlings, which may be desirable to smaller growers. They are inexpensive or even free if you're fortunate to know someone who buys baby food. In contrast to a pint or quart Mason jar with 25 or more seedlings, a baby food jar with 5-7 small seedlings will sell for less, and not overwhelm the grower with a large number of seedlings, which must be attended.

Baby food jars and other small vessels may be used to quickly and efficiently test seed for germination. With only about 25 ml of liquid in each jar, a liter of media will easily prepare three dozen jars. These jars may be used to bring seeds to the early protocorm stage, and then replated. Although special plastic lids are available, the lids these jars come with are also suitable. If using aftermarket lids for seed germination or seedling growth, it is recommended that the gap between lid and jar be wrapped in plastic wrap to prevent water loss.

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Torres, K. E-mail correspondence with the author, 1999.

Chapter IV: Technique

4.1 Preparation of the Sowing Area

The techniques by which orchid seed is placed *in vitro* vary between growers, and depend upon experience and what is available. There are several different factors to consider before proceeding with seed sowing, starting with a sterile work area. This is one suggested method, with different routes offered to get to the same destination. The reader must modify the technique to work best with his or her own conditions, materials and preferences.

Glass Sheet Technique

As mentioned in Section 3.8, it is possible to perform flasking under a single sheet of glass. Glass that is ¼" thick (for strength) and has the edges ground (for safety) is perfectly satisfactory. Four blocks or stands of equal height are required to raise the glass over the work area. Enough space must be left between the glass and the equipment to avoid constantly bumping into its underside.

It is possible to use two glass sheets for this technique, with one underneath and one above. Either way, the grower should place the work on a bench that is non-porous, such as a plastic counter top. The important thing is that this surface will be bleached or otherwise chemically disinfected, and therefore must not be damaged by the chemicals used.

Place two layers of white paper towels across the entire working surface. Saturate these with the disinfection solution (5-10% bleach or saturated calcium hypochlorite). Turn off any air circulation that may disrupt your work or cause contamination. Place the glass sheet on its spacers over the workspace, and allow 10 minutes or more to sterilize. At this point in time, it may be considered clean enough to start work.

Glove Boxes

When working with a glove box, there is the distinct advantage in that you may feel free to create more of a mess, and not have to be overly concerned about it. If well-constructed so that leaks will be prevented, a glove box may be situated almost anywhere, and the inside sprayed with bleach or other sterilant. Once left to sit for an appropriate period of time (5-10 minutes), the user can perform their work inside of the box.

One should be careful when opening glove boxes, as the accumulation of chlorine gas or other fumes that accumulate during work can be overwhelming, and even dangerous, when opened. Proper ventilation and a cautious attitude will go a long way toward preventing harm.

Laminar Flow Hoods

Laminar flow hoods may be sterilized with either ultraviolet light, or with chemicals. Surfaces should be sterilized only with chemicals approved by the manufacturer, but generally bleach solutions are recommended. For more information see Section 3.8, Aseptic Sowing Area.

Hands

Human skin is alive with organisms. These bacteria and fungi may readily infect your flasks. There are several ways to keep this from happening.

One of the most effective techniques is to wear gloves. Gloves made of latex are very useful in that they may be chemically sterilized and prevent contamination of your work, although sensitivity to latex (allergies) have become more common as a result of their use. Other rubber gloves are available, and the reader will have to determine which gloves are best suited to their applications.

For those with tough skin that is not adversely affected, direct disinfection and exposure to the chemicals used to sterilize surfaces is also an option. Begin by washing the hands and arms with an antibacterial soap or povidone iodine solution for at least 2 minutes. Scrub vigorously, removing any loose skin cells or hairs that may later contaminate the workspace. Use a nail brush to scrub under the fingernails (a toothbrush will also work). Allow your hands and arms to air dry, or wipe dry with a clean towel. Before starting work, spray hands and forearms with disinfecting solution.

This technique is rigorous and most disinfectants are not good for the skin, often causing severe drying. Be certain to use an appropriate moisturizer regularly. If so desired, the wash may be reduced to a quick, but thorough, 15-30 seconds; with all sterile techniques, it is important not to drip or drop anything into an open flask. With proper technique, simply keeping your hands clean while working will be sufficient to keep contamination to a bare minimum.

Bleach vs. Alcohol

Bleach is generally better because chlorine deactivates more organisms more effectively than alcohol. Alcohol is flammable and evaporates quickly. Hypochlorites are dissolved in water, which does not evaporate as rapidly, and is known to be very effective at deactivating a broad spectrum of active and dormant organisms. Hypochlorite solutions at a reduced pH (around 6.0) are most effective.

4.2 Disinfection of Seed

Having satisfied our requirements for sterile flasks, tools and a working area that will prevent contamination, we face the single greatest difficulty: disinfecting the seed itself.

As we have already seen, orchid seed is remarkable in its construction. Unique in its diminutive size, orchid seed is fragile, with the embryo itself being afforded little protection and therefore vulnerable to physical damage.

Despite such apparent fragility, orchid seed is capable of undergoing chemical surface sterilization that kills (hopefully) all of the microorganisms present, without damaging too much of the seed. There are a couple of solutions that satisfy the requirements for conditioning seed that will effectively sterilize the exterior of the seed without killing the embryo itself, and some degree of research is going on that may prove useful some day in terms of presenting us with options.

The most desirable solution to use is one of calcium hypochlorite, which serves several commercial purposes, including being the active agent in HTH pool chlorine, as well as many bleaching powders. It is chemically unstable, and must be stored cool and dry in a tightly capped container. It is corrosive, quite toxic, and the fumes it produces are irritating. Therefore, some caution must be used when dealing with the dry solid. It is also a powerful oxidizing agent, and very corrosive. The dry solid itself smells strongly of chlorine, and is powerfully destructive to many plastics. As a result, one must be careful to choose an airtight storage vessel capable of withstanding attack from the dry chemical. Also, the solid compound must be kept from contact with organic compounds, such as oils, paper, and wax.

There are three chemical solutions commonly used for disinfection of seeds: sodium hypochlorite, calcium hypochlorite, and hydrogen peroxide. The latter of these three is discussed in Section 6.5, New Directions in Seed Disinfection. Of these, probably the most commonly available to the home grower will be sodium hypochlorite, or common household bleach, which will be discussed below. Nevertheless, calcium hypochlorite is probably the most desirable compound for disinfection of seed, and the most worthy of discussion. For those that wish not to be bored with details, a saturated solution of calcium hypochlorite will be the most useful. This is prepared by placing about 10% calcium hypochlorite, by weight, in a volume of water. An example would be to put 10 gm of the solid in 100 ml of distilled water. This recipe produces a mixture that is less than 10%, but water is not capable of carrying this concentration of hypochlorite. There has been some discussion with respect to the amount of calcium hypochlorite that it takes to produce a saturated solution, but a small amount of undissolved solid it the bottom of a flask will assure that the solution is near or at saturation. This solution can then be used either by decanting clear liquid off of the top of the solution, leaving behind the solid, or by passing the liquid through a piece of filter paper or a coffee filter. There has been much discussion as to the longevity of calcium hypochlorite solutions. Early researchers sometimes stored the solution refrigerated for weeks, and recent work (Bergman, 1996c) indicates that filtered, refrigerated calcium hypochlorite solutions retain their potency for at least a year. It should be kept in a clean, stoppered bottle that prevents exposure to light–either wrapped in aluminum foil, or the bottle made of amber glass. This liquid can then be used to disinfect seed (see below for technique).

For those that wish for a more in-depth discussion about calcium hypochlorite, it will be covered more thoroughly under Section 6.5, New Directions in Seed Disinfection.

Probably the most commonly used solution is common household bleach, which consists of sodium hypochlorite; sodium hypochlorite normally exists only as a solution, and not a solid. Sodium hypochlorite is produced when gaseous chlorine is passed through a sodium hydroxide (caustic lye) solution, and its strength gradually decreases as the chlorine degasses from solution. Thus, although the label on most household bleach gives the concentration as 5.25% sodium hypochlorite, brand-new bleach may be stronger than this, and older bleach may be weaker. As the headspace grows larger in an increasingly empty bottle, chlorine is lost to the atmosphere and the strength drops, leaving behind a solution of sodium chloride (table salt) in water. Steele (pers. communication) notes that fresh bleach is important for consistent disinfection of *Cypripedium* seeds. One other consideration is that the concentration varies depending upon the season, to accommodate for temperature and other factors (Mike Herauf of the Clorox company advises that it is made to contain a concentration no less than 5.25% at the time of use, to comply with federal regulations, due to changes that result from shipping, storage, etc.). As a result, we can only guess at the exact concentration of free chlorine in a given sample of bleach, which may be a consideration with your work.

Household bleach is used to disinfect seeds in concentrations from 1 to 10% by volume (an example of a 5%

solution would be 5 ml of bleach made up to 100 ml solution by adding water); there has been some discussion as to the effect that the high pH of the solution may have upon seed, and it seems likely that it does not play a significant role in viability of most orchid seed. The seed of some native American cypripediums may benefit from this treatment, and display increased germinability as a result. In fact, some growers have advocated disinfection times as long as 6 hours, or that they be removed from the disinfectant solution only after they have blanched from prolonged exposure. A 5% solution of bleach is a good starting point.

Whichever solution is used, a tiny amount of wetting agent should be added; there are many solutions that will help with the task of disinfecting the seed, including most dishwashing detergents. In any case, only the tiniest amount of wetting agent is required (much less than a tenth of a drop per 100 ml). Other agents, such as Tween 20, are commercially available and perform the same job. Too much wetting agent will make it difficult to sow the seed, as you will need to wash the seed until the foaming stops when you agitate it. One solution I have used with success consists of two drops of Palmolive antibacterial diswashing detergent, dissolved in 100 ml of distilled water. Two drops of this solution are added to up to 5 ml of disinfection solution for seeds. A little experimentation before using live seed will go a long way in helping determine how much of a given detergent or other wetting agent to use. Also see Section 6.6, Practicing with Inert Compounds.

One other example for seed disinfection suggested by Wellenstein (pers. communication) is that disa seed may be disinfected with a solution made with 3.5 gm of "Super Shock" (78% available chlorine) in 600 ml of sterile water; 0.2 ml of Tween 80 (polyoxyethylenesorbitan) is added to this solution, and the seed is suspended in this for 20 minutes and rinsed once. Repeated and aggressive agitation may be required during disinfection and rinsing, as this seed tends to float. Vacuum treatment (see Section 5.1, Dealing with Contamination Problems) may be of use with seed of this genus.

The following material discusses several different techniques by which orchid seed may be disinfected. It is suggested that the reader try at least two or more techniques to determine what works best for them.

Technique A: Batch Washing

In order to disinfect the seed, a small test tube is used. Add a small quantity of seed (smaller than a grain of rice) to the tube (Figure 4.2.1). If using a wetting agent, add it now (step 1); a small amount of a dilute wetting agent may help wet the seed to make it sink, or at least evenly saturate the seed with disinfectant even if it floats. Add 5-15 ml of the hypochlorite solution into the tube (step 2). Seal the tube with a stopper, and agitate (step 3). If the seed has not been pre-soaked in sugar solution, some may float.

Disinfection times range from 5 to 15 minutes, and sometimes much longer for terrestrial species. A good starting figure for most tropical epiphytic genera, such as cattleyas would be to use saturated calcium hypochlorite (or 2 to 5% bleach solution) for 15 minutes. Try to keep the seed suspended within this solution for as much of this time as possible. It has been suggested by a number of authors that *Phalaenopsis* seed does not respond well to high concentrations of chlorine, and this too will be discussed in Section 6.5, New Directions in Seed Disinfection. Occasionally loosen the stopper, pull it out, and make sure the area where the stopper seals the tube is wet with disinfectant, and then reseal the tube, and resume agitation (step 4). Repeat this check at least once or twice. Resume intermittent agitation (step 5).

Shortly before your disinfection routine is done (say, after 14 minutes), stop agitating and let the seed settle out of solution. If using a flat-bottomed vessel, tip it to one side to trap the seed in one corner. It is essential to remember that everything entering the tube from this point onward must be sterile.

At this stage, you will want to wash your seed, which may be done one of two ways. The first way is using a dropper or pipet to remove the solution above the seeds (step 6). The solution is then replaced with a wash of sterile distilled water (step 7), and then agitated. Remove the water above the seeds. You can then wash again, or deliver the seeds into your flasks (see below).

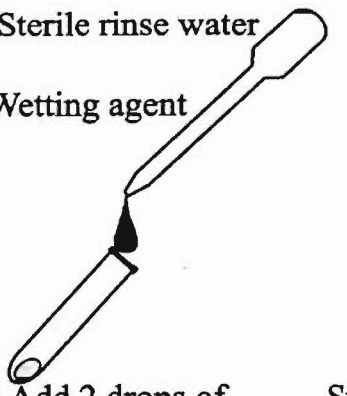
Instead of removing the disinfecting solution from the seeds, a pipet or dropper may be used to remove the seeds from the solution; you will carry a small volume of sterilant solution along with it, but this will be diluted when the seeds are washed. Draw up as many of the seeds as you reasonably can, and deliver them into a flask of sterile, distilled water. Agitate, and wash again, if so desired before flasking.

It is not essential to remove every last trace of disinfection solution. Conventional wisdom has been that chlorine is a very reactive molecule, and virtually all the available chlorine will react with the media to form relatively inert compounds. Although this concept seems to be theoretical, excess hypochlorite at the time of sowing does not seem to poison the developing seedlings. The chlorine will convert to compounds that are less prone to cause damage to developing seedlings than reactive chlorine. Also, some media incorporate finely powdered activated charcoal as a darkening agent or to remove certain waste products from the flask as seedlings develop. Activated charcoal scavenges chlorine with great efficiency. However, no research to date has been performed to determine if this is the case

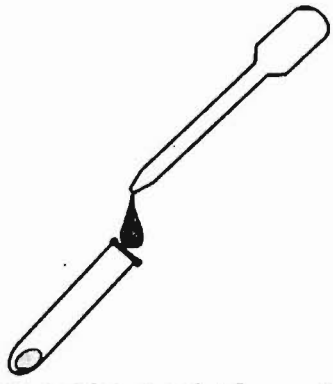
Green = Sterilizing solution

Blue = Sterile rinse water

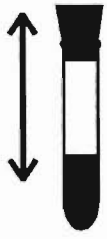
Red = Wetting agent



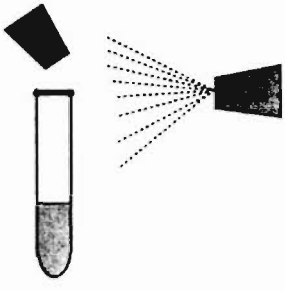
Step 1: Add 2 drops of wetting agent to seeds



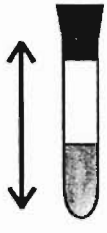
Step 2: Add 1-5 ml of disinfecting solution



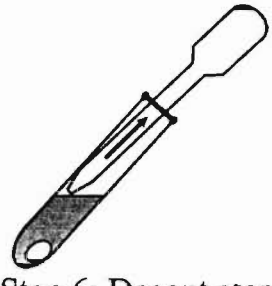
Step 3: Stopper and shake



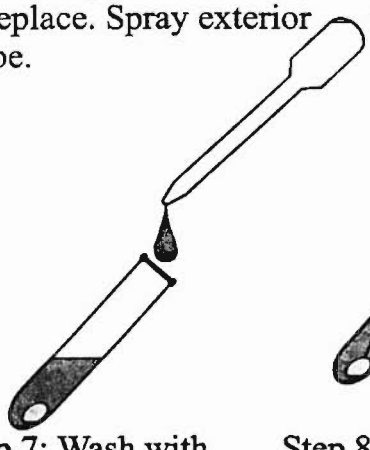
Step 4: Remove stopper, spray all exposed surfaces, and replace. Spray exterior of tube.



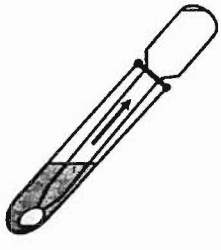
Step 5: Agitate intermittently until time is up.



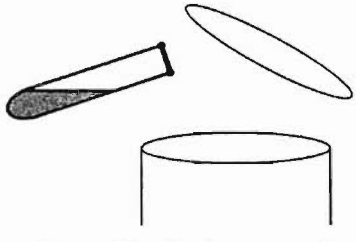
Step 6: Decant sterilant or remove with dropper after seed has settled.



Step 7: Wash with sterile water.



Step 8a: Seed may be drawn off with dropper and then sown.



Step 8b: Re-suspend seed with a quick shake, and deposit seeds and wash water into flask.

Figure 4.2.1: Technique A, Batch Washing.

in vitro. What is certain is that there will be more chloride left behind, which may inhibit germination.

At this point, you will have a small amount of wet, sterile seeds. These may be delivered onto the media in several ways. The best way (provided they are still wet) is to simply draw them up with a pipet or dropper (step 8a) and hold them carefully with one hand as you open the lid to the flask with the other. Open the mouth of the flask only far enough to insert the tip of the pipet. Being careful not to touch the inside of the flask, squeeze the bulb and eject the seed and the wash water into the flask. Try to expel as little air as possible along with the seed. Another technique is to use a sterile inoculating loop to retrieve seed from the tube, and deposit it on the media.

The other technique is to deliver seed and wash water together onto the surface of the agar. Shake to resuspend the seed, remove the stopper, open the flask, and deliver the entire contents to the media (step 8b). Recap the flask and tighten down the lid. Replace the foil, and set the flask aside. When you are completely done with your flasks, you can take each one and do your best to evenly disperse the seed by rolling it around so that the accumulated liquid (condensation plus wash water) inside the flasks will distribute the seed over the surface of the agar. It is relatively important that the seed be distributed evenly to avoid congestion from poor distribution. Still, not every seed will germinate, and transferring numbers of small seedlings to another flask when they are mature enough (see Replating) is an option when flasks have achieved maximum density.

Technique B: Filtration

Another way to separate seeds from the disinfection solution consists of using a filter funnel, or a Büchner funnel, and filter paper to remove one from the other (Figure 4.2.2). With this system, the filter and paper must be sterile prior to introducing the seeds (the paper may be autoclaved, wrapped in aluminum foil, and the flask may be chemically sterilized). The user should be familiar with the assembly and capable of using a filter flask.

Assemble the filter in your sterile work area, and if using vacuum filtration, turn on the aspirator and squirt a small volume of sterile water or hypochlorite solution onto the filter paper to wet and seal it. When ready to drain your seeds, agitate the seeds and sterilant, and dump both into the filter. The size of the filter should be proportional to the quantity of seeds to be cleaned. Once the disinfection solution passes through the filter, use a sterile dropper or pipet to deposit some sterile wash water onto the seeds, and let this drain. Repeat if so desired.

At this point, you will have a quantity of sterile seeds on a piece of sterile paper. There are several options from here, including:

- •Remove the filter paper and seeds using sterile tweezers and depositing them directly onto the media, allowing condensate to wash the seeds off the paper and onto the media.
- Remove the filter paper with sterile tweezers and place it inside a sterile Petri dish. Add some sterile water to re-suspend the seed, remove them with a dropper, and eject them onto media in a flask.
- Transfer the seed from the wet filter paper with a sterile inoculating loop, glass rod, or steel microspatula.
- Bergman (pers. communication) suggests moving the filter unit over the mouth of the flask and poking a hole through the filter paper while using a conical filter. Using a squeeze bottle, dropper, or Pasteur pipet filled with sterile distilled water, the accumulated seeds may be introduced into the flask by washing them off the filter.

When using this technique, it is important that the funnel be placed inside the glove box or (preferably) inside a laminar flow hood, as the funnel will also aspirate air, and the filter paper can gather dust and spores present in the air onto its surface, leading to contamination as it is introduced into the flask.

Coffee filter paper may be substituted for Whatman filter paper, and certain brands may work better than others. Look for a smooth finish that is capable of retaining seed of the size you will be disinfecting. Whatman manufactures a 25 mm diameter filter funnel that may be autoclaved, and works in a similar manner. It does not require a vacuum source, and lends great efficiency to the flasking process. Purchasing plenty of 25 mm filter papers of the correct grade is recommended so they may be reused indefinitely. They may both be purchased through Marskon LabSales (see Appendix III: Sources).

Technique C: Dropwise

This technique is a bit more involved than the previous two, but is more efficient and can be set up as a semi-automatic technique when done properly.

With the dropwise technique, the seed is kept stationary and the sterilant solution passes over and through the seed. There are a number of variations on this technique; the reader will be advised to customize one based on available materials.

A small siphon tube delivers liquid onto the seed; small vinyl tubing works well and is inexpensive. A small pinch clamp and the vertical distance between the reservoir level and the end of the siphon regulates the flow. It is

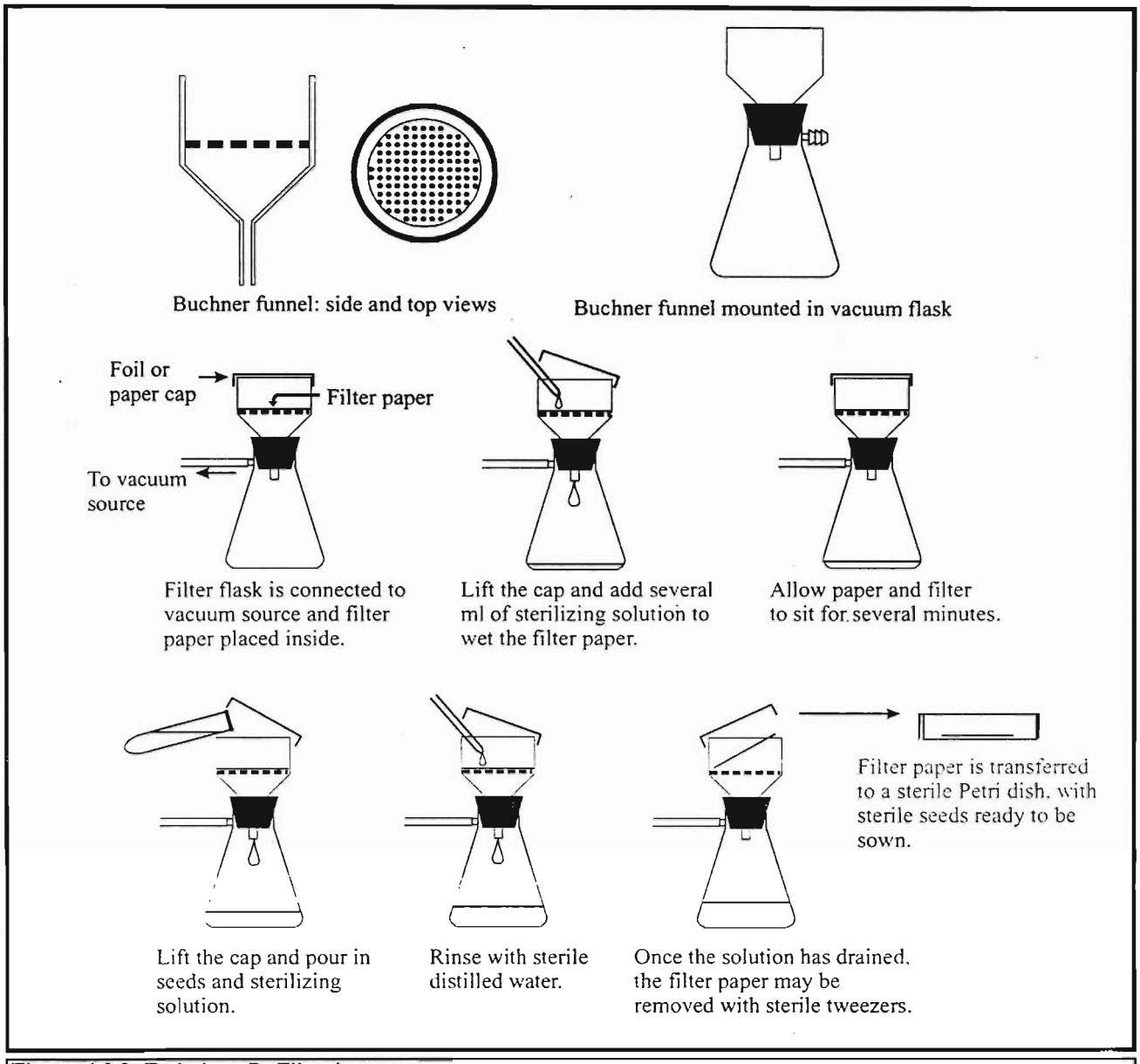


Figure 4.2.2: Technique B, Filtration.

important that the drops do not scatter the seed, or cause the fluid to build up faster than it can filter through. Experience will help determine the optimal flow.

The material from which the column that supports the seed is not critical; the rigid plastic tubes designed for aquariums as lift tubes would work well, but a small bottle would also work. The support simply provides for drainage, and keeps everything upright.

The assembly is designed to hold the seed in filter paper that is already nested inside of a filter funnel. Before adding the seed, a small amount of water or hypochlorite solution is added to get the filter paper to stick to the funnel walls, and then the seed is added to the bottom of the funnel. The flow is then started, and monitored until the user is satisfied that the liquid is draining from the tube faster than it goes in; thus, the seed is kept in a semi-wet state, but constantly bathed in sterilant. After the desired period of time, the sterilant is replaced with sterile distilled water. It is important to remember that the tubing and everything else that comes in contact with the liquid also remain sterile, and that once the sterilant is removed, that the atmosphere surrounding it must also be sterile.

The seed is then treated as above, in technique B, to sow into flask.

There are many variations on these techniques, and a little experience and ingenuity will permit the reader to find the most efficient procedure for your own facilities. It is suggested that Whatman #1 or #4 filter paper will be the most effective when it comes to trapping seed; an appropriate size (diameter) will have to be selected for the funnels being used. Whatman has introduced an acid-washed paper whose strength is greater than "standard" Whatman

paper. However, scrapings of filter paper, although out of place in the flask, do not increase the chances of contamination, and are common when using a microspatula.

If filter paper is not available, try using coffee filter paper, as mentioned above.

Technique D: Decanting

Advocated by Bergman (1996c), it is possible to mix seed plus disinfectant solution (total of about 3-5 ml), shake for about 10-15 minutes, and then decant the mix without filtration onto the media. Tilt the flask to one side, and decant the collected liquid, leaving the seed behind on the media. This technique works well, but may require practice; it does not work terribly well with "floating" seed, but can be used anyway. My experience has been that this technique works best with larger seed that sinks.

Technique E: Rapidfire

Probably the quickest way to deliver seed into flask was told to me by Fred Bergman.

Funnels that may be sterilized via autoclave or chemical disinfection are used. If possible, funnels should be lined with filter paper, wrapped in foil, and autoclaved. Otherwise, funnels may be disinfected chemically, and lined afterwards with autoclaved filter papers.

Several small funnels (one for each species) are lined with filter paper and supported in some kind of stand. After seed has been disinfected for the required period of time, the seed and disinfectant solution is dumped into a funnel, and allowed to drain (Figure 4.2.3).

If so desired, a rinse may be performed with sterile distilled water (Figure 4.2.4). The seed should now be in the very bottom of the funnel; if not, add water dropwise to help flush it into the bottom of the cone. Then the funnel is picked up and moved over the mouth of the open flask. The tip of a pipet or dropper is used to perforate the bottom of the funnel (Figure 4.2.5), and a small amount of sterile water is used to deliver the seed directly into the flask (Figure 4.2.6). The water may be delivered with a wash bottle, dropper, pipet, etc. Repeat with each funnel, making sure to change the dropper or other delivery tool between different batches of seed. Note that when sterilizing wash bottles with liquid in them, the top must be loose to allow for expansion; otherwise, your wash bottle will be empty after autoclaving. Cover the top with aluminum foil.

A plastic container, such as a butter tub or a sandwich container, can be used to hold several funnels by punching holes in the lid. Further, several plastic droppers are available from different sources, and these can be used to perforate the filter paper directly. These are often called transfer pipets, and Sigma (see Appendix III, Sources) has several models, many of which are available as individually wrapped, sterile tools.

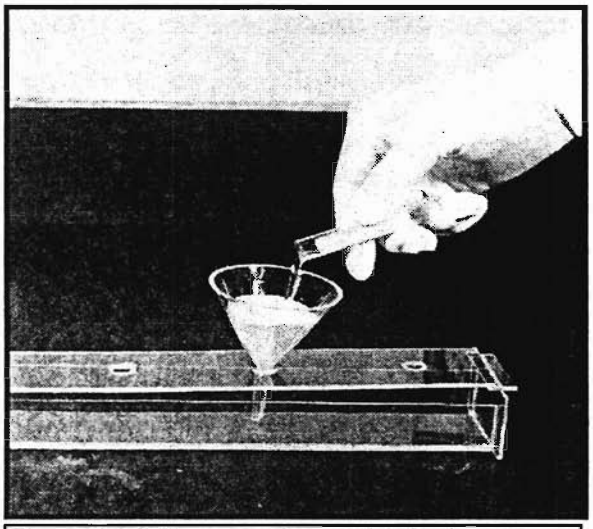


Figure 4.2.3: Dump seeds and disinfection solution into funnel for filtration.

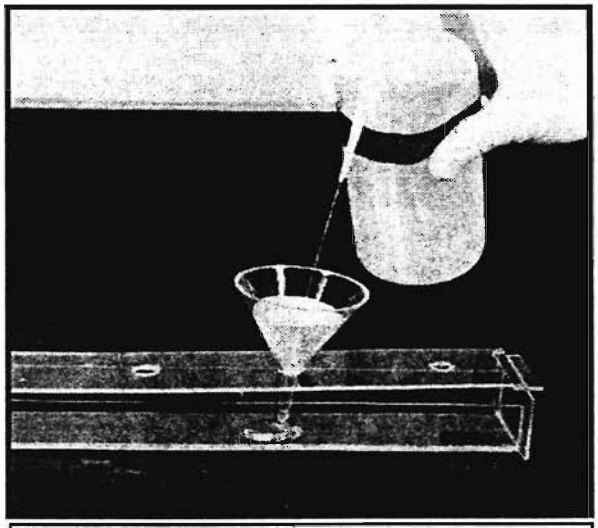


Figure 4.2.4: Wash with sterile distilled water

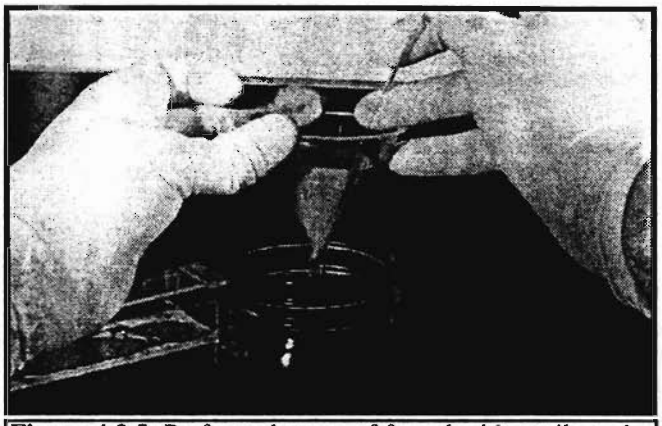


Figure 4.2.5: Perforate bottom of funnel with sterile tool.

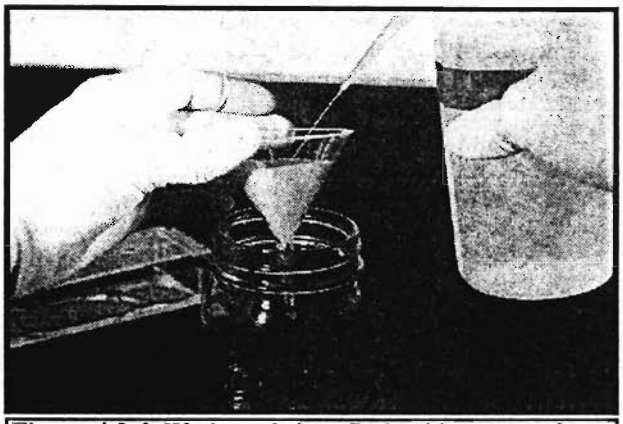


Figure 4.2.6: Wash seeds into flask with stream of sterile distilled water.

This technique is very efficient because several flasks may be sown in a matter of minutes. An alternative technique is to filter, rinse the seeds, and then re-suspend the seeds in a small amount of sterile distilled water. This may be removed with a dropper, or poured into the open flask. Otherwise, the funnel may be tipped on its side, and the seeds washed into the mouth of the flask with a small stream of sterile water.

Each technique has its own advantages and disadvantages. For flasking several species in a row, it might be best to use the filtration technique, and then store the filter papers as each one was completed in sterile Petri dishes. Transferring the sterile dishes to the flasking area would allow for several species to be completed in a very short period of time.

Some genera that tend to produce very tiny seed, such as oncidiums and vandas, make it very difficult to claim most of the seed from the filter paper, and may be best dealt with while suspended in a solution. Other genera that produce very large seeds that are easy to work with, such as cypripediums, stanhopeas, and some dendrobiums, are very handily removed from filter paper due to their large size. Many seeds are cream-colored or white, and may be difficult to see against pure white filter paper.

Note that with almost all of these techniques, the condensate that wets the surface of the media permits greater distribution of the seed. Before opening a flask, it is possible to shake a flask from side to side to encourage "pooled" condensate to evenly cover the surface, thereby facilitating seed distribution.

One last variation on this technique utilizes capillary action to germinate seeds. Small flasks (pint Mason jars) may be used, with a small (about 15 ml) amount of liquid media in them; no agar is required. The filter paper is removed from the funnel with sterile forceps, and placed inside the Mason jar. The jar is tipped on its side, and the filter paper is stuck to the wall of the jar while it is still wet. The level of the nutrient solution should just barely touch the bottom edge of the filter paper. No seeds should be washed away if the level of the nutrient solution is low enough. In this manner, the filter paper absorbs just enough nutrient solution to permit germination of the seeds. Once germination has occurred, they may be removed from the filter paper with a replate fork.

Chlorine Gas Sterilization

Although recommended only for the laboratory and individuals with training and skills proportional to the hazard, the use of chlorine gas to disinfect seeds may be of use on occasion. Arditti and Ernst (1992) suggests the following technique.

Chlorine gas may be generated in the lab by acidification of household bleach solution (recall from Section 3.6 that chlorine gas is stabilized in solution by the presence of sodium hydroxide, a strong base, in household bleach) by adding 3 ml of concentrated hydrochloric acid to 100 ml of bleach. This gas is then captured in a glass jar inside the glove box, with the seed or other material to be disinfected in the air space above the liquid. Materials to be sterilized are to be treated while held in porous material such as paper while being exposed. An exposure time of 5 to 30 minutes is suggested.

Great care must be taken while using this technique as chlorine gas is corrosive, toxic and hazardous to use. A fume hood is required for safety purposes, and this technique should never be used in the home, for obvious reasons.

Floating Seed

Some disagreement exists over what is indicated when seed floats. Some growers have characterized "floaters" as infertile seed, or seed without an embryo, and although this may be true, there is also live and viable seed that

floats. If you have access to a microscope, examine your seed before you sow it in order to determine if it has an embryo or not (see Section 6.8, Viability Testing and Seed Analysis). Bear in mind that an embryo does not guarantee viability. Even nucleated seed eventually loses its viability when desiccated, and some species may require elaborate stratification[17] techniques to germinate.

One suggestion is to make certain that your seed has been treated with an appropriate wetting agent such as detergent or a product like Tween (see Section 4.2, Disinfection of Seed). If the seed is particularly resistant to wetting, and it has proven to harbor embryos, then aggressive agitation or possibly a pre-soak that enhances the ability of the seed to absorb water may be in order. Another option is to use the "dropwise" technique mentioned above, but in order to wet all surfaces of the seed, it is necessary to "cap" the seed with a piece of cotton or filter paper, sandwiching it between two layers. This layering is used throughout the wash, and then removed just prior to sowing.

Seed that has been pre-soaked in sugar solution with a small amount of wetting agent will almost always sink, or at least have much of the initial buoyancy removed from it, making it easier to handle.

One problem with floating seed is that it is a certainty that the seed contains void spaces, which means that disinfection may be difficult. Vacuum techniques, as noted below, may be required to effectively disinfect floating seed.

Disinfection Times

Opinions vary, but 5-15 minutes for most disinfection routines seems to be adequate. Longer times may or may not damage seed; Northen (1990) notes that soaking *Cattleya* seed for several hours did not cause excess seed mortality.

Vacuum Treatment

There are some techniques that call for the use of special vacuum treatment of seed. Steele (1995) notes that drawing vacuum on seeds of many terrestrial orchids while they are being bleached enhances germination. There are several theories as to why this is so, including wetting the seed, and that it may destroy germination-inhibiting compounds in the seeds. This technique is also useful for seed that is thought to be heavily infested with contaminants. By invading the seed with disinfectant solution while drawing vacuum, it is possible to saturate the entire seed, effectively destroying bacteria, fungi, and their spores. Vacuum treatment is highly recommended with any seeds that float in order to reduce the chance of contamination.

Vacuum treatment may be performed using an apparatus similar to the one depicted in figure 4.2.7; the seed is added along with the disinfectant solution, and then stoppered. The glass frit serves as a robust filter that will permit gas (and liquid) to pass through while retaining seed. The nipple is attached to a vacuum source, and the valve turned to draw vacuum on the solution. The apparatus must be inverted, to keep the liquid from being drawn through the frit. The valve is turned, and vacuum may be disconnected. If so desired, the pressure may be restored, and the technique repeated. For a specific technique by Dutschka (pers. communication), see Section 5.1, Dealing with Contamination Problems.

When satisfied the seed has been disinfected, vacuum may be used to remove the disinfectant through the frit, being certain to use a trap if necessary to prevent liquid from entering the pump. The seed may then be washed with sterile water, and sown in flask.

This apparatus is not commercially available. A heavy-walled test tube with a single-hole stopper may be attached to a vacuum source for a much more simple apparatus, or a glass-blower should be able to make a complete one. A chromatography column may be quickly and easily modified for this purpose, and assemblies complete with stopcock and frit are commercially available, but expensive ($45). They can be cut to the proper length, then have the cut end flame-polished, and stoppered for use. It must be explained to the glassblower that the apparatus must be capable of withstanding vacuum. Moreover, whenever drawing vacuum on ANY glassware, it is important to protect the eyes and skin from injury in the event of failure. Caution must be taken not to drop evacuated glassware, or to use damaged glassware when drawing vacuum, to pre-

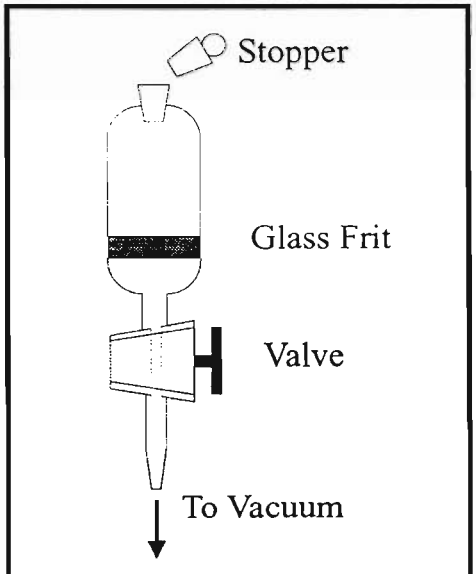


Figure 4.2.7: A small vacuum-sterilization apparatus, after Dutschka (1999).

[17] Stratification involves the moist preservation of seeds in or on media for a period of time, which may enhance germinability when used alone or in conjunction with cold treatments, pH changes, etc.

vent catastrophic failure and potential injury.

Documentation

Once the flask has been sown, it is crucial that the contents be properly identified. If possible, print up labels beforehand, attach them to the flasks either right before or immediately after sowing. Another solution is to paint a white rectangle on the flask using commercial typewriter correction fluid, and writing on it when it is dry. All pertinent information, such as the species or cross, date, and type of media should be on these labels. Some growers prefer to use a code, and then write the details in a lab notebook. In either event, a lab notebook with meticulous notes as to all of the elements involved will be invaluable weeks, months or even years later when they are required. "Was this flask *Cattleya aurantiaca*, or *Cattleya amethystoglossa*...?" is not a question the grower should have to ponder for any longer time than required for a glance at the label or look into a well-kept lab notebook. It cannot be stressed enough that careful note-taking and maintenance of a notebook are some of the most important aspects to proper horticultural technique when it comes to orchid seed germination. After all, how are we to know if a particular genus does or does not fare well on a given nutrient media? The collection and exchange of successes, and particularly failures, makes our work more powerful.

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4.3 Green Capsule Technique

This section on the use of green seed pods (more correctly: capsules) is provided for those who wish to use these techniques. It is suggested that until there is additional evidence that shows whether or not viruses are transmitted from parent to seedling in this manner that these techniques be used only out of necessity, i.e., species that are remarkably sensitive to chlorine or other disinfecting agents.

Green capsule technique, or embryo culture, presents the orchid grower with a number of advantages. Its proponents state that it is much easier, more reliable, and yields higher germination rates than techniques involving the use of dry seed. Also, embryo culture may circumvent the requirements for cold treatment of some temperate-growing orchids (Light, 1990).

Still, this method is not without disadvantages. The most important issue is when to harvest the green capsule, because if it is taken from the parent plant too early, the embryos may not be viable. There are general guidelines for the maturity of seed capsules, but these are just that: guidelines. Given the variability in culture, climate, and other factors, capsules may mature much more quickly or slowly than tabular data may indicate. It is clear that capsules for use with this technique must be carefully examined prior to utilizing them, and cracked or opened capsules should be relegated to techniques using disinfectants.

Thus, timing is an important issue when performing embryo culture. Fortunately, although maturation times are important, they are not critical for most species because slightly immature seed may germinate as well or better than dry seed. Therefore, we may successfully deal with these small differences that are present in many cases.

Technique:

As mentioned above, the whole purpose behind using green seed capsules is to avoid the disinfection of dry seed via a dunk, wash, and rinse cycle. Subsequently, the outside of the capsule is quite "dirty" from a microbiological perspective, and must be disinfected.

Capsules must be labeled for identification once removed from the parent plant. "Sharpie" permanent markers, with their indelible ink, tend to work well for these purposes, and you may write directly on the capsule if it is large enough. The capsule is removed from the parent plant before it reaches maturity, using a sterile knife blade. This capsule is then moved into the flasking box, where its exterior must be disinfected chemically.

Techniques vary, but either alcohol (70% ethanol is best) or bleach (30% bleach solution in water) may be used. Soak the capsule in either solution for 5 to 15 minutes, scrubbing periodically with a toothbrush. If using alcohol, the capsule may be flamed. Some growers use one solution to disinfect the exterior of the capsule, peel off the skin and then place the peeled capsule in the other solution. Remove the capsule from the solution, and place it on a sterile surface, such as a glass sheet that has been soaking in sterilant, or a sterile layer of aluminum foil.

Use a sharp, sterile blade to cut the end off the capsule; particularly tough capsules may require the use of a scalpel, X-Acto, or other hobby knife, or even a razor blade clamped in the jaws of a hemostat for greater safety. Another option is to cut a "window" into the side of the capsule. The seed will be visible inside. Hold the capsule up, with the open end directed downward to prevent any solution from running inside. Use another sterile tool such as a microspatula to remove a tiny amount of seed; depending upon the size of the seed, an amount from the size of the head of a pin to a small pea may be removed. Use the tool to transfer it to a flask, and drop it inside. Repeat with subsequent flasks.

If using tools that are chemically sterilized, be certain to direct them into the capsule pointing upwards to prevent liquid from entering.

Another technique involves cutting the capsule along its length, into four or more sections. From these sections, it is possible to remove seed, which is then placed into flask. The former method (cutting off the end) may be easier for species with small capsules, whereas the latter technique may be best for those with larger capsules. Be careful how aggressive you are with removing seeds; some have suggested that scraping seed from green capsules may be a source of virus transmission from parent to seedling.

Some growers take the sections, and then chop these into pieces, and place these pieces into flask. If centered in the middle of a sterile flask, the seed may disperse themselves, but it will be very uneven, and the center portion of the flask will be very crowded. This technique is very simple, with the benefit of being quick and easy.

With any of these techniques, a small sample of seed should be recovered for microscopic examination to determine if it is fertile (i.e., that embryos are present). If so desired, a tiny amount of seed may be transferred to a microscope slide, which is then handed to a helper either directly (if using a laminar flow hood) or through an air lock, if using a glove box. This way, if embryos are absent, materials and effort may be saved.

Those species that require cold dormancy *in vitro* should be stored in refrigeration as long as is required.

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4.4 Variations on a Theme

Up to this point, a number of concepts have been introduced and covered to a certain degree. This next section is not meant to confuse, nor is it necessarily suggested that growers "must" or "have to" adopt these variations. Orchid growers and, indeed, horticulturists in general are fickle yet precise and creative individuals: dissatisfied with available or "works for me" techniques, flaskers have developed their own techniques that suit their own talents and tools that are available. This section is meant to introduce some of these concepts.

Use of Steam During Flasking

Jordan (1965) reports success with the use of steam as a sterilizing agent with respect to open flasks and stoppers. Specifically, the author suggests that a "common water kettle" used as a source of steam could provide as a sterilizing "blast" within which the stopper, once removed from the neck of the flask, could be moved. Much in the same way that some growers will pass the neck of a flask through a flame (literally 'flaming' it) quickly in order to assure sterility when it is being opened or resealed, the author suggests that the neck of the bottle can be steamed while

introducing seed with a sterile tool. As a bonus, the resulting condensation is said to help distribute the seed across the surface of the agar.

Richter (1969) suggests that it is possible to sow seed over a pan of boiling water, such that all work is performed in the steam. Still air is required for this technique, as is caution while working with hot water and steam.

Techniques for Introducing Seed

There are several techniques reporting the simplification of the flasking process by facilitating introduction of the seed. Obviously, reducing or eliminating contamination routes during this step would be desirable.

Higgins (1960) suggests that seed be introduced into flask while suspended in a volume of hydrogen peroxide sufficient to produce a sort of slurry–a high concentration of seeds suspended in a nominal amount of liquid. This is then drawn into a 20-gauge hypodermic needle, and injected into the flask through the cotton plug. This technique may be enhanced by phlegmatizing the seeds with sterile ten percent orchid agar solution (a solution of 1:10 agar gel in water, not 10% agar in water), added to the liquid within which the seeds are held. This step prevents seeds from clumping and obstructing the hypodermic. At elevated temperatures to decrease viscosity, as much as 50% agar solution may be used.

A 20-gauge needle is quite fine, and individuals may have greater success using larger needles with larger seed. Although 20-gauge may pass very fine seed such as that of oncidiums and vandas, it will almost certainly clog with some of the *Dendrobium* and *Epidendrum* species that can be much larger.

Higgins also mentions that using a 30 ml wash bottle, into which seeds and disinfection solution are injected, will help when seeds need to be washed. Once the seeds have settled out of the solution, the solution on top is removed via needle, and distilled water with 10% agar solution is injected, homogenized, and then extracted by needle for injection into flask. It goes without saying that the wash bottle and implements that are introduced once the disinfection solution is removed (such as the needle) must be sterilized as well. Before injection, a "small quantity" of chlorine solution is to be added to the cotton plug of the flask.

Fanchaly (1961) uses a similar technique, but instead of contending with rubber stoppers and cotton plugs, he suggests the use of a commercially available foam plug, which survives autoclaving and is capable of performing the tasks of other stoppers. This information is from 1961, and it is unclear whether similar products exist today for cell culture.

Houck (1979) goes a step further, fashioning his own septa from patches of rubber, which are in turn cemented over holes punched in the lids of flasks such as canning jars. This patch of rubber replaces the cotton plug, and is glued over the hole such that a syringe needle can be used to introduce the seed into a flask, similar to the techniques used by Higgins and the technique used by Leroy and Pike (below). A 3 mm (⅛") thick rubber or neoprene disc is cemented to the outside of the flask, using a rubber to metal cement. filled flasks must be sterilized with the lids "loosely tightened," and further tightened immediately after the pressure cooker is cool enough to open. Houck mentions that he also swabbed the rubber patch with 10% bleach solution before injection.

Rodder (1998) suggests using a syringe for disinfecting seeds in the following manner. Remove the plunger of a small (5-20 cc) syringe and add seeds. Replace the plunger and bring it down to the 1-2 cc line. A small ball of cotton is placed in the hub of the syringe needle with forceps, and placed on the syringe. In this manner, the seed is retained within the barrel of the syringe, and may be exposed to disinfectant and wash water by alternately drawing it up through the needle and expelling it. When done with disinfection and wash, the needle is removed and the seed is expelled into the interior of the flask.

A technique similar to Rodder's involves the use of a small tube that is capable of being fitted tightly to a syringe. A length of flexible vinyl tubing about 2-5 cm (1-2 inches) long is used to hold a small quantity of seed sandwiched between two small balls of cotton. A syringe is filled with disinfecting solution, then coupled to the tubing. A small amount of solution is pushed through the tubing, saturating the seeds and cotton. More solution is periodically advanced through the tubing until the desired disinfection time is achieved. The syringe is then removed and replaced with a sterile syringe carrying sterile water. The seed is then washed by forcing this water past the seeds. Once washing is complete, one of the balls of cotton is removed with a sterile tool, and the seed is forced out of the tubing. This extraction may be done either with wash water, or by removing the syringe and using a small, sterile tool to push the seed and remaining cotton ball out.

OSP Technique

At the OSP, we use a slightly different technique. Although any of the sowing techniques specified above may be used, we have been satisfied with the system described below, which permits sowing between five and ten species at a time with relative ease. Vacutainer or similar small test tubes are selected, one for each species. The tubes and

stoppers are left to soak in 50% vinegar solution between every use, which removes calcium hypochlorite stains, and prevents contaminating flasks with foreign seed by destroying any that might remain. After washing and drying, several tubes are alphabetically labeled using a chemical-resistant pen. One species of seed is added to each tube and the name of the species is carefully recorded in the lab notebook next to the letter on the tube.

Pint jars or baby food jars are used as the mother flasks; a baby food jar uses about 30 ml of media, allowing over 30 mother flasks from a single liter of media. Moreover, it is possible to fit many more baby food jars in an autoclave or pressure cooker than other sizes of flasks. Although crowded, protocorms will not be lost if replated promptly.

Flasks to be sown are placed in the glove box or flow hood, and carefully covered in disinfectant spray. The spray is made with 16 parts distilled water, 2 parts bleach (5.25% sodium hypochlorite), and 1 part vinegar (5% acetic acid). After spraying the flasks with disinfection solution , about 1-3 ml of saturated calcium hypochlorite solution is added into each test tube. Two drops of a dilute wetting solution is added to each tube. The tubes are stoppered, and shaken to wet all seed. When the last tube is sealed, a timer is started.

Every minute or two, each tube is inverted or lightly shaken. While disinfection takes place, the stoppers are removed from the tubes, sprayed with hypochlorite solution, and returned. This is repeated a few minutes later to assure sterility of the stopper. Midway through the procedure, the tubes are placed into the glove box, and the outsides of the tubes are sprayed. At this point, the tubes can be kept in a water glass or half-pint Mason jar to keep them together.

Once the desired disinfection time is achieved, the solution is removed with a sterile dropper, and replaced with sterile wash water. If the seed settles quickly, the precipitated seed may be drawn up with the dropper and ejected into a sterile flask. If it does not settle quickly, the liquid is generally homogenized, drawn up all at once, and ejected into flask. If so desired, some liquid may be drawn off later when the seed has settled.

Before opening flasks for seed introduction, rings are removed from the jars, and the neck is washed down with disinfectant solution. A different dropper or pipet is used with each species of seed to prevent cross-contamination.

This technique has proven to be quite satisfactory for us, with a low contamination rate. When contamination occurs, it is generally because the seed was heavily contaminated to begin with (most often from capsules that had cracked open, and were kept moist too long). A second sowing, preceded by a sugar soak of the seed (see Section 5.1, Dealing with Contamination Problems) may remedy the problem.

Small-volume Flasks

Vacutainer tubes are commercially available at the time of this writing. They are commonly used in hospital labs for blood draws. Leroy and Pike (1976) suggest that they may be used for flasking orchid seeds in the following manner.

Vacutainers are sterile when new, and have much of the air removed from them before shipping (which is what permits blood to be introduced without having to remove the lid). The authors suggest filling each Vacutainer with a small amount of nutrient agar, which varies depending upon the size and volume of the tube, which is then autoclaved. A syringe needle is to be placed through the lid of each tube to serve as a pressure release valve as the pressures change. Cool the tubes at an angle, to increase the available surface area, and remove the needles when cool enough to do so.

For introduction of the seeds, a syringe without a needle is sterilized and used to draw up a volume of seed, suspended in sterile wash water. This is then injected through the Vacutainer lid, again piercing the rubber stopper with the needle to do so. The author makes no mention of sterilizing the exterior of the lid. The number of seeds that can be sown at one time is relatively small. This may serve as an acceptable technique for determining viability.

In this age of drug paranoia, it may be difficult to get suitable syringes and needles, and moreover, they are a dangerous presence in any household, particularly with children. Still, tuberculin syringes can be obtained with a prescription, and larger syringes may be available from veterinary supply stores with absolutely no paperwork at all.

Tubes are excellent for the propagation of orchids, but Vacutainers may prove to be too small for many species. If you wish to use larger tubes, a number of manufacturers supply tubes suited for plant tissue culture, including Sigma Chemical Company. Sigma's C-1048 and C-5916 glass tubes are 20mm and 25mm in diameter, respectively. They are to be used in conjunction with the correct closures, whose stock numbers depend upon diameter and color (see Appendix III, Sources). Using a cotton plug in conjunction with the plastic cap and an aluminum foil cap on top of that will significantly reduce contamination.

Also suitable as culture vessels are baby food jars. They are available in at least two sizes, and can be purchased from suppliers by the hundred if you don't already have a friend or relative whose infant is going through bottles. Magenta manufactures a special lid for these, called "B-Caps." These lids are prone to losing water rapidly, and cultures kept in baby food jars tend to dry out quickly with these lids. Baby food jars capped with B-Caps are also

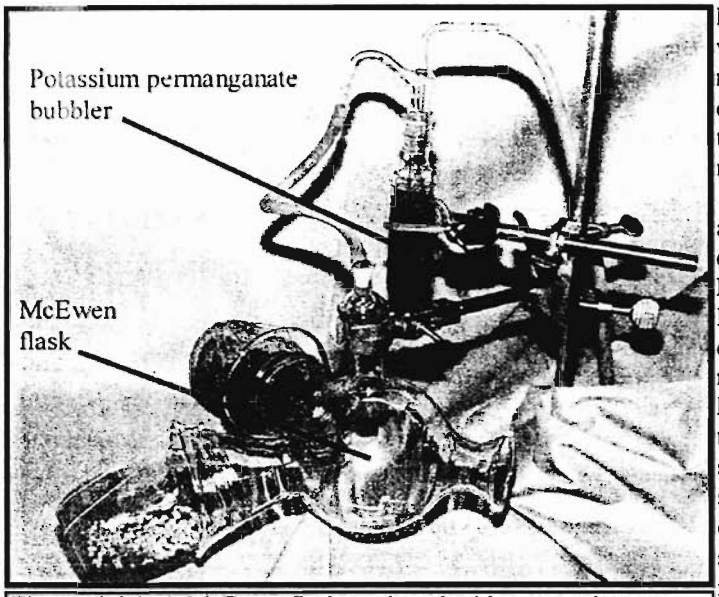

Figure 4.4.1: A McEwen flask equipped with a potassium permanganate bubbler.

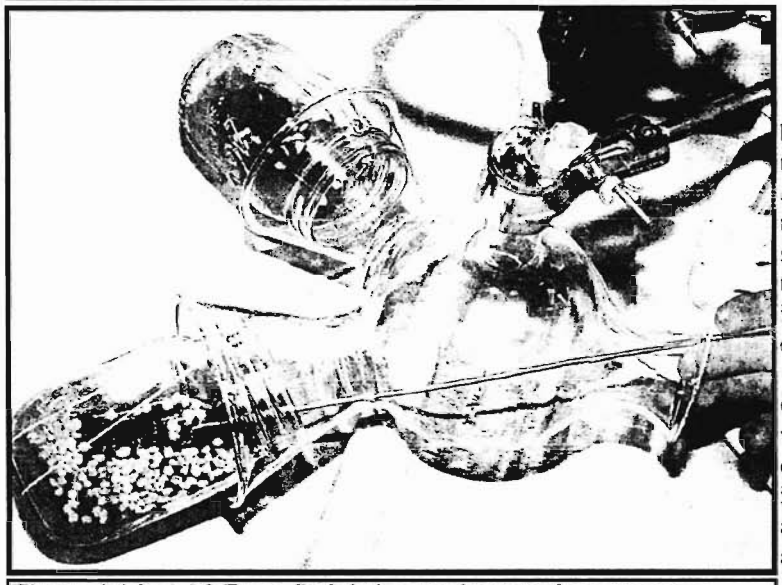

Figure 4.4.2: A McEwen flask being used to transfer protocorms (simulated) from "mother flask" to "replate flask."

liable to become contaminated. Sealing the flasks with a bit of plastic film such as that used on refrigerated food is suggested to keep them from drying prematurely and help prevent contamination. Petri dishes may be secured in a similar manner, greatly extending their usable life.

Other Magenta products include polycarbonate squares (GA 7), special closures, and trays for different purposes. Both Sigma and PhytoTechnology (see Appendix III, Sources) sell Magenta components; although these are often an expensive alternative to glass, they come highly recommended by Kuhn (1981), a director of the Eric Young Micropropagation Centre. Their properties include superior growth performance due to size and shape of the vessels, as well as being strong, durable and "have taken hundreds" of cycles of autoclaving, culture, and reuse without significant change in properties. They also optimize use of space due to their square shape and are commonly used in commercial micropropagation of many plants.

Sterile Area Alternatives

Certainly the construction of a glove box or laminar flow cabinet may be prohibitive in terms of cost and space, and not everyone has a 20-gallon long aquarium sitting around the house. Thompson's "glass sheet" technique has its place for growers that wish to try it, but there are those that will insist on home flasking within an enclosed environment.

One alternative presented by Bryder (1961) is the use of a polyethylene enclosure, which can be sterilized so that the contents can be kept sterile much in the same way as in a glove box. In essence, it is a "glove bag," and the technique is similar to working inside a dry-cleaner's bag.

Bryder suggests that polyethylene bags of the type she uses will not withstand autoclaving, but will survive boiling; another option would be to chemically disinfect, either by itself or by working with a wet sheet soaked in disinfectant at the bottom. Bryder's technique involves manipulating the contents inside the bag from the outside–admittedly a clever adaptation, but this is not very practical for those that would wish to do any significant amount of flasking over the long run.

One company, called I^2R (see Appendix III, Sources), produces several different types of polyethylene glove bags that are made with integral gloves. They are reusable, but expensive (available as a box of six, the bags run from $97 to $198 a box, depending upon the size and models desires, 1998 prices). They are typically used for air- or moisture-sensitive work, and are accordingly produced with gas couplers and a large equipment sleeve, through which equipment passes before it is clamped shut. Although I have not seen these used for flasking, their being constructed of inert polyethylene plastic should permit chemical sterilization. Having used these glove bags, it is worth noting that delicate manipulation is made very difficult by the size of the gloves, as well as the slickness of the plastic.

Jensen (1963) adapts this idea for replating, by using a plastic bag that has been soaked in bleach as a chamber for replating. The grower works from the open end, while two flasks are "docked" through holes in the opposite end,

allowing for sterile transfer from one flask to the other. Northen (1990) and Fitch (1978) suggest the use of a McEwen flask (Figure 4.4.1), a piece of glassware used to transfer seedlings from mother flask to replate aseptically when docking Erlenmeyer flasks. The McEwen flask consists of a sphere with three ports: two accommodate flasks (one replate, one mother), and a third for the operator to insert a tool. From this third port, the operator can move seedlings from mother flask to replate without undue exposure to potential contaminants (Figure 4.4.2). Fitch (1978) notes that positive pressure is maintained inside the flask, with air having been passed through a solution of potassium permanganate before entering the McEwen flask to further reduce contamination. The McEwen flask is available through the Orchid Seedbank Project (see Appendix III, Sources), customizing if necessary to meet your requirements. The McEwen flask in Figure 4.4.1 is fed air, bubbled first through a disinfection solution of potassium permanganate, producing slight positive air flow that helps ensure the sterility of the air within the transfer flask, as well as the two flasks docked to it. Although not required, it may enhance the quality of work.

One major drawback of the McEwen flask is that it requires the use of very long tools- upwards of 18 inch (45 cm) replate forks if used with quart jars. As these are not commercially available, it will be necessary to build them yourself, or have them custom-made. Nickel, stainless steel, and glass are all acceptable construction materials, with metals being the best choice as they are not as fragile.

Lid Seals

We have mucked our way through many variations on how to equalize pressure on a flask despite the rigors of thermal sterilization while preventing contamination of a flask. There are two basic types of vents: tortuous pathways (such as cotton), and membranes. Stoppers and lids wadded with cotton or rubber are satisfactory. Mason jar lids are easily vented by perforating the lid with a punch or awl, and then tightly stuffing the hole with cotton. Other seals, such as membranes or polymer strips, are available. Unfortunately, few of these are inexpensive and sufficiently versatile to use in plant tissue culture applications.

Band-aids that allege to be "breathable" have been used by some growers. It has been my experience that some of these do not breathe, for several reasons. When flasks are sealed with these band-aids, autoclaved and lids screwed down while still hot, flasks may be stored for several weeks, after which they still retain a low pressure when opened, indicating to me that the "vent" has not permitted any air to get into the flask. While these bandages may indeed be "breathable," they do not exchange significant quantities of gas after having been autoclaved.

Others have used different types of bandages with varying degrees of success. Different types of surgical tape evidently exclude contamination, but it is not clear how well these breathe, if at all. One tape recommended for this purpose is Leukopor surgical tape, which allows at least some gas exchange. Several growers have suggested that 3M Nexcare Active Strips may be used as vents. These are round foam bandages about 25 mm (1 inch) in diameter, sold as "Sterile Spots" in most supermarkets. Some suggest using one on the inside and one on the outside (Fournier, pers. communication). These have been in use at the OSP for over a year without problems.

We await the production of an inexpensive, autoclavable, adhesive vent for plant cell cultures. Although similar products are available, some are expensive, and do not fit all the requirements. Sigma sells a "sun cap" closure designed for baby food jars, either as foil or polypropylene film with a 0.02 um filter disc in the center, and while these are not very rugged, some growers report success with using them as vents.

The importance of venting flasks is not clear. It has been my experience that at least some flasks seem to do fine without them, but this is admittedly in the absence of an experimental control with vented flasks. It seems unlikely that vents move a great deal of air, given that the only significant exchange will occur only when the barometric pressure or temperature changes. Even then, the volume exchanged is not large. People have suggested that carbon dioxide is essential for growth; if this were the case, the small (<400 ppm) concentration of carbon dioxide in the air would quickly be exhausted as the carbon were incorporated into plant tissues. Orchids in flask clearly obtain the majority of their carbon from the media, not from atmospheric sources. Whether carbon dioxide is a requirement for any orchids in flask is not known. Arditti and Ernst (1984) note that germination of certain species is as good or better in unvented flasks, while venting produces better seedling growth. Buildup of other gases (ethylene, for example) that may be detrimental may occur without venting.

Flasks should be vented, except for unusual situations such as an experimental group with certain terrestrials, for example.

Miscellaneous

An interesting article from a German flasking perspective by Lucke appeared in the February 1975 issue of the AOS *Bulletin*; although perhaps not particularly relevant to modern techniques with respect to this date of this article, it makes for an interesting read. Techniques have changed somewhat since then, but it is still of value.

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Chapter V: Post-Sowing

5.1 Dealing with Contamination Problems

As discussed before, contamination is a problem best dealt with at the source. If you are having problems with a large number of your flasks being contaminated, it is important to carefully review your procedures. Note that growers new to aseptic technique will have problems with contamination; do not be discouraged if your initial attempts lead to little more than mass propagation of large quantities of fungi and bacteria. It happens to everyone, and by modifying techniques and with a little practice, the likelihood of contamination can be reduced dramatically. Contamination may reveal itself in many different ways. Several exciting forms of bacterial or fungal growth may manifest themselves in the form of powdery, green-gray growths similar to those found on bread or old food in the refrigerator. They may be slimy, powdery, lumpy, or smooth, and can be any color of the rainbow. In any event, they will certainly show themselves long before any seeds will germinate; colorful raised welts that expand rapidly will indicate something unpleasant has happened, and that you will lose the flask in all likelihood. Particularly nasty is the "white slime," which resists even the most aggressive decontamination techniques. Some potential solutions to contaminated flasks will be presented, but these are last-ditch efforts, and should not be tried unless the seed is particularly unusual or valuable.

The first source for contamination is improper sterilization of the flasks. If possible, flasks should be autoclaved several days prior to use because the components in media do not decay with any great speed when stored in a sterile environment. Accordingly, flasks can be prepared several days or even weeks in advance, and stored prior to use. In this time frame, any contamination should become evident. Inspect a flask carefully before introducing seed: one does not wish to go through the trouble of sterilizing the surface of the seed and flasking it, only to find out that the flask was contaminated from the onset.

It is worth noting that maintaining a variety of flasking media may be very desirable if you are doing work with particularly rare or valuable seed. Since seed whose germination characteristics are poorly known may germinate readily on one media, and poorly on another, it will often be of use to a good flasker to have on hand a variety of media. Thus, preparing flasks reasonably far in advance of their use (weeks) is not only prudent from a microbiological standpoint, but with respect to good culture as well.

If flasks are contaminated from the start, one must first check to make certain the seals on the flask are intact. If the cotton plug is secure and the foil caps have not been breached, then it is most likely that something is wrong with your media preparation regimen. Provided that thermal disinfection is the only method employed to achieve sterile media (which will almost always be the case), then you should make certain that your autoclave is achieving a sufficiently high temperature under adequate pressure for a long enough time. Check your times and temperatures again. You might even consider using autoclave verification strips, available from lab supply companies, that change color after a cycle has successfully been completed. Make certain that you are not attempting to sterilize large volumes of media that were too cold when placed in the autoclave. Media should be in small volumes in individual flasks if possible. If autoclaving a large volume of media, it should be very close to boiling when placed in the autoclave. However, this technique is less desirable than having it dispensed and sterilized in individual flasks. Make certain you are sealing the flasks shortly after they are removed from the autoclave, since you must leave them loosely capped or stoppered during sterilization.

Most contamination occurs shortly after sowing, for obvious reasons. Dust or spores may be entering the flask in air currents; if not already using a box or a laminar flow hood, one should make reasonable attempts to stop all air circulation across the work space, and to insure that the room is as free of dust particles as is reasonably possible. If using the glass plate technique, one should expect the contamination rate to be higher than with a glove box or a flow hood; all the same, if you are willing to accept a certain amount of loss just for the experience of sowing seed, then this may be what you need. If any tools are being used to disperse the seed, or to transfer them into the flask, they must be absolutely sterile prior to introduction into the mouth of the flask.

The most likely source of contamination is from improperly disinfected seed. Make certain that your disinfection solution is of the correct strength, and that your disinfection times are adequate. If present, separate chaff (such as pieces of the seed capsule that may have fallen in) from the seed; this may require a well-lit area with a magnifying lens. Ultra-fine tweezers and the very tip of a sharp hobby knife will help remove these unwanted pieces.

Some batches of seed may prove to be heavily contaminated, or have organisms that are resistant to hypochlorite solution. This seed can be pre-treated with a short (overnight) soak in a sucrose (table sugar) solution, and incubating the solution at 37° C, or about 98° F, will help. Incubating at room temperature is also acceptable. Dissolve 1 teaspoon (5 cubic centimeters) of table sugar in 1 cup (227 milliliters) of water. Place the seed in a small test tube with a small volume (1-2 teaspoons, or 5-10 ml) of this sugar solution, and let it sit overnight. Rinse once with dis-

tilized water, and then proceed normally with the hypochlorite solution. If so desired, increase the duration of exposure to the disinfection solution.

Another technique, suggested by Zytaruk (pers. communication), is to soak seeds in a half-strength solution of saturated sugar, i.e., producing a saturated solution of sugar, and then diluting it 1:1 with water. A tiny amount of wetting agent is added, usually in the form of a small amount of a dilute mild dish soap solution. A few drops of this liquid in a test tube plus the seeds is enough. Zytaruk then suggests stoppering the tubes, and then mixing and leaving for 2 hours. Agitating occasionally (three or four times) before disinfecting is suggested. This technique has proven reliable for Zytaruk, who notes that seed almost always sinks after this treatment, making it easier to handle.

What sugar treatment does is cause recalcitrant spores to germinate, and make them more vulnerable to the hypochlorite. As a result, many more bacteria and fungi will be killed, instead of being introduced into the sterile confines of the flask. It is worth noting that some growers pre-treat their seed using this technique for most or all of their flasking, particularly with seed that must be subjected to a lower concentration of chlorine due to sensitivity. Techniques involving sugar pre-treatments are highly recommended for the novice, or when using dried seed of unknown origin that has been shipped or previously handled.

Some contamination may occur weeks or months after a flask has been sown; this is an unusual occurrence, and there are several different explanations. It is possible, but unlikely, that spores may remain dormant for this period of time, and "just happen" to germinate for whatever reason. It is also possible that spores may be within the confines of a flask, such as the top of the inside of the flask, and detach or drift from wherever they are, and possibly land on the media, where they sprout. Infection due to mites (see below) may cause this type of contamination.

This is one reason that flasks that are shipped via the mails should have only seedlings that are capable of surviving outside of a flask: flasks often receive rough treatment during shipping, and occasionally become contaminated as a result. If they do become contaminated, de-flasking the seedlings is probably the only option available, which will require fat, healthy seedlings capable of surviving outside of the flask. As discussed before, slants (particularly those prepared with a slightly higher amount of agar, say 9 or 10 gm per liter) may survive the mails a little better than larger flasks. An exception to the above rules would be when shipping immature seedlings destined for replate at the receiving facility. Even if jumbled, plantlets may be replated upon receipt.

As mentioned above, mortality in contaminated flasks is exceptionally high. By the time you first see the unsightly contamination, the critters involved have almost certainly gained a foothold and started to reproduce. Often, this involves ejecting spores or live organisms that will happily colonize the nether regions of what would otherwise have become a happy family of orchid seedlings.

Still, desperate times call for desperate measures; if the seed is particularly valuable, and the colony is small and slow-growing, it is possible to attempt to excise the contaminated media and treat the area with a disinfectant in the hope that all is not lost. It is worth reminding the reader at this point of the old adage where you should not place all of your eggs in one basket; valuable seeds should be spread among multiple flasks using a variety of techniques, if possible, and distributed upon several different media such that desperate measures need not be taken. Test a small amount of seed first in order to determine if it is heavily contaminated before sowing the entire lot.

To excise contamination using your flasking box (laminar flow hoods may not help the situation any, particularly if the colonies have set spore), open the lid and insert a microspatula with the blade bent to help form a cutting tool that works like the mechanical scraper on an ice cream scoop. Remove the colony, as well as a block of media around the colony itself. It is no different than treating an infected plant in that the larger the block you excise, the more likely it is you will succeed in defeating the infection. Be careful not to drop any pieces of infected media on the way out.

At this point, you will probably need to treat the hole that has been produced. Introducing a 10% bleach solution will help destroy any remnant critters, and the bleach will slowly produce sodium chloride (table salt) as a result; although the salts are not conducive to seedling growth, a small amount will not hurt the seedlings to an appreciable degree (Arditti, 1967). Calcium hypochlorite will also work. Hydrogen peroxide has the advantage of decaying to nothing but water and free oxygen, and is harmless to the seedlings that will appear if your strategy succeeds. Despite this, hydrogen peroxide is less aggressive (particularly in its domestic 3% strength) than either hypochlorite solution.

Consan 20 and Physan, which contain quaternary ammonium compounds or "quats," are said to have a disinfecting capacity, and may be used in a similar manner to treat the agar to try to stem the infection. Virkon S, a relatively new product in the United States, has been used to decontaminate flasks. Visibly contaminated material is excised, and a small amount of 1% Virkon S is added to the flask, and then decanted after most of the seed has settled out (see also Section 6.5, New Directions in Seed Disinfection).

I have had good success disinfecting contaminated flasks by excising the affected section, then misting the interior of the flask with a spray bottle filled with 1.4% Virkon S solution. Decant any gross excess, but leave enough solution to fill any holes. Inspect the flask every 8 hours afterwards, and repeat if necessary.

Another option that may be of use with widespread, slow-growing infections, is to take Virkon S powder (2-3

cubic mm, or literally a pinch) and add it to the media. Using liquid present on the surface of the media, dissolve the powder and slosh it around until the entire surface is saturated. As this will certainly spread contamination, make certain the liquid turns orange-yellow with the powder, and get it up onto the walls of the flask. If there is not enough liquid, use a spray bottle to deliver a small shot of 1-2% Virkon S solution to get it going. When repeated 24-72 hours later, recovery of contaminated flasks is generally successful, but often kills the developing protocorms or seedlings.

It has been reported that a solution of 2.0 to 5.0 grams of dichloroisocyanuric acid (DCCA) in 1 liter of water may be used to salvage contaminated seedlings. Deflask the protocorms or seedlings into a sterile glass container with this solution. Gently agitate the vial every few minutes for 10-15 minutes, and then replate the plantlets onto fresh media. The low concentration of DCCA is low enough that many seedlings are not harmed, and there is not a radical pH swing as there is with calcium or sodium hypochlorite. Much lower concentrations of DCCA (0.75 grams per liter) may be used if the exposure time is increased.

Another technique that has been used in the past for destroying individual bacterial cultures is to take the flask outside on a bright, sunny day and sterilize the affected areas by focusing sunlight using a magnifying lens and burning away the organisms (Yearrow, 1979). It has also been suggested (possibly even seriously) that a laser could be used to do the same thing. It is worth noting that this technique would best be used on flasks that are constructed of high-quality, smooth glass such as Erlenmeyer flasks, rather than Mason jars, due to the distortion of the focused light by irregular glass.

Aggressive Efforts

When first starting out, or if you encounter significant problems during flasking, you can use a spray bottle with 5 or 10% bleach solution (or 1-2% Virkon S) to keep down contamination. For example, before opening a flask, the entire work surface should be coated in a thin layer of liquid disinfectant. Between removing the ring and the cap from a Mason jar, you may wish to spray or wipe the newly exposed surfaces with disinfectant. While doing replate, it is acceptable with some types of plants to use the spray bottle to deliver a single shot of disinfectant to the flask before closing it back up. Similarly, if the inside of the cap or the stopper to a flask touches a non-sterile surface, it is acceptable to spray the potentially contaminated surface[18]. It should be noted that some orchids dislike this, and may be burned by the high concentration of chlorine. Plants in later stages of development with large (>1 cm) leaves can be damaged by this treatment. Plantlets in flasks that are dry, and have little or no moisture riding on the surface of the media, may be injured more readily than those in flasks that are wet.

For these reasons, batch washing with seeds where a large volume of disinfection solution is delivered onto the media with the seed may be useful for new growers before they perfect their technique. Compounds such as Virkon S, and calcium or sodium hypochlorite all decompose to add a little extra chloride into the media, which doesn't seem to harm most common genera. Salt-sensitive species may be put off by this, and the use of a rinse is advised when sowing unfamiliar genera.

Determining Origins of Contamination

How contamination manifests itself may tell the grower exactly what is wrong with the technique, and indicate how it should be fixed. Nutrient agar was used for culturing microorganisms long before its use as an orchid culture media, and as a result, different bacteria and fungi will display different growth patterns. First, examine the organisms present. If there is more than one point of infection in a flask, does it appear to be the same organism? If there are organisms growing within the media, under the surface, these are anaerobic organisms that somehow survived the rigors of disinfection: check your autoclave or other factors in media sterilization. If there are a multitude of foci, or centers around which infesting organisms have started to grow, then we must assume gross contamination.

Gross contamination indicates that either your seed is heavily infested (often from seed that has been left in green capsules for too long after they have opened), or that you have used a solution or tool that was improperly sterilized, or later became contaminated. With infested seed, it is sometimes possible to recognize colonies that started around individual seed–a strong indication that this type of contamination has occurred. With other types of infection, the results may be sporadic, or colonies will form in the spaces between seed on the agar. Contaminated wash water, for example, would do this.

One single organism, growing in several different flasks, will indicate something has gone wrong over a period

[18] Growers will be well-advised to keep a number of foil-wrapped, pre-sterilized replacement lids in the hood or glove box to efficiently replace potentially contaminated ones.

of time, and the grower would be well-advised to find whatever recurring theme is responsible. Obviously, seed from the same lot placed in several different flasks, resulting in remarkably similar contamination characteristics, should indicate something may be wrong with the seed. If a particular disinfection regimen is followed by high contamination, then the technique should be reviewed. If the grower cares to determine the source of contamination (and one should), deducing precisely what happened from the evidence presented will help prevent similar incidents in the future. Again, meticulous note-taking is your best tool.

It is worth noting that contamination in replate flasks occurs most often just inside the flask, where the media is closest to the cap. Caution should be exercised in future efforts if this is where your infection most often occurs, making sure liquid is not introduced from contaminated hands, gloves, tools or lids.

MacDonald (pers. communication) experienced problems with a red slime contamination. The problem was traced back to his seed disinfection solution, which was created with tap water containing chlorine-resistant organisms. He reports that using purified bottled water to make up his disinfecting solution rectified the problem.

Thompson (1996) suggests that the type of infection often relates to the source. Slimy, yellow-gray bacteria result from contamination from the hands, i.e., touching the media. Multiple tubes with the same type of fungus result either from "an intense local source of sporulating fungal hyphae" such as if your sowing area were to have been contaminated with a dirty flask from before, or from heavily contaminated seed. The occasional tube infected with several different types of organisms may result from airborne spores.

Treating Plugs

Bergman (1996) suggests adding a drop of copper sulfate solution to cotton plugs, as well as to "the crack between the neck of the flask and the rubber stopper," citing an inhibition of microorganism growth in this manner. The recommended solution consists of approximately 30 gm of copper sulfate added to 250 ml of water. Fitch (1978) notes that filters that have been wetted and dried with copper sulfate solution have been used as an interior filter on flasks commercially.

Sealing Flasks

There are differences in technique with respect to sealing flasks. The methods presented above all involve using aluminum foil caps, as they can be readily sterilized in the autoclave. Plastics should be tested before autoclaving, but may be used to cap flasks if they have been chemically sterilized.

In either event, it may be useful to cinch either foil or plastic seals over the ends of the end of the flask. For this, a knot like the constrictor knot, as described by Ashley, may be of use (see Appendix IV: Miscellaneous).

Mites

There have been recent reports of flasking facilities that demonstrate unreasonably high rates of contamination despite aggressive measures to the contrary, many of which come from flasks that have been sown many months prior. These reports also state that the flasks may succumb in large numbers, seemingly as if it is a disease, spreading throughout a collection.

In these cases, contamination decreased radically when controls for mites were instituted. These controls, consisting of the application of a miticide on working surfaces as well as where the flasks are stored, along with increasing hygiene inside the facility, bring the matter under control in short order. Evidently, mites can infest a whole flask collection by being small enough to make their way through a cotton plug. In this manner, they can infect the media as they come and go. Use of the copper sulfate solution suggested by Bergman to treat plugs (see Treating Plugs, above) may help prevent contamination via mites; similarly, the use of breathable tape or band-aids may restrict the movement of these tiny arachnids.

Vacuum Treatment

It has been suggested that vacuum treatment of seed may be useful for disinfection of seed (Dutschka, pers. communication), or for inducing germination in stubborn temperate terrestrials (Steele, pers. communication).

Steele uses a small vessel with a single port for admitting seed and disinfection solution. After mixing, a small vacuum pump is used to reduce the pressure in the flask. The air is re-admitted after a period of time, and the cycle repeated until most of the seed sinks. In this manner, chemical solutions may be taken deep within the seed coat, penetrating it and making disinfection more effective. Steele's technique is primarily to wet the seed of cypripediums, and destroy germination-inhibiting compounds, but the modification by Dutschka is used to destroy bacteria and fungi.

Dutschka uses the apparatus depicted in Figure 4.2.7 for disinfection of seed. His "vacuum frit" is approximately 14 cm overall length. To use, the seed is added and wetted with 0.5 ml of ethanol and shaken for 3-5 seconds. Sodium hypochlorite disinfection solution is added, leaving approximately 0.5 ml as air space inside the tube. The assembly is inverted so the air bubble is at the stopcock, and vacuum is applied with a 10 ml plastic disposable syringe. The valve is then closed, and the solution shaken to remove small air bubbles from the seeds. The valve is then opened to return it to atmospheric pressure, closed, and the assembly is shaken again.

Reducing and returning pressure is repeated several times, until satisfactory results are achieved (i.e., the seed sinks). After this, the stopcock is removed, the solution drawn off with a syringe, and new sodium hypochlorite solution (without alcohol) is added to disinfect the seeds for the desired period of time. Afterwards, the disinfection solution is removed, and the seeds are extracted with a sterile spatula with the tip bent into a tiny "L" shape, approximately 3 mm x 4 mm.

A syringe will work to wet seed using this technique; large-volume syringes are often available through veterinary supply stores. Efficiency is enhanced by trying to evacuate a relatively small air space (the 0.5 ml air space, as above). With larger air spaces, a small vacuum pump such as the "Mityvac" produced by Prism Technologies may be of use. The Mityvac may be used for evacuating test tubes with a small volume of liquid, and relatively large volumes of air. Maximum vacuum of 23-25 inches of mercury are possible with this device, which may be purchased through automotive supply stores for bleeding air from hydraulic brakes.

See also "Vacuum Treatment," in Section 4.2, Disinfection of Seed.

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5.2 Care of the Sown Flasks

As mentioned above, one should monitor the surface of the agar in freshly sown flasks for signs of contamination. After a week to ten days, if signs of contamination are not present, it is much less likely that a flask will ever show them. Despite this, contamination as late as seven months after sowing may spontaneously manifest itself. Therefore, close monitoring of sown flasks, regardless of their age, is called for. Moreover, not all contamination will be visible; although fungi may grow very quickly, latent bacterial contamination may not be detectable by commonly available means. If desired, indexing techniques have been published in texts on plant tissue culture.

Flasks may be kept in the dark or in dim light, until the seeds start to germinate because light is not a known requirement for any species of orchid (Light, 1990). However if there are several flasks, different light levels might be used to determine optimal conditions for germination. The first sign of germination will be when seeds start to swell (Figure 5.2.1), and sometimes turn green. After this, tiny white to pale green spots will appear on the media. Occasionally these spots, called protocorms, may be brown. After this, flasks may be moved to higher light, but light levels are generally to be kept low relative to what mature plants receive. Many growers germinate and maintain seedlings under lights. The fluorescent lights available at the hardware store are commonly used for such culture; purchasing specialized grow lights is not required (Figure 5.2.2).

The amount of light is important because too much light will produce seedlings that are pale, or have bleached leaves. A good starting point is from 10 to 12 inches under a single 40-watt fluorescent tube, and from 12 to 18 inches underneath twin 40-watt tubes. The seedlings do not require a great deal of light. Some growers report that 100 to 150 footcandles (lumens per square foot) of light is sufficient under artificial illumination, while between 300 and 500 footcandles of natural light at its greatest intensity (noon, in general) will be adequate. Although these relatively precise measurements will be of value for comparison between growers, it is important that the grower rely more upon the product (i.e., how well the plants are growing) than on measurement instruments. Experience will be the greatest factor when optimizing light intensity. A long day, consisting of 16 hours on and 8 hours off, is acceptable

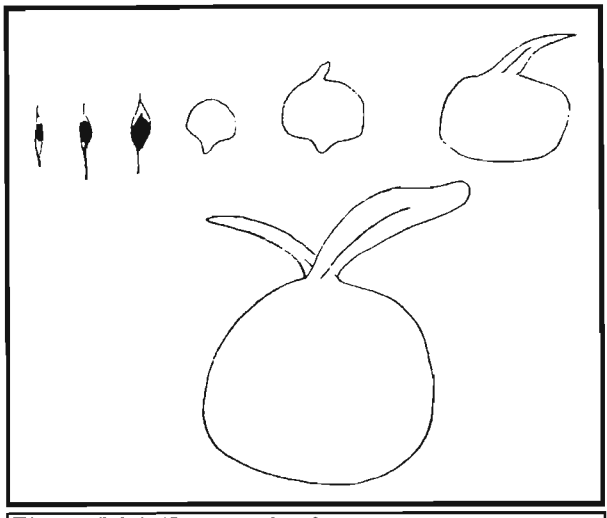

Figure 5.2.1: From seed to late protocorm stage.

for most species. Lights tend to generate significant heat; those in temperate climes may conserve energy by running light arrays out of phase with daylight (i.e., running lights mostly at night). Orchid cultures may also be grown with 24 hour a day illumination, but the benefits of this over shorter daylength are not clear.

Note that since the environment is sterile, when seedlings die they do not decompose. They will turn white or other colors, staying in place and leaving behind a frail tuft of leaves. Do not confuse this with bleaching from too much light, as this is common to flasks that are not gradually acclimated when daylength or intensity are increased. After all, in nature, only a tiny fraction of the total number of seedlings will survive; the number of seeds that germinate *in vitro* is much larger, and a certain mortality is expected. If the flasks are getting far too much light, it will be noticeable from the number of seedlings succumbing to injury from this kind of damage, and that the injury will be consistent between seedlings. Other mortality, such as the contents of an entire flask perishing simultaneously, may be caused by the absence of gas exchange, desiccation, waiting too long to replate, or environmental extremes such as exposure to heat or cold.

Flasks of tropical species should be kept on the warm side (65° F at night, 78° F or so during the day) as they develop, and control of the temperature to prevent rapid temperature swings will maximize seedling growth. Despite the usual caveats to keep flasks from high temperatures, work done both here in New Mexico and in Florida have shown that seedlings in flask are capable of tolerating temperatures in excess of 95° F (Hicks, unpublished; MacDonald, pers. communication). The limiting factor seems to be the amount of light. High temperatures in conjunction with high light will quickly cook seedlings. If the amount of light is restricted, temperatures up to 100° F can be tolerated for short periods of time at the hottest time of the day. Other measures may be taken with non-ideal genera, such as cold-tolerant species from North America, Europe, and elsewhere, which should be dealt with independently.

Some growers suggest that higher temperatures may cause protocorms to grow, rather than encouraging differentiation. If protocorms refuse to develop into plantlets, try dropping the temperature.

Dry Flasks

Occasionally a grower will be presented with the problem of a "dry" flask; sometimes media with too much agar in the mix will stress plants by desiccating them. The primary cause is when flasks are kept too long, or the seedlings are slow to develop, water escapes the system via the vent plug. This evaporation is compounded by the problem of seedlings incorporating water from the agar into their tissues as they grow. Seedlings will demonstrate the usual symptoms of dryness found in mature plants. For plants *in vitro*, desiccation will advance only very slowly, as a limited amount of water is still available from the media. The plants in a flask that is slowly getting drier are not in immediate danger, but unless the problem is corrected, they will certainly die. More symptoms of a dry flask include no visible amount of condensation visible on the media and on the inside of the vessel, or the media appears to pull away from the glass, often leaving a "watermark" or "bathtub ring" of dried media indicating the original level. Dry flasks will cause the media to become more concentrated, slowing plant growth and causing roots to slow or halt their development. Experience will indicate how much fluid should be inside the prepared flask.

There are three options with a dry flask, the first of which is the most straightforward: simply deflask the seedlings, provided they are old enough to survive. They may require a little extra care, and will probably be difficult to handle, being flaccid and easily damaged if the desiccation is advanced. Particularly valuable seedlings will benefit from rehydration prior to removal, so add several ml of sterile distilled water several days before de-flasking. Even if the flask is accidentally infected during this step, they will be removed from flask shortly, and more good than harm will result by being able to work with stronger plantlets.

The second is to replate the seedlings. In general, as with having seedlings that are old enough to survive independently of the flask, a dry flask can be stale, with the water having left the flask in vapor form through the stopper (it is not clear to what degree extra layers of foil slow the progression of desiccation, if at all) over time, and the seedlings may be replated if so desired. Still, many kinds of seedlings are touchy about being replated once they have passed out of the protocorm stage, and the first option may be the best one.

The third technique is to add extra water to the flask. This is a simple proposition, not much more difficult than sowing the flask, or replating seedlings. The flask should be prepared in a manner similar to replating, but rather than removing anything, sterile water is added to the flask (10 ml or more, depending upon conditions and the size of the flask). The flask should be watched carefully over the next few days to note any contamination; in the event the flask is contaminated, the seedlings should be de-flasked immediately, taking care to discard any contaminated media and possibly the seedlings involved.

In general, flasks run to dryness because they have been around for a while, and the seedlings within have already probably consumed a fair amount of the available nutrients, as well as sponged up available water for their growth. Flasks with cotton plugs are particularly prone to drying out; flasks that are not vented, or plugged with membranes, tend to stay very wet no matter how long they are kept. This may be of use for individuals wishing to grow species with long developmental periods. If necessary, wrapping the area between plug and seal with several layers of plastic food wrap may help delay desiccation by retaining water.

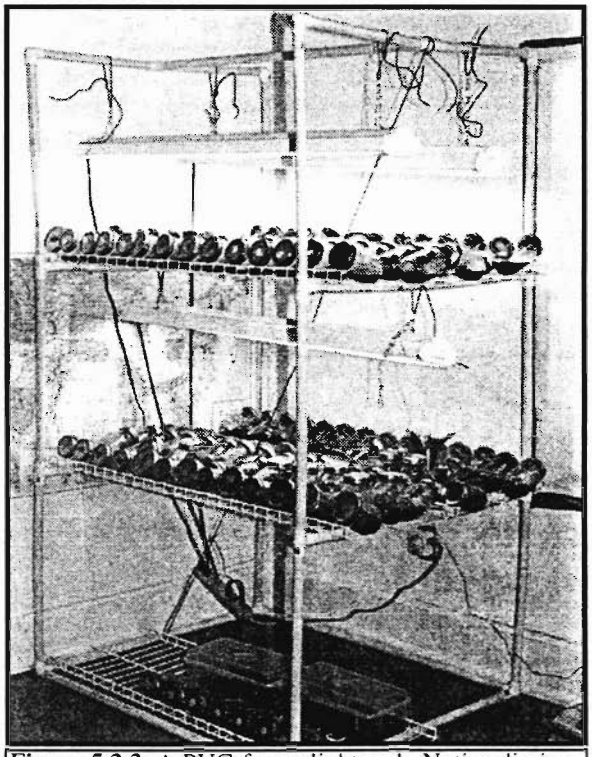

Figure 5.2.2: A PVC-frame light rack. Notice diminishing light intensity from top to bottom.

Flasks with plants that are slow to develop and require rehydration may also be "topped off" with nutrient solution in lieu of plain water, called "overlays." A small amount of sugar, banana puree, and other components may be autoclaved separately and added in small concentrations to dry flasks. It is important that these solutions not be very strong, as these will make the situation worse. This solution may also be used to change a mother flask into a replate flask if there are only a very few seedlings in the flask: adding a banana puree overlay that will ride on the surface of the agar can sometimes facilitate the growth of seedlings.

It is also worth noting that changes in the properties of the media such as pH, formation of waste products, etc., may be so high that topping-off may be ineffective. Nutrient deficiencies may be remedied through the addition of specific or general overlays by adding iron, calcium, magnesium, or other components.

Root Growth

Roots may develop at any time on protocorms; wet conditions may inhibit root growth, as plantlets have water immediately available to them. As flasks start to dry out, and plantlets begin to mature, root growth progresses rapidly. Roots that travel along the surface may indicate that the gel is too firm, or this may be normal. Roots that grow straight up are a sign that the plants do not like the media; for example, *Dendrobium falcorostrum* refuses to grow normally on Phytamax P-1056. It will grow normally on blotter paper/perlite saturated with fertilizer-based media as described elsewhere. The exact reasons for these differences are unclear. Moving seedlings to other media to optimize growth may be the best option.

Disposal of Flasks and Conditioning

Occasionally, seeds may germinate only after a long time on agar. One collection of *Zygopetalum mackyi* that had been in refrigeration for over 2 years germinated very well, but only after 90 days on agar (Hicks, unpublished data). Flasks should be disposed only after 4 months (for tropical epiphytic species), or even longer when experimenting with species whose natural environments may require climactic changes. Light (pers. communication) notes that germination *en masse* may occur as late as 6 months after sowing pleurothallid species and hybrids. Ewert (pers. communication) has retained flasks as long as 9 months awaiting germination of paphiopedilums.

Encouraging recalcitrant seed to germinate may require sowing multiple flasks, and exposing each one to different conditions: varying levels of light (from none to bright), temperature (from multiple chillings at 4° C or even freezing cycles *in vitro* to 32° C or warmer), nutrients, and even gasses for some species. Other than variables involv-

ing light. these differences pertain primarily to terrestrial species, and not epiphytes. For example, it is generally recognized that *Cypripedium* species germinate best in the dark (Weber, 1998; Reyburn, 1978), and this may be true of some *Paphiopedilum* species as well.

Ewert (pers. communication) notes that chilling some paphiopedilums to 45° F after sowing will enhance germination. Chinese and Indian species, particularly those of the *Paphiopedilum insigne* group, prefer this treatment. Addition of coconut milk may enhance or terminate seed germination.

Weber (pers. communication) notes that some species of *Liparis* may require two seasons of dormancy to germinate. Seed that was sown from green capsule material onto agar was put into chilled dormancy in the fall, and removed in March. If no signs of germination are present by fall, the procedure is repeated, by which time seed should germinate in the second spring, if at all.

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5.3 Replate

As has been mentioned before, eventually the stage is reached where protocorms may be replated. Replating (Figure 5.3.1) may be viewed as a kind of "repotting." Protocorms are removed from the nutrient agar in which they have depleted many of the nutrients and removed much of the water, as well as placed waste products into the media, and placed on fresh, new media that is often formulated to maximize seedling growth.

Media must be selected carefully for replate. Plants that grow rapidly and aggressively, such as stanhopeas, must be provided with plenty of nutrients. Because they grow quickly, there are few concerns about the flask running out of water, but nutrient deficiencies may result. Plants that grow more slowly must be paired with the appropriate media, which may require experience and experimentation to determine the best parameters for optimizing growth. In some cases, it may be desirable to replate onto several strengths of media. For example, doing a primary replate onto full-strength, 85% strength, and 70% strength media may help determine the best concentration for a secondary replate, if so desired.

Until this point in the text, the grower has been given only vague figures about the amount of seed that must be placed onto the agar. Ramsay (pers. communication) suggests that sowing too thinly is better than sowing too heavily; experience and a little luck will guide the grower to estimate the amount of seed used. Among other factors, viability will provide great variation in the amount of growth in a flask. One cut of seed may demonstrate virtually 100% viability and rapid growth, and quickly overwhelm a flask with protocorms and seedlings. Yet another will demonstrate scant viability, slow growth, and other factors that will indicate that replating may not be the best option.

Depending upon whom you ask, timing is either critical or unimportant with regards to replating. The only hard and fast rule is that protocorms should be replated before roots develop. Although it may be traumatic on developing plantlets to move them once they are past this stage, it can certainly be done without too much trauma. Some consider it important to replate while protocorms are still brown or white, and that unnecessary delays may cause high mortality. This rule may particularly hold true for some genera and certain species. Others wait until protocorms are up to 1 or 2 mm across. Individuals will have to try techniques suited to the genera and techniques with which they are working.

Replating a flask requires tools that are generally expensive or custom-made. In fact, microspatulas available through commercial laboratory supply companies are good for replate procedures. They come in a variety of shapes and sizes, but generally consist of a metal rod with a thin, flat scraping end that is ⅛" to ¼" wide, and about ¾" long. The tool is generally about 8" long, and constructed of stainless steel. Microspatulas are expensive, but durable. Cohen suggests making "replate forks" out of stainless steel welding rod, using a hammer to flatten one end. It may be necessary to heat the end of the rod with a torch to facilitate this procedure. They can also be made out of nickel wire (available through jewelry supply stores at a reasonable price) in the same manner.

The instruments should be soaked in a 30% by volume solution of bleach or 1-2% Virkon S for half an hour or more while preparing the flasks. Trays for these instruments are hard to find, but custom trays may be produced by taking a length of 50 mm (2 inch) diameter PVC pipe and gluing on two endcaps. This is then run through a bandsaw

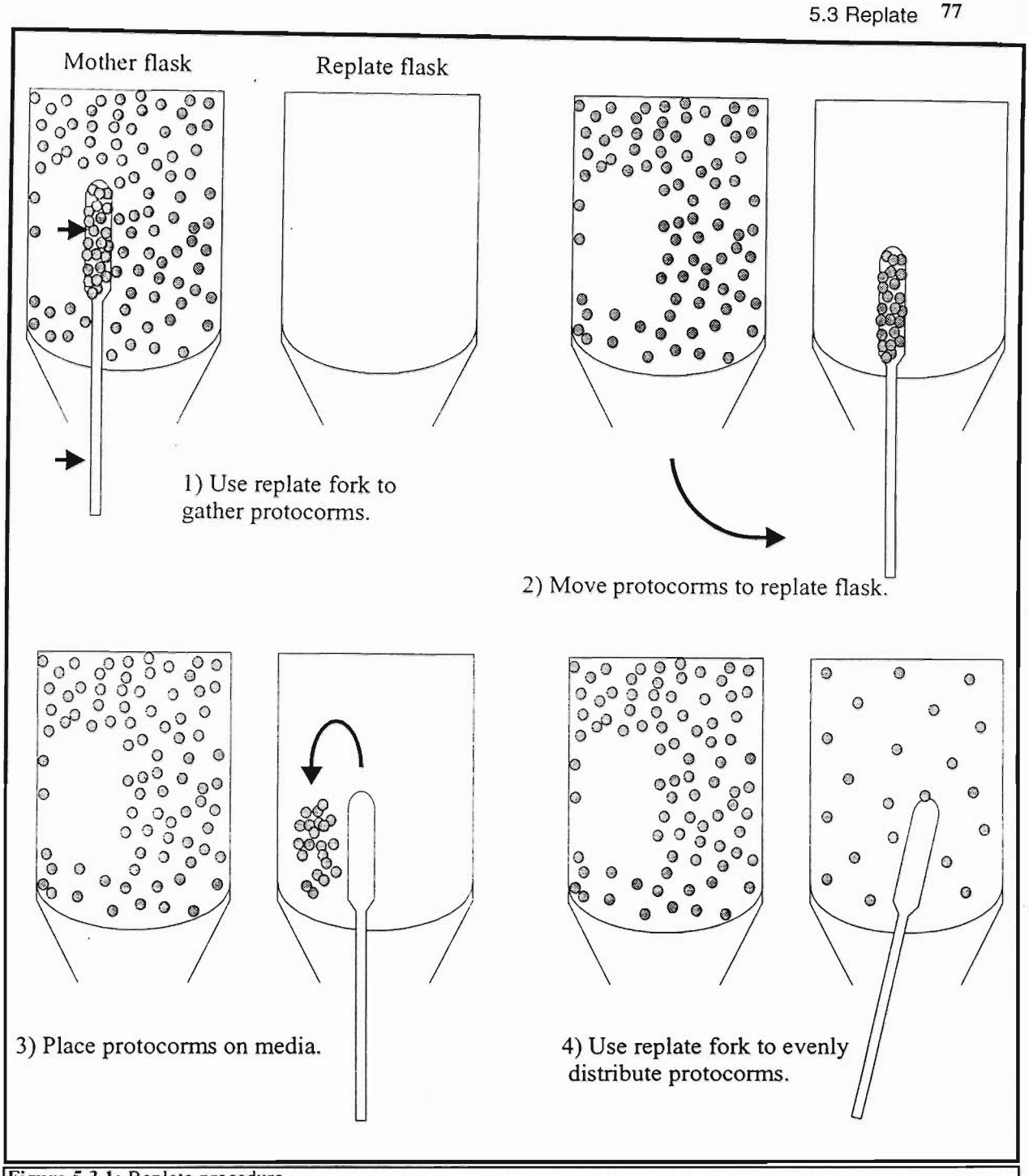

Figure 5.3.1: Replate procedure.

lengthwise to produce two trays, each half of a cylinder, of whatever length may be desired. These are then filled with the disinfection solution of choice.

Spray down your replate area with the same solution of bleach.

Since we are working with flasks of live seedlings, some consider it fairly important to remove excess bleach from the tools before using them to handle seedlings. A wash bottle of sterile water should be used to clean the tools after the rinse in bleach, and before putting them inside the flask. Others do not consider this important, and even spray bleach solution into the flask (see below). As noted before, wash bottles must be autoclaved with the top loosened, and preferably be covered with aluminum foil.

Carefully open the empty replate flask and the mother flask that carries the protocorms. Take the lids off, and place them sterile side up on a clean, dry surface within the flasking box. If there is a lot of liquid on the surface of

the replate flask from accumulated condensation, pour it out now. With the mouths of two or more flasks open, it is important that the grower be very careful of any actions from this point on: nothing that has not been sterilized should be placed into or above the flask. If using a HEPA filter, do not place any unclean tools "upwind" of the open flask. If any tools touch anything that is not sterile, they should be set aside, and another tool picked up and washed to replace it.

Use the flat end of the tool to pick up protocorms, either by skimming them off the surface of the agar, or by wedging them against the glass. This is why some growers prefer to use very hard media (i.e., high in agar) for mother flask so that it is easier to nick them off the surface of the media; others prefer to use a softer media, which allows the grower to make a "divot," removing a negligible amount of media with the protocorm. In either event, count the number of protocorms transferred, to prevent the replate flask from becoming overcrowded. Depending upon the size of the protocorms, the anticipated seedling size, and relative value of the plants, the number of specimens placed on replate can vary widely, but 20 to 40 protocorms in a quart Mason jar is a good starting point. Some consider it important to gently press protocorms into the surface of the media to get them started; others do no more than drop them on the surface of the media at about one per square inch or so.

One other technique, which may be used if the protocorms are small and float freely upon the media, is to use a pipet or dropper large enough to accept the protocorms. Add a small amount of sterile distilled water to the mother flask, and slosh it around to gently dislodge the protocorms. Cant it to one side, and then use the sterile dropper to suck up a small quantity of liquid with protocorms. Tip the dropper to one side to keep the protocorms from forming a "logjam" at the tip, making it difficult to expel them once again. Then simply open the replate flask, and add the protocorms and liquid. Use the dropper to remove up any gross excess of liquid on the surface of the media. Sterile, disposable polyethylene transfer pipets may be of use, and are available through chemical and research suppliers (see Appendix III, Sources).

With either technique, if so desired, the inside of the lid may be sprayed with disinfectant solution before resealing. It is also possible to spray a small amount (less than a ml) of disinfectant directly into the flask before sealing it. This may help kill any number of organisms incidentally introduced to the flask, but some plants are remarkably sensitive to chlorine and will burn. However, Virkon S does not seem to burn plantlets quite as badly as bleach.

After replating the protocorms, drop the replate tool into sterilant solution, and pick up another tool. If the instrument has become contaminated, this switch will reduce the number of flasks that are contaminated from flask to flask. It is also acceptable to periodically disinfect the tool you are using with whatever solution is at hand: dunking it in bleach or Virkon solution and then letting excess drip off works well.

For chlorine-sensitive species, it may be best to thermally disinfect replate tools. Forks may be wrapped individually in aluminum foil, and several of these forks may be bundled and wrapped in a larger packet of foil and autoclaved. When a fork is needed, it is removed from the packet, used and set aside when finished. I have found this technique to be preferable to chemical disinfection, but this requires that you purchase a fair number of expensive microspatulas at a time.

It is also worth mentioning that when replating, it is often advisable to set aside protocorms which you intend on sharing with others. Often we will have hundreds or thousands of protocorms, and few growers have the capacity (or desire) to bring this many propagules to maturity . These protocorms can be set aside in sterile tubes and shipped to other growers. The easiest technique by which this may be performed involves sterilizing a number of tubes, complete with cotton plugs and aluminum foil caps. These in turn are wrapped in aluminum foil, and grouped 7 at a time to form a hexagon. This is then wrapped in heavy freezer foil, and autoclaved.

When done with replate, open up an individual tube, remove the cap, then the cotton plug with sterile forceps and set it on a dry, sterile surface. Using a replate fork, place a generous quantity of protocorms in the tube. If desired, add a small quantity of sterile water; the condensate from the inside of sterile flasks is perfect for this. Replace the cotton, replace the foil lid, and return to its foil wrapper. Seal and label. In this form, they are stable for at least a week, and may be shipped as required. NOTE: to meet CITES regulations, sterile protocorms without media are not sufficient. A small quantity of agar must be supplied to be exempted under these laws. This agar may be solid, as with a slant, or it can be a loose slurry. The important thing when shipping either in liquid or in gel is that there not be an excess of liquid such that it will leak from the flask, potentially contaminating it.

Upon receipt, the foil is opened in a sterile workspace, the tubes externally disinfected, the plugs removed, and the plants inside are transferred to sterile media to resume their growth.

These procedures are also satisfactory for plantlets in later stages of development, all the way up to small seedlings, depending upon the size of the tube. However, if plantlets are large and have grown together into a mass, they may be removed from the flask and separated carefully with replate forks or other tools, and then placed back into flask. It is sometimes useful to work on a sterile work surface that has no chemicals on it that might damage the seedlings. For this, a large square of aluminum foil may be folded or rolled, and sterilized in an autoclave. When

ready for replate, the foil is opened up. The seedlings are then removed onto the foil for separation, and subsequently placed into replate flask. Use of the foil reduces the chance of chemical injury to seedlings of sensitive species.

Liddell (1980) reported a high level of contamination among his replate flasks (25%), and solved his problem by immersing small seedlings in 1% bleach solution. Seedlings are removed from mother flask, left in the solution for "several minutes," after which they are removed and placed onto replate media. This technique is rather extreme, and in the presence of a controlled, clean workspace, is not necessary. It is mentioned primarily for those that would choose to work in the open air, and not as a routine technique, although the author reports that some 32 species and hybrids were replated with no differences in growth between the test group and a control group. Some damage was reported, but was thought to be mechanical, and not chemical, in nature as those species damaged had thin leaves and were treated roughly. The author notes that "there was no mortality" as a function of these techniques.

References:

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5.4 Deflasking and Seedling Care

Seedlings that are yet to be removed from flask are denizens of a cushy environment. The flask is at 100% humidity, with absolutely no chance of rot from infectious agents, and the temperature is relatively stable, as is the nutrient and water supply. There are no predatory insects or slugs, nor any of the other hazards normally associated with greenhouse life. Removing them from this heavenly abode is a proposition that incurs the wrath of seedling mortality, the management of which is the subject of this section.

Deciding whether or not seedlings are large enough to survive is primarily a matter of opinion and experience (themes repeated often enough throughout this text). Nevertheless, it is important that we give seedlings every reasonable opportunity to survive and thrive outside the flask. Therefore, we must bear in mind several issues. Seeding mortality is commonly caused by dehydration, because seedlings have little capacity to store water, nor an adequate root system with which to accumulate it. Some plants are simply poor at generating roots, particularly when living in such a wet, humid environment. All the same, examining the seedlings for a developing root system is very important. Next, we must look at other aspects of the seedlings, such as size and developmental stage. Unless the members of a flask include a high proportion of plantlets that are slow to develop, or seeds that were very slow to germinate, there should be relatively few plantlets in the protocorm or undifferentiated stages of growth. Although these certainly may survive when de-flasked, they are not particularly vigorous growers, and will have a high mortality rate. Flasks with large numbers of green, growing protocorms that also have numbers of seedlings with large, healthy leaves and roots may be sent to replate the protocorms before deflasking so as to increase the yield if desired.

Having decided to de-flask a particular batch of seedlings, we must decide which technique is most appropriate for removing the seedlings. If the bottle neck is particularly small (such as with Erlenmeyer flasks), or the seedlings are very large and we do not wish to damage them, it may be appropriate to smash the bottom of the flask. Wrap the flask in a towel and protect eyes and hands; use a hammer to deliver a sharp blow at and parallel to the base of the flask in an attempt to knock it off cleanly, in one piece. In the unlikely event that you succeed in doing so, you will have a few, large glass shards, one of which is the very bottom. If you don't, you will have many smaller ones, and the remains of what was once the bottom of the flask.

Wellenstein (1999) suggests using a drift or a punch, inserted through the neck of the bottle, to deliver a firm blow to the center of the flask from the inside. Using a bolt or another strong metal punch, carefully push it through the opening of the flask until it contacts the bottom. Wrap the flask in newspaper and tap the end of the bolt until the glass shatters.

Remove the agar and seedlings as one unit, being careful not to cut yourself in the process: shattered glass looks very similar to fragments of agar. Place it in warm water to let it soak, and follow the procedure given below for cleaning up seedlings for planting.

Otherwise, if you are working with Mason jars or similar wide-mouthed bottles, remove the lid and fill the flask with warm water and allow the agar to soften. Remove the softened agar with seedlings using tools such as spoons, forks, butter knives, chopsticks, and whatever else comes handy. Float the seedlings in warm water to help further soften the media; some growers add "SUPERthrive" or similar vitamin and nutrient complexes to help mini-

minimize transplant shock. As with any potion in a bottle, some growers swear by it, others claim "it can't hurt," and yet others scoff at the idea.

At this point, it is good to clean up the seedlings you have; nutrient agar, as we have discussed, will grow an unhealthy profusion of bacteria and molds if allowed to remain. Floating the plants in a bowl of water permits the agar to soften, as well as to let glass fragments that you may have missed drop to the bottom of the bowl. Use forceps, your fingers, or chopsticks to gently remove the seedlings from this agar, and wash off any remaining media. Be careful to minimize bruising and damage to roots and leaves. If using forceps, you may find it useful to bend one of the tips out 5 or 10 degrees, or far enough to give a small gap to keep from crushing seedlings. Many growers skip this, and are less accommodating to the seedlings, and treat them more roughly; losses are probably slightly higher this way, but it could be argued that you will be salvaging only weak specimens anyway.

The media into which you place the seedlings is very important; recall that seedlings *in vitro* have yet to be exposed to desiccating drafts and low moisture. Therefore, a mix that holds lots of water (or, alternatively, will be exposed to frequent wet-and-dry cycles) is important. Pure Sphagnum moss is a good starter mix, but personal preferences and local conditions will guide the grower to the most suitable media. The containers are also important; larger pots hold water longer, which may stave off dryness if one should forget to water occasionally. Compots, or community pots, will hold 20 or more seedlings with ease. Using a wide, shallow pot, fill it with media and sow with recently de-flasked seedlings. Place in a humid area, or inside a large plastic bag. Such community pots make it easy to work with large numbers of seedlings.

Another option is to plant the seedlings in "six packs," such as those in which garden plants are often purchased. Small mini-greenhouses that neatly support 6 or 8 of these six-packs are also available, and will support a fair number of seedlings in a humid environment.

If you so desire, you can place small numbers of seedlings in ice cube trays. Use a soldering iron or a sharp knife to make a hole about ½" up the side of each well; this will permit a small amount of water to remain in the bottom of each well after you are done watering, which may help serve as a reservoir if you neglect to water regularly. Further, ice cube trays are inexpensive, and you can always cut off any number of wells if you wish to send seedlings off somewhere else. They fit neatly inside of plastic bags that will help keep them humid.

One other trick is to use the clear plastic trays in which food is sold at the supermarket; place 2.5 to 5 cm (1-2 inches) of vermiculite in the bottom half, and distribute your seedlings on this. Close the lid, and you have a mini-greenhouse in which you can happily maintain even the smallest of seedlings. These trays must be inspected periodically to make certain that they are not overwhelmed by ferns, algae, and other organisms that may overgrow the seedlings.

Since bacterial or fungal infection may destroy seedlings at an alarming rate, it is important to monitor community pots and seedlings very carefully. Nevertheless, this threat is no reason for prophylactic spraying of fungicides or other chemicals because this practice breeds strains of resistant pathogens. Some scoff at this idea, but in twenty years, when chemicals used today are either illegal because they may destroy the environment, or are no longer effective, we will be out of alternatives, wondering why the miracle sprays of the last century no longer work. Worse, this practice may breed weak plants, susceptible to disease when passed on to greenhouses that do not practice prophylactic spraying.

If your pots are in the greenhouse, beware of slugs; placing the compots on special benches designed to ward off slugs, such as by isolating the entire bench with the legs in water bowls, protected by copper flashing or other techniques, is highly recommended. Slugs, cockroaches and other insects make a hasty meal out of soft, succulent seedlings in short order. Cockroaches may be dealt with very efficiently through the use of boric acid; commercial preparations that contain almost pure boric acid are highly effective at ridding areas of these pests. It is soluble in water, and should not be applied in areas which are washed down regularly, but can be put in other areas which are frequented by roaches. One little tip: it must be distributed wherever possible for it to be effective. Dust the areas around baseboards, under sinks, behind counters– roaches love water, so perform your dusting with this in mind. Boric acid has low toxicity (MSDS sheets state that the LD_{50} is about 2660 mg/kg in rats; compare this with about 3000 mg/kg for sodium chloride, or table salt), and can be used as an ophthalmic solution (eye drops). As with any chemical, exposure should be minimized, but boric acid is one of the safest solutions to household pests, including ants and roaches.

Protect your seedlings from extremes of temperature, humidity, and light because it is easy to harm seedlings which have not had the opportunity to harden off. Remember that seedlings are not capable of taking the temperature swings that mature plants often can, and that small seedlings have next to no water storage capacity–they don't have pseudobulbs, and have not yet developed the ability to store water in succulent leaves and tissues. Be careful not to change conditions on them rapidly, with no chance to acclimate.

If the seedlings are very large, but without substantial root systems, it may be necessary to prune the leaves to minimize losses. Cutting the leaves to minimize transpiration and preventing water loss may increase seedling sur-

vival. Loss of half the length of the leaf may be sufficient in many cases.

If you have a relatively small number of seedlings, one option is to build a small climate chamber out of some scrap plastic or glass. Placed under fluorescent lights and controlled by a timer, this structure becomes a haven for small seedlings that might not otherwise survive. As with other systems, this is not a "fix-all," and seedlings are still not immune to the effects of fungal or bacterial pathogens; although it may be a "closed system" to begin with, it is important to slowly wean the seedlings off their high-humidity diet, and eventually bring them to a point where they can be sustained indoors, or at least in a greenhouse. A large sheet of Lexan or other plastic over the top of the climate chamber can be incrementally removed from the top, increasing air circulation at a slow rate. Carefully monitor the seedlings for any signs of dehydration or other ill effects, and completely replace the sheet if necessary.

Seedling mortality is to be expected, but can be minimized by careful observation and treatment of symptoms at this phase. Seedling growth varies; the occasional robust *Phalaenopsis* can be brought to flower within a couple of years of being removed from flask. Others, such as cattleyas, may take five years or more in order to bloom.

Patience, as always with the Orchidaceae, is called for.

Bob Gordon's book, *Orchid Seedling Care*, is an excellent text on the subject of tending to the needs of orchid seedlings after being removed from flask, and is highly recommended.

References:

Gordon, B. 1991. Orchid seedling care. Laid-Back Publications, Rialto, California.
Wellenstein, B. 1999. Web URL http://www.ladyslipper.com/compot.htm

Chapter VI: Advanced Techniques

6.1 Media Modifications

As has been mentioned before, there are several different components in nutrient agar for plant culture. Plants may have specific nutritional and physiological needs, and certain components can help provide for these requirements. This section will provide a brief discussion on some of the most common additives.

Charcoal

The most basic addition to nutrient media is charcoal. Ernst (1974) found that its use produced "remarkable" enhancement in root and shoot growth in *Paphiopedilum* seedlings, with better results when used in conjunction with banana (see Fruit Juices and Other Extracts, below).The mechanism by which charcoal functions is one that is still under debate. Arditti and Ernst (1993) suggest that it may serve to adsorb ethylene produced by seedlings during their growth, or that it may remove (l) 5-hydroxymethylfurfural (produced from sugar during autoclaving), or possibly phenolics or carboxylic compounds also produced during seedling development.

Charcoal also has the disadvantage of chemically binding some complex compounds that are added for the enhancement of plant growth. Finely powdered vegetable charcoal can be added at 2 gm per liter, and may settle out prematurely unless agitation is maintained before the media gels.

One theory as to the potency of charcoal is that it functions as a darkening agent. One possible substitute for powdered charcoal is powdered graphite, which is added at a similar rate, or lampblack. However, neither compound has proven to be as satisfactory as vegetable charcoal, and experiments with *Paphiopedilum* seedlings where the flasks were masked to exclude light found no significant difference (Ernst, 1974).

Water

In some areas, tap water will suffice in place of distilled or deionized water. Most water contains well below 400 ppm of TDS (parts per million of total dissolved solids), which includes cations and anions such as sodium, magnesium, calcium, potassium, chloride, fluoride, sulfate, carbonate, and so forth. Within reason, most plants will do very well in the presence of moderate quantities of these ions. If you have a water softener (which is simply exchanging sodium chloride for carbonate hardness), use un-softened water because the extra carbonates will not harm them.

One caveat is "ready-mix" formulations with a pH buffer set as part of the media that are designed to be dissolved in distilled water. If these are mixed with tap water, the user will want to monitor the pH, and adjust it if necessary.

It is also important to bear in mind that most growers have used distilled or deionized (DI) water because "everyone else does." The main reason we use DI water is to standardize our work, thereby producing a control. For our purposes, DI water will be the same everywhere, and therefore (in theory) its use allows researchers and horticulturists to produce consistent results across the board. If you are a researcher, or will be doing work that is eventually intended for publication, it is critical that your results can be duplicated by someone working at another facility. However, if your commercial lab finds that tap water is an acceptable substitute for DI, and you do not intend to permit anyone else to duplicate your results, then tap water may work well for you.

Remember that the composition of tap water may vary considerably between seasons and even between different times of any one day. Therefore, it may not be acceptable for use in your laboratory work. The nominal cost of distilled water should not be a factor when sowing orchid seed. The value of your time and energy far exceeds the cost of water that will produce consistent, reproducible results.

Iron

Arditti (1967) noted that lower phosphate levels were possibly responsible for better growth in some experiments, but insoluble ferrous phosphate possibly rendered the iron unavailable. Today, iron is generally complexed, using EDTA, to increase its availability, so the lack of this element is apparently not as much of a concern as it once was. It is worth noting that despite improvements, iron deficiencies may still occur. Iron may be made available to plants in many forms; generally, it is chelated. Chelation is most often performed with EDTA, but other complexing agents such as DTPA may be used.

Iron may also be made available to seedlings as iron citrate, or iron oxalate.

Sugars

Sugars serve as a carbon source for the developing seedling.

The most readily available sugar is sucrose, which is a disaccharide composed of the monosaccharides alpha glucose (also known as dextrose) and fructose (also known as levulose). When we autoclave sucrose, it will hydrolyze to fructose and glucose, particularly in an acid solution. Arditti (1967) citing Ernst (1967a) notes that different sugars have different effects upon the growth of *Phalaenopsis* and *Dendrobium* hybrids *in vitro*. In both cases, fructose proved to be a superior sugar in terms of seedling weight, and leaf and root development.

The average weight for *Phalaenopsis* hybrids grown on fructose was 211.9 mg/seedling, followed by xylose (194.11 mg/seedling), beta glucose (190.5 mg/seedling), mannose (172.0 mg/seedling), sucrose (163.8 mg/seedling), and alpha glucose (162.9 mg/seedling).

With *Dendrobium phalaenopsis* x self, the average weight of seedlings grown on fructose was 117.295 mg. This was followed by alpha glucose (83.25 mg/seedling), mannose (77.775 mg/seedling), and sucrose (56.875 mg/seedling). Xylose produced inferior results, at 13.85 mg/seedling.

Fructose is commonly available as high fructose corn syrup (HFCS), which has largely supplanted the more expensive cane or beet sugar (sucrose) in commercial products. For those that wish to experiment, honey should prove to be an excellent commercial source of fructose, as virtually all of its sugar will be in this form. Due to differences in osmolarity (water balance), a different amount of fructose will have to be used (Arditti and Ernst, 1993), as described below.

For those that wish to experiment, sucrose (a disaccharide composed of glucose and fructose, with a total of 12 carbons) will replace maltose, cellobiose, trehalose, and turanose on an equivalent weight basis. This is to say, 20 grams of sucrose can be replaced by 20 grams of the above sugars. Each has 12 carbons and may be considered equivalent for carbon balance.

For other sugars, osmolarity must be considered. Glucose, fructose, and mannose each have the same molecular weight, so 5.623 grams of any of these will have the same osmotic strength as 10 grams of sucrose in the same quantity of water. For xylose, 4.386 grams will replace 10 grams of sucrose, and 5.322 grams of mannitol has the same strength as 10 grams of sucrose.

To replace each with an equivalent quantity of carbons, glucose, fructose, mannose, and mannitol all have 6 carbons, and the quantity derived above must be doubled for sucrose equivalency. Xylose has 5 carbons, and must be multiplied by 2.4 for equivalency.

Karo Light Syrup, a commercially available HFCS, is 76% (by weight) carbohydrates. Of this, more than half (38.5%) consist of polysaccharides, and 8.7% trisaccharides. The balance consists of maltose (9.5%), dextrose (16.0%), and fructose (3.3%) according to the parent company Bestfoods.

Vitamins

The role of vitamins in orchid media has been explored. Vitamins such as thiamine (B_1), niacin (nicotinic acid, or B_3), and pyridoxine (B_6) are currently used in commercial media. As noted in section 3.7 Preparation of the Media, under Improvised Media, B vitamin supplements may be used for this purpose. Others have recommended the use of biotin, folic acid, and pantothenic acid, but the role of these vitamins is not as clear-cut as it is with B_1, B_3, and B_6 (Arditti and Ernst, 1993). It is also worth noting that vitamins are not thermally stable and decompose when heated. In fact, thiamine is particularly unstable, and high concentrations before autoclaving (10 mg/liter for some media) may be required to allow significant quantities to survive.

No requirements for these vitamins have been established for asymbiotic cultures of orchids. Concentrations of vitamins at the 1 ppm level are not uncommon in media formulae, and thiamine, as mentioned above, may be higher to make up for what is destroyed by heat. Glycine has been used at 2 ppm (M&S media), and 5 ppm (Arditti and Ernst, 1993).

Nitrogen

The use of nitrogen by orchid seedlings has been discussed in the literature, and urea, nitrate, and ammonium may be used by growing seedlings (Arditti,1967; Arditti and Ernst, 1984). For those plants that grow better on ammonical nitrogen, ammonium sulfate may be used as the source. Ammonium nitrate will provide both forms, and compounds such as potassium nitrate will provide only nitrate.

There has been some discussion as to the variability of each of these nitrogen sources. Nitrate reductase, an enzyme, may be present in the seedling only after a given point in its development, permitting the use of nitrate only later in growth. Exactly when nitrate reductase is generated may vary, so the presence of both ammonia and nitrate

for seed germination is recommended.

Raghavan (1964) researched germination and growth of *Cattleya* seedlings on media utilizing different organic nitrogen sources to determine growth effects when used singly and in combination with ammonium nitrate. Compounds included amino acids, urea, and urea compounds. It was determined that amino acids related to the ornithine cycle (arginine, ornithine, and urea) could replace ammonium nitrate as a nitrogen source in *Cattleya*. Other compounds varied as to whether they enhanced or inhibited germination, and are listed specifically in his paper.

The use of urea has produced variable results, as have amino acids (Arditti, 1967). The latter group is discussed below.

Inositol

The role of inositol in orchid culture is poorly known. Also known as *myo*-inositol, it is generally recognized as a growth enhancer in plant culture media, but Arditti and Harrison (1977) note several reports where it failed to have significant effect on cultures of *Cattleya, Epidendrum tampense,* and *Goodyera repens*, although it was reported as possibly stimulating the germination of *Cattleya* seeds.

Amino Acid-Based media

Malmgren (1996) developed a formula for the micropropagation of terrestrial orchids; the disadvantage (for most growers) is that, being from Sweden, his use of certain proprietary solutions that are not widely available internationally have made it difficult to reproduce his work. He reports that "All *Ophrys*, *Orchis*, *Gymnadenia*, *Platanthera*, *Nigritella*, and a large number of *Cypripedium* species and hybrids" are capable of being germinated on his media.

For those interested, his formula is reproduced here; this and several suggested variations can also be found in the NANTOC Proceedings (see Appendix III. Sources).

Calcium phosphate [$Ca_3(PO_4)_2$]	50-100 mg
Magnesium sulfate [$MgSO_4 \cdot 7H_2O$]	50-100 mg
Potassium phosphate, monobasic [KH_2PO_4]	50-100 mg
Sugar	10 gm in sowing media, 15-20 gm in growing media
Agar-agar	Approximately 6 gm
Pineapple juice	10-25 ml
Vamin (amino acid solution)	Approximately 5 ml of solution
Soluvit (vitamins)	0.1 to 0.3 amp
Tap water	1 liter
Charcoal	Approximately 0.5 gm
Kinetine	2-5 mg, added for some species of *Cypripedium*

Malmgren goes on to note that his local tap water has approximately 13 mg of calcium per liter, and about 22 mg of sulfate (SO_4^{-2}). The pH of the media is to be set at 5.5 to 6.0. Interestingly, the two amino acid components are used in Sweden in solutions for intravenous injection, much like we use dextrose solutions in the United States. Ironically, Vamin is manufactured in North Carolina, but is unavailable except in Europe.

Margaret Ramsay, head of the Micropropagation Unit at the Royal Botanical Gardens, Kew, adapted Malmgren's formulation using a commercial amino acid formulation plus Nitsch and Nitsch vitamins. The following formulation is currently used by Kew for germination of cypripediums.

Amino acid solution	Either 10.0 ml RPMI-1640 (Sigma R7131) **or** 7.7 ml 'Vaminolac.'
Vacin and Went powder (Sigma V5505)	0.41 grams
Nitsch and Nitsch vitamin solution (Sigma N0390)	1 ml
Pineapple juice	25 ml
Activated charcoal	1.0 gm
Sucrose	10.0 gm
Agar (Sigma A7002)	6.0 gm

Everything but the agar is mixed and brought up to 1 liter total volume using distilled water. The solution is mixed for 15-20 minutes, and the pH adjusted to 5.7 using potassium hydroxide solution. Agar is added, and the solution is then microwaved to heat it. The solution is stirred between microwave cycles. Solution is then dispensed

and autoclaved for 15 minutes at 15 psig. This was published in the Botanic Gardens Micropropagation News, August 1992, Volume 1, Part 5, on pages 59-63. The article is "Large scale asymbiotic propagation of *Cypripedium calceolus*- plant physiology from a surgeon's point of view."

David Mellard (pers. communication) has made efforts to formulate a mixture comparable to that of Malmgren's, using components available in the US, aided by some suggestions passed on by Ramsay. His current formula is as follows:

Sucrose (table sugar)..... 10 gm
PhytaGel..... 2.5 gm
Charcoal..... 0.5 gm
Nitsch and Nitsch vitamin powder, 1000x (Sigma N-0390)..... 0.1 gm
RPMI 1640 amino acid solution, 50x (Sigma R-7131)..... 5 ml
Calcium phosphate [$Ca_3(PO_4)_2$]..... 200 mg
Magnesium sulfate [$MgSO_4 \cdot 7H_2O$]..... 200 mg
Monobasic potassium phosphate [KH_2PO_4]..... 200 mg
Pineapple juice..... 20 ml
Tap water..... 1 liter

Mellard notes that the vitamins are "not an exact match for what Malmgren uses," but that it is probably of little consequence in any event. He suggests that other substituents, such as coconut water or potato cubes, can be used in lieu of pineapple juice. Some experimentation may be called for, because the concentration of cations seems to effect the gelling action of the PhytaGel. Therefore, it may be desirable to replace the gelling agent with agar, or an agar/PhytaGel combination.

A specific list of amino acids of use is not available at this time. However, Malmgren (1996) notes that with 18 amino acids, the number of permutations is not small. The following components are recommended by Malmgren as a variation of the Norstoog media, created by Haugli:

Glutamine.....400 mg
Alanine.....50 mg
Cysteine.....20 mg
Arginine.....10 mg
Leucine.....10 mg
Phenylalanine.....10 mg
Tyrosine.....10 mg
and "Potato instead of pineapple, kinetine, NAA and 2,4-D."

In lieu of RPMI 1640 amino acid solution, analagous formulations may be used instead.

Amino Acid	Vaminolac (grams/liter)	RPMI 1640 (Sigma R7131) (grams/liter)
L-alanine	6.3	-
L-arginine (free base)	4.1	10.0
L-asparagine (anhydrous)	-	2.5
L-aspartic acid	4.1	1.0
L-cystine and cysteine	1.0	2.5
L-glutamic acid	7.1	1.0
Glycine	2.1	0.5
L-histidine (free base)	2.1	0.75
Hydroxy-l-proline	-	1.0
L-isoleucine	3.1	2.5
L-leucine	7.0	2.5
L-lysine • HCl	5.6	2.0
L-methionine	1.3	0.75
L-phenylalanine	2.7	0.75
L-proline	5.6	1.0
L-serine	3.8	1.5
L-threonine	3.6	1.0
L-tryptophan	1.3	0.25
Taurine	0.3	-
L-tyrosine	0.5	-
L-tyrosine • $2Na_2H_2O$	-	1.4415
L-valine	3.6	1.0
Total nitrogen	**122.46**	**112.18**
Organic nitrogen	**76.99**	**66.71**
Inorganic nitrogen	**45.47**	**44.47**

(From Botanic Gardens Micropropagation News, August 1992, Volume 1, Part 5, on pages 59-63. The article is "Large scale asymbiotic propagation of *Cypripedium calceolus*- plant physiology from a surgeon's point of view.")

It has been suggested by Martin (pers. communication) that almost any intravenous solution of amino acids will suffice for use in asymbiotic culture of terrestrial orchids; he suggests using 1.5 ml of virtually any such solution per liter of media. There are several such products on the market, whose trade names will undoubtedly change over the years. At the present time, these compounds include Travasol 10% (Baxter), Aminosyn (Abbot), Nephramine and Heptamine (by Kendall), and Pharmathera of Memphis, TN produces an 8% amino acid solution for intravenous therapies. The use of amino acids in culture may not be limited to circumboreal terrestrials; they may find applications in tropical terrestrials as well, and their use in culture of the epiphytic species is unknown to the best of my knowledge.

Weber (pers. communication) has used commercial health food store preparations including amino acids as the nitrogen source, noting that food supplements from collagen and whey were found to be acceptable. Concentrations were not specified, but he notes that short roots are produced when the concentration is too high.

These media, when used in conjunction with a dark dormancy period after sowing, can often produce seedlings *in vitro* in a short period of time, but experimentation is almost always required. Several species of *Cypripedium*, including *C. passerinum*, are readily germinated via this route.

Steele (pers. communication) has noted that Harvais medium (from 1982, not 1974), modified through the addition of ammonium nitrate, has proven to be superior for germination and growth of cypripediums. See "Terrestrial Orchids," in Section 6.2.

Making Your Own Media

Doug Martin, of Biosource Orchid Labs (pers. communication), suggests that a spreadsheet may be used to help determine the total concentration of each ion used in the formulation of your own media. Thus, if you desire a fixed quantity of calcium in your media, you can play with the concentration of different compounds (calcium phosphate, calcium nitrate, calcium chloride) to get the right amount of calcium. While tabulating these numbers, the spreadsheet can also determine the concentration of phosphate, nitrate, chloride, and other anions. Through the use of a spreadsheet, it is possible to attain set goals for the concentration of each anion and cation, but a little chemistry is required. If you are unfamiliar with the methods, an introductory chemistry text will be of use.

Fruit Juices and Other Extracts

Pineapple juice has served as an excellent additive to media: 50 ml or so does not appear to inhibit germination, and has little impact on pH. Fig and tomato fruits may serve to enhance growth as well. Other fruits, such as grapes and raspberries, have toxic effects upon developing seedlings to the point of killing most of the population in some cases (Ernst, 1967b). Coconut milk has been known to inhibit the germination of some species, but growth is unimpeded in older seedlings (i.e., replate). Coconut milk (also called coconut endosperm) may be added at up to 20%, and sometimes more.

Banana is a popular ingredient and sometimes part of the formulation as it comes from the supplier. Banana powder can be purchased from Sigma, or you can make your own extract with a blender. Its properties are not affected by autoclaving. Different literature cite different results with regard to whether or not banana inhibits germination. The presence of banana certainly has a negative impact upon germination of some species, but it is not possible to make generalizations as to which species are or are not affected. Arditti (1968) used whole banana, water-soluble extract, water-insoluble extract, ethanol-soluble extract, and ethanol-insoluble extract, during *Cattleya aurantiaca* germination and growth. Whole banana was found to enhance growth for the first 89 days, but the control (without banana) surpassed the whole banana by day 140. The water-soluble fraction did not develop shoots and roots as well as the water-insoluble fraction. Interestingly, the ethanol-soluble fraction strongly inhibited germination, but the ethanol-insoluble fraction germinated and grew well, surpassing the control plants at 140 days.

Today, as mentioned elsewhere in this text, banana is routinely used to enhance seedling growth. Its addition, either as ripe, fresh bananas, or from baby food, is recommended by many growers. However, if from baby food, it should have no added sugar.

Tomato juice was once touted as satisfactory for media, and Arditti (1966) found it to promote germination. Unfortunately, *Cattleya* seedlings perish or develop abnormally after 80-100 days on such media.

Ernst (1967b) summarized previous literature and new experiments regarding *Phalaenopsis* germination and growth in the presence of several different additives including banana, pineapple, fig, mango, tomato, raspberry, Concord grapes, kiwi fruit, mushrooms, autolyzed fish, tryptone, peptone, and coconut milk. Banana was found to have the best results. Pineapple was found to have a "significant promoting effect," and fig and tomato also produced

positive results. Grapes and raspberries produced growth abnormalities and seedling death. Coconut milk "induced strong proliferation," but inhibited differentiation and did not significantly enhance growth. Mango, Kiwi, fish and mushroom extracts were detrimental. Peptone and tryptone (derivatives from enzymatic digestion of meat proteins) caused modest enhancement. Peptone today is used in some commercial media at 2 grams/liter.

Ernst (1975) found substantial growth enhancement with the addition of 0.2% powdered charcoal, as well as 10% banana homogenate when added to Knudson C media. Fresh and dry weights of *Phalaenopsis* were reported to have been tripled in the presence of these additives over the control group. Growth in *Paphiopedilum* cultures were similarly enhanced.

The use of beer as a growth enhancer *in vitro*, described as "exotic" (Arditti and Ernst, 1984), may have some basis in fact. Yeast from brewing contains B vitamins, which are commonly added in pure form to media formulations today (see Section 6.1). Thus, unfiltered beer with "body," composed of yeast and yeast fragments, and particles of hops from brewing, could serve to enhance growth; since these vitamins are available in purified form, this is more of a curiosity than a recommended additive.

Anticontaminants in Media

The use of compounds that may be added to prevent media contamination has been suggested. It is possible to construct an essential orchid media for seed germination or nodal propagation that contains certain components to inhibit bacterial and fungal growth. One early solution, as suggested by Johnson et al. in Spencer et al. (1979/1980) contains benlate, Nystatin, Penicillin G, and Gentamycin. Other suggested compounds include Amphotericin B, Dowicide A (an "agricultural fungicide and bactericide" produced by Dow Chemical at one point in time), Ethirimol (fungicide), PCNB (a "soil and seed fungicide"), sodium omadine (an "industrial fungicide and bactericide"), and Vancomycin.

Improvements in techniques for seed disinfection have resulted in diminished contamination and therefore reduced demand for anticontaminants. There have been concerns with respect to phytotoxicity although effective combinations that do not appear to harm developing seedlings have been created. Unfortunately, the overuse of antibiotics has become a very serious concern as more and more resistant strains of microorganisms have developed, but commercial propagation techniques often make use of anticontaminants to increase productivity, use relatively unskilled labor, and decrease the requirements for clean workstations.

Further, the addition of anticontaminants hardly means that one can conduct flasking with total disregard to sterility and other factors. Arditti (1982) describes how the glassware must be washed with alcohol and stored in a "clean, dust-free area" before coming in contact with the prepared solution and how the media must be prepared with water that has been boiled. He goes on to say that media incorporating anticontaminants as discussed in *Orchid Biology: Reviews and Perspectives II* are "not suitable for use in seed-germination media," but for seedling culture (or micropropagation via vegetative techniques) only. Sigma Chemical Company's catalog suggests that "antibiotics in tissue culture protocol [are] not intended as a substitute for proper aseptic technique," and adds that toxic reactions may occur in the cells being cultured. Unfortunately, many growers have come to rely upon anitcontaminants for all phases of their production.

One relatively recent addition to the anticontaminant realm is PPM, or Plant Preservative Mixture. It is composed of isothiazolone biocides, developed to optimize its disinfective properties while minimizing phytotoxicity. Its components defeat potential infection by acting on key enzymatic pathways in potentially infectious microorganisms, helping maintain sterility with plant cultures *in vitro*. Although widely touted for general use, its presence should be the exception, and not the rule. One possible use is in education, where those unfamiliar with sterile technique may learn the essentials while using media incorporating PPM while perfecting their efforts. PPM is to be used at a concentration from 0.5 to 2.0 ml per liter, but testing to determine the concentration best suited to individual needs may be required. It is available through several suppliers (see Appendix III, Sources). The use of microwaved media (see Section 3.6, Media, Sterilization, and Bacteriology 101 under Microwaves) generally involves the use of PPM to reduce the chance of contamination.

It is worth noting that PPM is expensive (about a dollar per ml at the time of this writing), and the general use of such preservatives is to be discouraged for the reasons given above. All the same, its use may be desirable for particularly rare or unusual material. It has been successfully used in flasking orchid seed without first disinfecting it. However, when combined with using baby food jars for germinating orchid seed, PPM may be very effective at inexpensively reducing contamination in mother flasks. At 25-30 ml of solution per baby food jar, 30 to 40 mother flasks can be made with a liter of media incorporating PPM.

To produce media that did not require thermal sterilization, Yanagawa et al. (1995) incorporated 0.01% sodium hypochlorite solution or 0.01% hydrogen peroxide solution. It is worth noting that these compounds both preclude the use of complex components, such as banana, activated charcoal, etc., as these will either bind the chlorine or

introduce overwhelming numbers of organisms that will almost invariably produce contamination.

The basic media for use with these additives is described in Section 3.7, Preparation of the Media, under Improvised Media. Media were prepared to produce a 0.1% and 0.01% active chlorine concentration. Both produced good germination, but at the 0.001% level, contamination was too high. Hydrogen peroxide, at 0.1% and 0.01% concentrations, was also acceptable.

For direct application of disinfectants to plant tissues, Yanagawa et al. determined that benzalkonium chloride was toxic at 0.2%, and ineffective at 0.02%. Several other solutions, 10% and 20% kitchen bleach and 0.5% and 0.05% active chlorine (sodium hypochlorite), were found to be effective in controlling contamination without mortality in several genera. Oxidol, a commercial brand of hydrogen peroxide, was ineffective at 0.35%, and toxic at 3.5% concentrations.

See also 6.5 New Directions in Seed Disinfection for more on the use of hydrogen peroxide for seed sowing and replate.

Nutrient Deficiencies

As has been noted before, proper nutrition is critical to maintaining orchids *in vitro*. We have learned much about proper nutrition of orchids in flasks, the product of over 8 decades of experience. Commercially prepared media are such that they have been formulated to provide optimal growth without deficiencies. Despite this, it is still possible for deficiencies to appear. Moreover, there is no assurance the media has been prepared correctly, and subsequent diagnosis may be essential if salvaging a critically important flask becomes necessary. Occasionally, efforts must be made to determine the cause of problems in media that are in use, whether they are formulated in your own lab, or purchased commercially.

Nutrition of plants is a complicated subject, and the reader is advised to seek a good library for clarification of these issues if so desired; it is simply not possible to cover more than the essentials here. Nutrition requires consideration of essentiality (a requirement for survival), deficiency (not enough of the element), and excess (toxic or antagonistic effects caused by too much of a compound). Some elements, such as iron, are well-studied, and their properties are clearly defined (required for photosynthesis). Others, such as nickel, are poorly understood, and their essentiality is unclear. Moreover, the presence of one element may make up for the absence of another by substitution; Rendig and Taylor (1989) note that manganese may substitute for magnesium in certain enzymatic reactions, for example. Interactions such as antagonism (where one element inhibits the function of another) and synergism (the combined action of two elements is greater than the expected sum) may cause unexpected effects.

To make things worse, orchid nutrition is in its infancy. Moreover, even if it were much more advanced, it is doubtful that the generalizations made on a handful of plants could be applied to more than a few other genera. Thus, orchid nutrition is largely based on extrapolation from what is known in other plants. Many of the characteristics of toxicity or deficiency listed below have been noted in food crops; specific interactions with orchids are not well documented, so the reader is advised to take the symptoms described with caution. These caveats should not be complete without reminding the reader that knowledge gained from mature plants may not always be applicable to seedlings, and applying them to seedlings *in vitro* may be impossible.

What is known is that certain elements are required for plant growth. Nitrogen, phosphorous and potassium (the N, P, and K of fertilizer) are macroelements. Sulfur, calcium, magnesium, iron, zinc, manganese, copper, molybdenum, boron and chlorine are also required, but in much smaller quantities. Requirements have not been established with cobalt, sodium, nickel, silicon, and vanadium, but they have been shown to play a role in accelerating the growth of some plants (Rendig and Taylor, 1989). Recent work has been such that nickel is accepted by some as being necessary to plant growth, probably in small quantities.

When a nutrient deficiency is suspected, the easiest solution is to simply transplant the seedlings to fresh media that is known to possess the elements required for growth. A determined hobbyist or researcher may decide to seek out the nature of the deficiency through trial-and-error, applying liquid overlays that have different concentrations of the elements suspected of causing the deficiency. In this manner, future media formulations may be improved. Some of this may be absorbed by the media. Plants directly absorb some of the rest. A layer of liquid several millimeters thick on top of the agar does not seem to affect orchids adversely.

The concentration of the elements used in the overlays must be chosen carefully. Too much may cause toxic effects, and too little will not have the desired result. Careful review of the concentration of the element thought to be in deficiency used in commercial media will help the grower determine how much should be used in an overlay. Then, if one wants to add, say, 10 ml of solution, this solution can be made up at several times the strength normally used in media. If media normally contains about 1 ppm of the element, the overlay might contain 2, 5, or 10 ppm, which can then be administered to the surface of the media. Results should be obvious within a couple of weeks for most elements. The entire plant may recover, or it may be that just new growths dramatically improve. Deficiencies

either affect new growth only, or the entire plant. Plants may be capable of redistributing elements that are becoming scarce; if this is the case, the entire plant may show symptoms of deficiency. Other elements are difficult to move around once they are incorporated into tissue; thus, the newest growth will show the strongest symptoms.

When suspecting a micronutrient deficiency, iron is a good starting place. As has been noted in Section 6.1, Media Modifications, the availability of iron *in vitro* is generally poor. Iron is required for the proper formation of chlorophyll, which is responsible for the green color of plants. Its deficiency results in interveinal chlorosis, meaning that the space between veins is yellow or very pale green, while the veins themselves are green. It affects new growth. Iron deficiencies have been experienced in media used to grow stanhopeas in our labs when using P-1056; however, since virtually all media use iron sulfate chelated with disodium EDTA, it is unlikely that this problem is specific to P-1056. Sigma Chemical Company, which manufactures P-1056 and other orchid media, notes that their media are manufactured in mills using techniques that meet FDA guidelines, and media are subsequently analyzed via inductively coupled plasma (ICP) elemental analysis to assure composition prior to shipping. Seckinger (pers. communication) notes that orchid labs often use P-6668 and P-1056 as a "base medium," to be optimized via trial and error for particular species of interest.

With iron deficiency, initial symptoms consist of the leaf tips of new leaves appearing as if they were sunburned; eventually, the growing point dies. This has been seen in several members of the Stanhopeinae, including gongoras and *Peristeria elata*. Symptoms have also been seen in *Dendrobium falcorostrum*. Liquid overlays of chelated iron are generally successful in reversing the symptoms if they are not too advanced.

When formulating media, chelators may help an iron deficiency, but once media has been poured, it may be necessary to apply a liquid overlay to remedy the problem. Solutions of iron plus EDTA are autoclaved, cooled, and added to the surface of the media as required. Ferric (Fe^{+3}) iron may also be used, but it is not as available to plants as ferrous (Fe^{+2}) iron. A quick-and-dirty solution is to add autoclaved solutions of balanced fertilizer, or chelated iron plant "food." A better patch consists of dissolving 54.25 mg of mixed iron sulfate and disodium EDTA in 50 ml of deionized water. This is then distributed as 5 ml of solution into each of ten test tubes, which are wrapped in foil and autoclaved. A flask with 80 ml of agar in it will use 5 ml of this solution to replace the iron that is normally required for growth. The mixture listed above consists of 2.78 g of iron (II) sulfate heptahydrate and 3.73 g of disodium EDTA. Adding this solution of iron will quickly provide an answer as to whether or not iron deficiency is the problem. Subsequent leaves will be green and healthy, but old leaves with yellow coloration will generally retain their chlorotic appearance.

Although there are certainly other deficiencies that may occur, iron is the easiest to diagnose, and most readily compensated for. Unexplained illness, death, discoloration, or stunting of growth may be due to nutrient deficiencies. The subject is too complicated to discuss here alone; further research may be required if symptoms persist.

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6.2 Specific Genera

In this text, we have discussed general parameters concerning the production of flasks for orchids only in broad terms. With respect to the proper storage of orchid seed, there is immense diversity within the Orchidaceae, and we can assume quite correctly that with this diversity we must face the fact that that no single set of parameters will satisfy germination and growth for all orchids *in vitro*.

Sadly, tragically, unfortunately, success in specific areas of orchid seed germination is kept as proprietary information by many growers. This secrecy is a function of economics, and it leaves others to reinvent the wheel each time one desires to master a given field.

What we have discussed so far will suffice for the majority of the epiphytic orchid species; fortunately, many of these germinate and grow relatively well using media based on formulae originally developed by Knudson decades ago (Knudson C was published in 1946). Terrestrials are another matter, but many still respond well to "traditional" media. Circumboreal (temperate) terrestrials, such as the cypripediums of North America, are quite different, and may require special treatment indeed. Some may require cold (Weber, pers. communication), and it is generally recognized that cypripediums require darkness for germination (Reyburn, 1978).

Some miscellaneous formulations:

Bob Hamilton's variation on Sigma Phytamax P-6668:

Sigma Phytamax P-6668, full-strength
Type "E" agar.....7 gm
Banana baby food (113 gm, or 4 oz.), free of added sugars.....1 jar
pH adjusted to 5.4, higher for paphiopedilums, if so desired.

This formulation is suitable for most epiphytic and terrestrial species.

For salt-sensitive species, modify by using Vacin and Went media as base. Then add agar, banana, adjust to pH 5.4, and go from there. Light (pers. communication) notes that Sigma Phytamax P-6668, full and half-strength, works well for pleurothallids and other salt-sensitive species, and coconut milk may be added at 50-100 ml per liter. G&B Mother Flask V has also worked well for her.

Torben F. Anderson's formulation for *Cypripedium*:

Murashige and Skoog Minimal Organics..... ¼ strength
Pineapple..... 25 ml per liter of mix
Sucrose..... 10-20 gm per liter
Gelrite..... 2.0-2.5 gm per liter

Some growers recommend the use of Dr. Morel's media (see Morel Chemical, Appendix III, Sources) for germination of *Paphiopedilum* seeds.

Terrestrial Orchids

The terrestrial Native American orchids, as well as other extratropical terrestrial species, enjoy a special place

in the hearts of many growers. This is a specialized field, and while there is an established background to the asymbiotic germination of tropical orchid seed, relatively little is known of propagation via this route with the extratropical species. The reader is strongly encouraged to read the pertinent literature, including the NANTOC Proceedings (see Appendix III, Sources), as well as Rasmussen's text on terrestrial orchids. There are a number of native orchid growers groups, which may specialize in your particular field of interest (European, Asian, Australian, or American orchids), whose expertise will prove to be invaluable.

The literature presented below may or may not have been superceded by more recent wisdom because it is difficult to tell what the "cutting edge" on terrestrial orchid seed germination may be at any given point in time. Some species are fickle when it comes to germination, and some have strange and curious requirements to get them to germinate. Worse, some germinate and then languish and eventually expire unexpectedly. Some, such as *Limodorum abortivum*, can be encouraged to germinate and occasionally grow. Unfortunately, this growth is often at an extremely slow rate, and it has proven difficult to get this species to do anything dramatic *in vitro*.

The following is a gross simplification, with a few pointers for additional literature. Remember: a few weeks in the lab can easily replace an afternoon in the library.

Many growers recommend the use of immature seed for flasking of these species. Arditti et al. (1981) note that "seeds from immature capsules germinated faster and in higher proportions than those from mature fruits," and Rasmussen (1995) indicates that many species grow better when seed removed from green capsules is used (embryo culture). Several growers have noted that embryo culture of many cold-tolerant species greatly enhances germination. Dramatic results include generally higher viability in *Cypripedium reginae* among seeds removed at 45 to 60 days after pollination, versus seed harvested before or after these dates. It is unclear whether this is due to one or more characteristics that change during late development of the capsule, but it has been suggested that late changes include differences in imbibition, or the absorption of water once the seed has been released. Thus, if this mechanism can be bypassed, treatments that affect imbibition are unnecessary.

Arditti (1982a) advocates the use of immature seed when sowing *Epipactis atrorubens*, *E. gigantea*, and *E. helleborine*, and media and modifications can be found in his paper on the subject. Rasmussen (1995) indicates that with *Epipactis palustris*, a cold treatment increases germination dramatically. He noted while almost no seed germinated after only 4 weeks at 5° C, germination was "significantly higher" at 8 weeks, and higher yet at 12 weeks. Many native species may require or be assisted by a cold treatment. However, it is a very complex subject, and certainly varies between genera, and often between species. Generalizations are not possible, and seeking previous literature on the subject will be important.

Arditti et al. (1982b) on the germination of *Goodyera* seed indicates that half strength Curtis media may be used for *G. oblongifolia* and *G. tesselata*. Also note that both require up to six and a half months to produce protocorms.

Arditti (1985) reported that *Calopogon tuberosus* germinated at "nearly 100%" using immature capsules (age not specified) on a modification of Curtis medium. Also discussed in this paper is the germination of *Calypso* ("did not survive transfer to fresh media"), *Cypripedium*, two species of *Piperia* ("have not developed past the protocorm stage"), and two species of *Platanthera* ("complete seedlings in 12-15 months"). Readers will be interested in this paper for the media modifications therein.

Cypripedium culture *in vitro* has been a series of hard-earned lessons, to which we add additional chapters every year. In contrast to the tropical epiphytic species, this is a much smaller market that has been realized only relatively recently. At the time of this writing, no fewer than five companies sell seedlings of native American orchids that are claimed to be propagated "in captivity," instead of having been dug from the wild. This presents a remarkable and admirable departure from the status quo, which until recently had consisted of providing plants that had been ripped up from the wild, with predictable survivability among the transplants.

Now, instead of purchasing plants with most of their root systems having been removed, the plants traumatized, and natural populations decimated or extirpated, we are able to purchase healthy, entire seedlings of known quality without having to wonder if laws have been broken in order to appropriate them. We are also able to purchase seedlings with specific qualities, known previously only from rare collections, without endangering the source population. Further, we can purchase plants whose parentage is well-known, and therefore be able to purchase a given species and be more than reasonably certain of its identity.

These remarkable advances should be recognized for what they are. This is one of the most sensible methods by which plants may be conserved, i.e., assigning a price tag to a plant in a manner that not only buffers wild material from dubious collection techniques, but also provides for a reservoir of plants to be grown in cultivation. In this manner, they can be conserved, their growth studied, and allow even more plants to be grown from ex wild material.

The North American Native Terrestrial Orchids Conference proceedings (see References, below, and Appendix III, Sources) is a good resource because it represents some of the latest work in the field, and makes an excellent reference. David Mellard's formulation that attempts to emulate Svante Malmgren's formula (as given in Section 6.1,

Media Modifications) is a good stepping-off point to more advanced work.

Light (1990) notes that *Calopogon tuberosus* germinates on "oats medium," composed of 25 grams of uncooked oat flakes (2 tablespoons or 30 cubic centimeters) and 12 grams of agar mixed together in a liter of water, boiled, strained, pH adjusted to 5.5, and then autoclaved. Other terrestrial species may be amenable to this medium.

Muick (1978) suggests that *Cypripedium calceolus*, *C. reginae*, and *C. macranthos* can be germinated in "mycelium-enriched humus without clay at a pH of 5.6," evidently using humus that was enriched with "coconut milk, banana milk, yeast, and sugar" in unspecified concentrations.

Steele (1995) notes that one or two cubes of potato about 5 mm on a side per 25 ml of culture media, will optimize culture of *Cypripedium reginae* in media with less than 1.2 mg/l of kinetin. In the absence of kinetin, a single cube of potato 10 mm on a side may be used. The culture media is simply poured into the tubes over the potato cube, leaving the cube at the bottom. Steele also discusses several important factors in the germination of cypripediums, and provides a formulation for stock solutions based on the media formulation by Harvais in 1982 that may be used to germinate this genus.

In a personal communication, Steele (1999) notes that he has used Harvais medium (see below), modified by adding 500 mg of ammonium nitrate per liter of medium. Germination and subsequent growth of protocorms was enhanced by reducing ammonium nitrate to 200 mg, and adding 200 mg of casein hydrolysate to each liter of mix. Steele reports that there may be benefits in eliminating the ammonium nitrate, replacing it with 400 mg per liter of casein hydrolysate. Steele's modification of Harvais' medium from a publication in 1982 is given below; ammonium nitrate may be used at different levels, but is given as 1400 mg per liter here.

Calcium nitrate [$Ca(NO_3)_2 \cdot 4H_2O$]	400 mg/liter
Ammonium nitrate [NH_4NO_3]	1400 mg/liter
Potassium phosphate [KH_2PO_4]	200 mg/liter
Magnesium sulfate [$MgSO_4 \cdot 7H_2O$]	200 mg/liter
Potassium nitrate [KNO_3]	200 mg/liter
Potassium chloride [KCl]	100 mg/liter
Ammonium citrate	19 mg/liter
Ferric ammonium citrate	25 mg/liter
Boric acid [H_3BO_3]	0.5 mg/liter
Copper sulfate [$CuSO_4 \cdot 5H_2O$]	0.025 mg/liter
Zinc sulfate [$ZnSO_4 \cdot 7H_2O$]	0.5 mg/liter
Sodium molybdate [$Na_2MoO_4 \cdot 2H_2O$]	0.02 mg/liter
Cobalt nitrate [$Co(NO_3)_2 \cdot 6H_2O$]	0.025 mg/liter
Potassium iodide [KI]	0.1 mg/liter
Manganese sulfate [$MnSO_4 \cdot H_2O$]	1.54 mg/liter

The NANTOC Proceedings (see Appendix III: Sources) outlines the various stock solutions for preparation of Steele's modification of Harvais medium. Durkee (pers. communication) reports that this formulation is used by him for commercial propagation of cypripediums with good to excellent results at the Vermont Ladyslipper Farm.

St-Arnaud et al. (1992) germinated *Cypripedium acaule* on Murashige and Skoog salt media with 5 mg/l thiamine, 10 mg/l nicotinic acid, 5 mg/l calcium pantothenate, 1 mg/l kinetin, 0.1 mg/l napthaleneacetic acid, 1.2 g/l Difco potato extract, 19 g/l dextrose and 7 g/l of Bacto agar. Seeds were started in darkness, then replated to identical media under 5.000 Lux light, 16 hours/day. Seeds used were harvested green at 30, 60 and 90 days after pollination; 60-day-old seed germinated the best at 15.8%, +/- 0.8%. 90-day-old seed germinated at 3.4%, +/- 0.8%, and the 30-day-old seed failed to germinate at all.

Ballard (1987) discusses factors in asymbiotic germination of *Cypripedium reginae*.

Pre-chilling of *Cypripedium calceolus* var *pubescens* at 5° C for 8 weeks greatly enhanced germination (Chu and Mudge, 1994).

Asymbiotic culture of several species of the genus *Cypripedium* with varying degrees of success are discussed by Hoshi et al. (1994).

For those that are determined to isolate symbionts and perform symbiotic germination, the literature by Breddy (1991) will prove to be of value.

Australian terrestrial species (*Pterostylis* species) are germinated via symbiotic technique in a paper by Clements and Ellyard (1979).

The effects of pH and darkness upon germination of *Cypripedium reginae* are discussed by Reyburn (1978). Harvais (1980) also discusses several issues on the germination of this species.

Disinfection of temperate terrestrial orchid seed seems to be a critical factor with regards to germination. Jürgen Böhm notes the orchids may be divided into five broad categories when it comes to pre-treatment of seed:

1) Those that bleach quickly, and may be sensitive to high concentrations of hypochlorite. Example: spiranthes species.
2) Seeds that respond well to a 30-minute exposure to a 5% calcium hypochlorite solution. Examples: *Ophrys, Dactylorhiza, Orchis morio, O. coriophora, O. palustris,* and *O. spitzelii.*
3) Species that require sulfuric acid treatment, or "optimized" hypochlorite exposure. Examples: *Gymnadenia, Anacamptis,Himantoglossum hircinum, Orchis purpurea, O. mascula, O. militaris,* and *Aceras.*
4) Seeds whose level of bleaching is difficult to determine as a result of an easily discolored embryo coat. Examples include *Nigritella* and some *Disa* species, which have seed coats that are difficult to bleach, and species whose seed coat remains yellow despite intense bleaching, including *Limodorum, Cephalanthera,* and some *Epipactis* species.
5) Species for which immature seed is required, as poor germination results despite best efforts to the contrary. Example: *Cypripedium calceolus ssp. calceolus.*

Böhm's protocols for seed germination are as follows.

Seeds are disinfected using a solution of calcium hypochlorite prepared by dissolving 25 grams of the solid in 500 ml of distilled water; after shaking to dissolve the solid, the solution is left to stand overnight, and the liquid decanted to leave behind any precipitate.

A small quantity of seed is soaked overnight in a small amount of water (1 ml) in a small vial (10 ml capacity). Add 8 ml of the calcium hypochlorite solution; if so desired, this may be preceded with exposure to a 1% sodium hypochlorite solution, but the two solutions should not be allowed to mix. If they do, a precipitate will form, and the seeds must be washed with 1% sulfuric acid if this happens.

The seeds must be exposed to the bleaching solution for at least 10 minutes. Böhm recommends monitoring the progress by examining under a microscope at 40x to determine the best duration of exposure, and notes that the time may range for minutes for sensitive species (such as *Spiranthes*, as above) to hours for some European species. With species whose seed takes hours to bleach, the progress may be accelerated by exposure to dilute sulfuric acid (1-5%), or by dual bleaching with calcium hypochlorite and sodium hypochlorite; either solution may be used first.

One technique used by Böhm is to expose seeds to 2% sulfuric acid solution for a few minutes, remove the solution and replace it with calcium hypochlorite and examine the seeds for swelling. When a "very small percentage" of the seeds start to swell, stop the reaction. Excess bleaching will cause the embryo cells to become more opaque, which is toxic. One alternative is to switch to a 0.5% calcium hypochlorite solution after initial exposure to the 5% solution but before swelling so that the seeds develop together. Böhm notes that this method, utilizing an optical technique to determine the progress in seed treatment, is unique.

Bleaching may be stopped immediately by flushing with 100 ml of sterile 0.01 molar hydrochloric acid, followed by 200 ml of sterile water. Seeds may then be resuspended in water, and poured into Petri dishes. Böhm seals these with Parafilm, and incubated at 25° C in the dark; a few species, such as *Disa uniflora,* may be germinated in low light.

Böhm uses an immersion filter to remove one solution before replacing it with another. This is constructed with a #2 porosity sintered glass disk, 10 mm in diameter, manufactured by Schott. This is fit into the barrel of a disposable syringe, cut at the 0.2 ml mark, with the Luer tip connected to an aspirator for suction. The filter is kept sterile by immersing in fresh calcium hypochlorite solution between uses.

For those species for which dry seed fails to prove reliable, green capsule technique may be used. Capsules should be harvested at 2/3 the time required for dehiscence, which is about 50 days after pollination for most species with a rhizome according to Böhm. Dry seed may take weeks or months to produce results. Poor results may require modification or experimentation on any one of a number of factors listed above. *Cypripedium montanum,* which had been reported to be extremely difficult to grow from dry seed, reportedly germinates and grows readily from immature capsules (May, pers. communication).

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6.3 Making Your Own Glove Box

As discussed before, use of an aquarium is satisfactory for performing small volumes of flasking. Indeed, a 20-gallon aquarium can be used to efficiently flask a fairly large number of bottles while effectively preventing contamination. With a helper to bring more flasks and take away freshly sown bottles, a small "assembly line" can be created that can prove to be quite productive.

All the same, it is easy to outgrow the requirements of an aquarium, so growers may be interested in producing their own glove box. It is worth pointing out that glass, despite its detractions, is probably the best material with which to produce your own glove box. It is heavy and fragile, but it is also chemically resistant, difficult to scratch, and capable of being sealed to other glass sheets with silicone caulk–something that untreated Lexan and similar plastics are not able to boast.

Arditti (1982) shows how to manufacture a simple flasking area from a cardboard box (Figure 6.3.1), some sheet plastic, and aluminum foil (foil corrodes away when exposed to bleach solutions for too long). If so desired, the

box can be made of glass replacing foil and cardboard, with the edges mated with silicone sealant. General Electric manufactures the best sealant for these purposes. It is a clear silicone rubber sealant commonly available at hardware stores for the purpose of sealing windows and around bathtubs. It has an extremely long life, and resists most commonly available chemicals with ease. It bonds to the glass with great strength and, if applied correctly, will not leak even under pressure. There are two grades of this material, one of which is for use with food surfaces, the other of which is not. Purchase the version that is rated for food surfaces or use in aquariums; it should have a stamp of approval stating that it meets NSF Standard 51 (Any-Type Food).

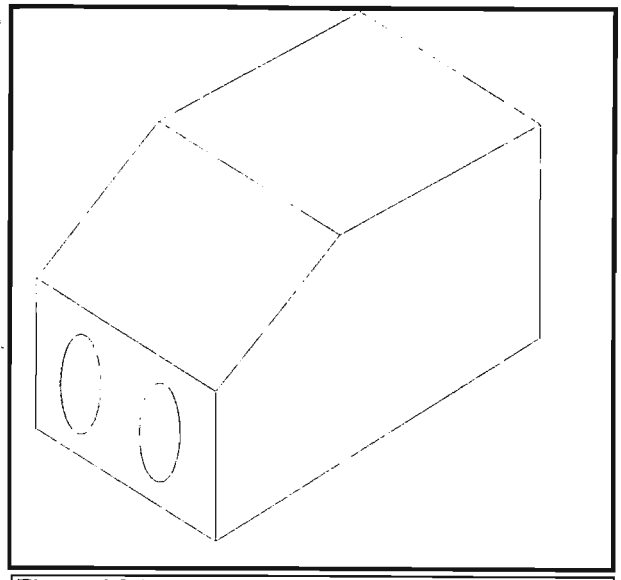

Figure 6.3.1: A very simple glove box. After Arditti (1982).

If you wish to construct a glove box using sheet plastics, it is best to first test a piece by placing a small strip of silicone caulk on the surface, and then trying to pull it off after it has cured for 24 hours or more. Most plastics that are available to the consumer will not allow silicone caulk to adhere to flat, untreated plastic. Nevertheless, there are two options available.

If you have access to proper tools, and can create very fine mating surfaces, plastics can be bonded together with solvents; dichloromethane will dissolve enough of the host plastic when it is added to Lexan, PVC, acrylic and some other plastics that it can be used to form a solid joint. This does take two very flat surfaces as well as some practice because an unsuitable bond is formed between surfaces that are not flat enough (note: flat does not necessarily mean smooth).

Most will have to make do with a much cruder technique, but it allows us to make use of silicone caulk by roughing the edges of the mating surfaces, where we would place the caulk. Using coarse (80 grit) sandpaper, or a small motor tool with a cylindrical sander, rough up a ¼" strip along the side of the plastic. This surface will hold silicone caulk with enough strength for our purposes, but one would be best advised to test whatever plastic you are using prior to construction to be certain. Silicone caulking will not adhere to smooth Plexiglas, for example, so it must first be physically abraded before bonding.

The construction of a glove box may be very much like that of an aquarium. All other factors being equal, it is not terribly important for the box to be constructed to do anything other than 1) maintain an atmosphere that will not openly be exchanged with outside air, eliminating that source of contamination and 2) provide a hard, non-porous bottom surface that will not leak sterilant solution all over the place, and will be readily disinfected to provide a good working surface. A short article by Roberts (1986) in the August 1986 *American Orchid Society Bulletin* is recommended reading for anyone who wishes to build their own glove box.

As a result, the reader will want to keep in mind the approximate working dimensions, and go from there. One of the most important tools that you can use is a 2x4 or other flat wood with two grooves in it, each set at 45 degrees relative to the length, and at 90 degrees to each other (see picture). The groove should be the thickness of the glass used, so when slotted over two pieces of glass, it will produce an adjustable jig that will maintain a precise 90 degree angle between two sheets of material such that they can harden in place (Figure 6.3.2).

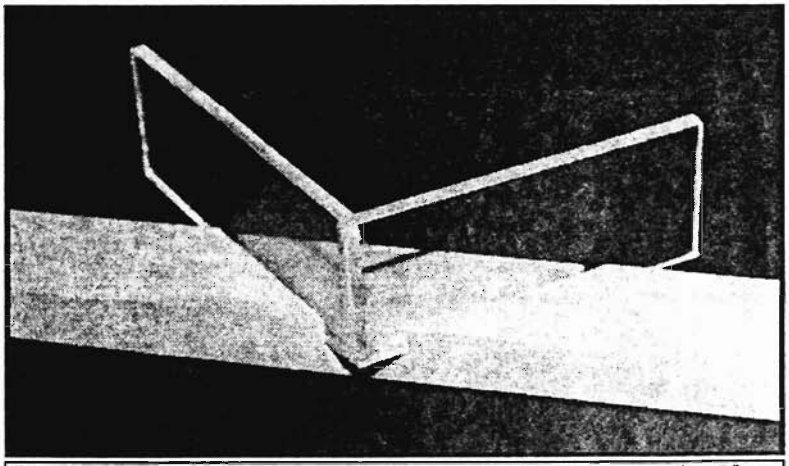

Figure 6.3.2: A simple wooden jig used to align parts at a precise 90 degree angle while bonding.

If so desired, it is possible to coat the bottom of the glove box with plastic by layering it with a solvent that is virtually saturated with plastic. Such commercial "tool dips" are available, but the most desirable color (white) is difficult to find. Permatex makes such a product, and Dri-Dek manufactures a special plastic mat that can be

used to keep your work out of a puddle of disinfectant (see Appendix III, Sources).

Another variation on the aquarium glove box theme is to seal the open side with a sheet of clear plastic. Taped over the front of an aquarium that is laying on its side, two holes are cut to admit the arms; this will form a tight seal. Use of long gloves may be in order, to keep from pulling out arm hairs.

One last note is that surfaces may be treated with silicone caulk to afford them protection against the caustic or corrosive substances used in flasking; even stainless steel may corrode when exposed to water under the right conditions, and bleach is particularly bad with its high concentration of sodium hydroxide. Still silicone caulk is very thick, and does not apply evenly to most surfaces. If so desired, it may be diluted in hexane or other non-polar solvents, which may then be sprayed onto the surfaces to be treated. Diluting silicone caulk, one part in five parts hexane, works fairly well. Apply lightly, and then apply additional coats as required.

It is worth noting that this technique is not without its hazards; hexane is very flammable and it is a powerful solvent, and exposure should be minimized. When carrying silicone caulk, it will deposit upon any exposed surface (including your skin, lungs, etc.). If you use this technique, take great care to protect the eyes, skin, lungs and respiratory tract. If you have any doubts about what you are doing, DO NOT DO IT.

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6.4 Manufacturing Baffle Ports for a Glove Box

This section is placed under its own category, since people may wish to retrofit an old glove box with baffle ports, rather than simply equip a new one with them.

Ports are invaluable to the flasker that wishes to have a tightly sealed glove box for whatever reason. As we have discussed before, it is not absolutely essential that a work space be air tight, but drafts are certainly not desirable. To prevent excess air leakage from a glove box, it is important to have a tight fit between the wrist and the box. This can be done either with large, bulky gloves that eventually fill with perspiration, or with modified ports that will allow the worker to have a tight seal while working either with bare hands, or with wrist-length gloves if one is sensitive to prolonged exposure to bleach or other chemicals.

Commercially available ports cost between $200 and $500, but it is relatively quick and simple to manufacture your own for a fraction of this cost. Other than a few readily available tools, the only material required is the sheet material from which you will manufacture the ports. Many different materials are commercially available, but the materials must be tough, durable, and chemically resistant. Some examples would be silicone rubber, and latex rubber, both of which are available in a variety of thicknesses. One commercially available product is the universal red silicone rubber used to produce large gaskets; check the telephone directory under seals and gaskets, and ask for this material. Many thicknesses are available, but around .070" seems to be a good working thickness. A sheet 36" x 36" cost $38 retail locally, and is enough to produce about 12 layers of port material for ports up to 9" diameter. This material is very soft and pliable, and is not to be confused with a very similar product that is somewhat more rigid and resistant. The gasket-making rubber that is referred to here will flop back and forth like a sheet of paper when held on edge, the rigid material will not.

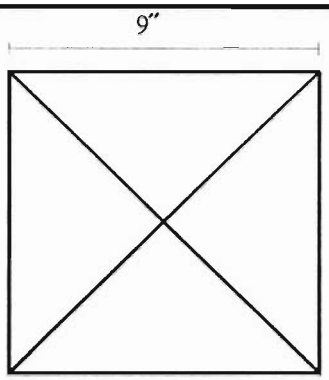
One tool that you will require is an arch punch, used for making holes in soft material such as leather and rubber. Size is relatively unimportant, and ⅛" or ¼" will be satisfactory. These tools can be found at most hardware stores.

It will also be important to have sealing rings, which will be discussed later.

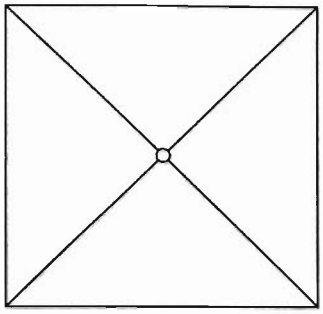
When making baffle ports, the most important factor is the size of the holes used for access into the glove box. Since most are around 6" diameter or so, we will work with this number as an example, along with a piece of sheet rubber material around 36" square.

The first task is to section the sheet rubber into squares large enough to accommodate a 6" diameter hole, plus a little excess for a sealing ring. If the ring is about 8" outer diameter (OD) and has a 6" inner diameter (ID) hole, we can cut our rubber into 9" squares (step 1). For the size of sheet rubber given, this allows us to make 16 squares of material.

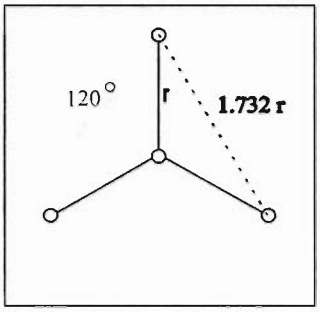
Use a ruler and a marker to lay out a grid on the surface of the sheet rubber. It is not terribly important to make the grid squares at exact right angles, since the "squares" will be reduced to circles when we are done and the excess trimmed off. Cut the sheet material into squares on a soft surface, such as several layers of cardboard, so that the cuts go all the way through. Use a sharp blade, such as a single-sided razor blade. As you will only need 6-10 squares for



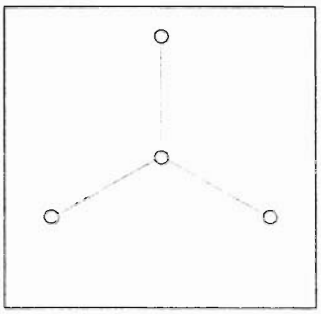
Step 1: Find the center of a square of the material.



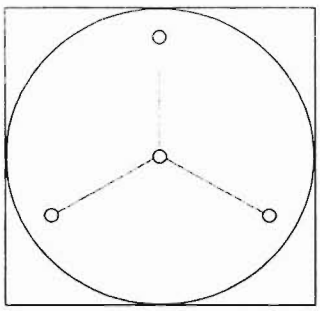
Step 2: Use an arch punch to punch a hole at the exact center.



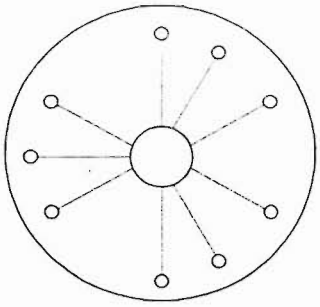
Step 3: Draw radius r to one edge, then two more radii which terminate as shown.



Step 4: Use razor blade to cut each of the lines traced. Each line terminates in a punched hole.



Step 5: Cut out circle of material to fit glove box ports.



Step 6: Stack several ports (three shown) and cut out center and bolt in place.

Figure 6.4.1: Cutting material for baffle ports.

a pair of ports, it will not be important to make them all perfect, so make some extras so that you can replace ripped or damaged layers later on.

Take a single sheet, and find the exact center by drawing an "X" between the corners of the sheet. This will be the center of an equilateral triangle which will be the center of the layer through which you will place your hands. Inscribe another "X", this time going from the middle of each side to the opposite side of each sheet. Along one of these sides, trace a line that goes ½ the diameter of your glove box holes; in this case, make the line exactly 3" long. From this line, you can use a protractor to measure 120 degrees to either side of this line, and make two more lines exactly 3" long as well. Or, if you would enjoy greater precision, the distance between the terminus of any two legs is

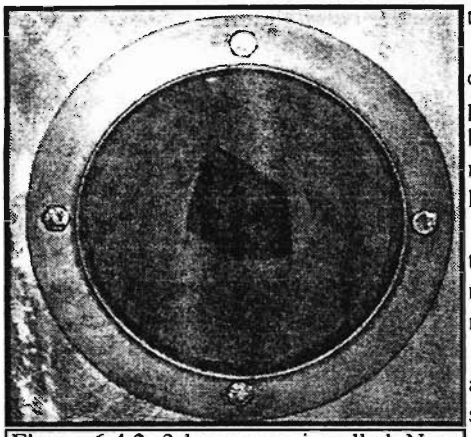


Figure 6.4.2: 3-layer port, installed. Note enlarged center, to accommodate arms.

the radius times the square root of three. Enjoy.

Place the individual sheet on two or three layers of cardboard, or some other soft, yielding surface that will not damage the fine edge of the arch punch. Practice using the arch punch on a small piece of scrap. Use a hammer to produce a hole at the exact center of the sheet (step 2). Then make a hole at the terminus of each of the three lines, producing an equilateral triangle with a center hole (step 3).

Return the sheet to the cutting board, and use the razor blade and a ruler to cut along each of these three lines (step 4). It is very important not to nick the opposite edge of the holes produced by the arch punch, as this may weaken the material.

When you are done, make sure each of the three flaps that are produced are separated, and that the holes at the ends are not damaged. The holes serve as strain relievers; they will keep the slits in the material from propagating and ripping the sheet.

Repeat this procedure until you have 3 or 4 sheets. Trim the material to fit your ports (step 5). At this point, stack them so that each consecutive sheet is 90 degrees out of phase with the previous one (step 6). When you have them stacked, push your hand through the middle; notice if the flaps secure around your wrist, producing a barrier that allows little air to pass by. If you are satisfied, go ahead and continue to make more sheets. If not, try adding another sheet or so until you are satisfied. Remember, you do not have to have an absolutely airtight seal; all you need to do is restrict circulation. The center may be cut now or once they are mounted to make access easier by enlarging the diameter for access.

Once you have two stacks of the appropriate thickness, you can secure them in place on the front of your glove box with an outer sealing ring and a few bolts. The material for the rings is not important; you can cut them yourself out of sheet steel, Lexan or similar plastic, or even have a machinist turn them from some flat stock. Drill three or four holes in them so they can be bolted through to the interior of your glove box (Figure 6.4.2). Punch holes through your sheet rubber very carefully so that you do not lose alignment when you seat them in place.

A good baffle port will obstruct air flow, and yet permit full use of the glove box. If the layers are sticky, use a silicone spray to lubricate them. Oils are messy, and will adversely affect some rubber compounds, particularly latex. If done correctly, they will allow your forearms to slide through with ease, without removing too many arm hairs (which increases risk of contamination). Keep a stock of extra layers handy in the event of a rip or a tear; the strain relievers might eventually give out, and it is very difficult to glue certain compounds like silicone rubber. All the same, it is worth trying to repair them in place with a bit of cyanoacrylate glue, such as SuperGlue. Clean the rip with a solvent such as acetone or ethyl alcohol, and apply the glue in small volumes. If this fails, you will probably have to replace the panel. Two ports with 4 layers each have been in use at the OSP for over a year with no failure or rips.

If needs be, you can get by with up to a 2-3" diameter hole in the center when modifying the ports for access, while still reducing air flow and potential contamination. Access is made easier by washing the hands and arms with soap and water (as they should be before using a glove box) and keeping the forearms wet while pushing through the ports.

If not available locally, sheet rubber and similar compounds are available through McMaster-Carr and similar suppliers. However, most are available inexpensively, so shop around if the prices seem too high.

6.5 New Directions in Seed Disinfection

There have been no major innovations in seed disinfection, and existing techniques remain essentially unchanged for over 50 years. Accordingly, some researchers propose that the existing techniques are sufficient, and that there is no need to change them. Of course, there really was no need to fly in planes that weren't made out of bicycle parts, used fabric for wings, and whose passenger list consisted of one man, lying down in front. Still, some modicum of comfort exists in the modern aircraft, and our problems have improved somewhat: for the Wright brothers, one concern consisted of not having steady wind. Today, our greatest concern is that the flight attendant might have a parachute, and we, the passengers, might not.

There is a great deal of room for improvement in orchid seed germination. It would be good to find a technique that does not require washing, or the use of any liquid at all. Some people would contend that green capsule sowing does exactly this, but the risks and benefits of sowing dried seed or green capsule seed are poorly known. See the section on orchid seed sowing for further discussion of this subject.

It would be difficult to argue that hypochlorite disinfection is not a successful route toward producing seed that is sterile without excess mortality. Still, we look forward to continued innovations in this field.

Thermal

In at least one other plant family (rice), *in vitro* cultivation consists of thermal disinfection of the seed. This occurs at a temperature akin to that of Pasteurization of milk products, which is about 72° C (161° F). Rice seed evidently maintains its viability despite brief exposure to temperatures this high. However, one report of *Orchis* seed being exposed to 50° C for just 5 minutes indicates that otherwise viable seed was killed by exposure to such high temperatures. There is one report (Pritchard, 1993) of *Dactylorhiza maculata* being stored at 62° C for 6 days, with 4% of the seed still germinating, so this is not necessarily a lost cause.

Gas

Other types of chemical sterilization may work; it has been suggested that gas disinfection of seed similar to that in which glassware and other objects can be sterilized with ethylene oxide (EtO) has potential as a disinfecting agent. See also Chlorine Gas Sterilization, in Section 4.2, Disinfection of Seed.

Peroxide

It has been suggested (Arditti, 1982; Zytaruk, pers. communication; Rasmussen, 1995) that hydrogen peroxide, in a 3% solution, will suffice as a disinfection agent, particularly when preceded by a 4-hour soak in a 4% honey solution at 37° C (98° F). Snow (1985) used a 24 hour presoak in a solution consisting of 1 teaspoon (about 5 cubic centimeters) of table sugar in a cup of water (about 237 ml), after which the solution is removed with a small bore hypodermic needle (22 gauge, small enough to exclude any seed) and replaced with about 2 ml of 3% hydrogen peroxide. The solution is agitated every 5 minutes for about 30 minutes and then the peroxide is removed in the same manner as the sugar solution. Snow recommends transferring the seeds into flask by means of a sterile wire loop after adding a few drops of sterile water.

It has also been suggested (Snow, 1985; Snow, 1987; and Yanagawa et al., 1995) that media may be prepared without autoclaving by adding small concentrations of hydrogen peroxide to the media during its preparation or to seedlings and protocorms during replate. Snow (1985) reports that a concentration of 0.005% (addition of 1.73 ml of 3% hydrogen peroxide per liter) prevents contamination of media when glassware, lids and utensils have been surface-decontaminated with a solution of Physan 20 (1 part in 256 of water), a quaternary ammonium compound. The media must be very hot when the peroxide is added, and distributed into freshly decontaminated flask. Media with a concentration of 0.1% was used to germinate non-disinfected seeds of several genera, often with acceptable seedling and protocorm development, usually without contamination. Seedlings of *Cattleya, Laelia, Encyclia, Dendrobium* and *Leptotes* species did poorly and usually perished when transplanted onto this media.

Concentrations below 0.05% were too low to be effective, but were still toxic to seedlings of the above genera.

Snow (1987) later presented an alternative by which the hydrogen peroxide is destroyed by the enzyme catalase. A 5,000 unit per ml solution of the enzyme is prepared in a buffer solution and preserved with 0.25 ml of a 37% formaldehyde solution (Warning: toxic and carcinogenic!), stored in a refrigerator between uses. One ml of this solution was added 24, 48, and 72 hours after sowing, or at 12, 24, and 48 hours after replate. Transplanting seedlings required a single addition of catalase after about a week, or sooner with more sensitive species.

Yanagawa et al. (1995) found that a 0.01% solution of sodium hypochlorite or hydrogen peroxide would serve to create media that did not require autoclaving for sterility. Spraying media with a 0.5% solution of sodium hypochlorite and spraying the media and plantlets with a 0.05% solution of sodium hypochlorite were effective for sowing seed that had not been disinfected, and replating respectively.

These techniques advocated by Snow and Yanagawa et al. are touted as being capable of being used in the absence of a sterile sowing area or other extreme techniques that are normally reserved for seed germination, replate and so forth.

Hypochlorites

There are a variety of hypochlorite salts that have been synthesized. Lithium, sodium, potassium, magnesium, calcium, strontium, and barium have all been used as the cation (Trotman-Dickenson, 1973). Of these, the sodium, calcium, and lithium salts are commonly commercially available. The use of lithium hypochlorite in the disinfection of orchid seed seems to be absent from the literature.

Chlorine in the form of the hypochlorite ion (OCl^-) combines the best properties for disinfection of orchid

seeds. It offers relatively low toxicity in conjunction with being relatively easy to work with and being readily available. Despite its long track record, the parameters for optimizing its efficacy for our purposes have only recently come to light. Discussion of the hypochlorite ion and its relationship with hypochlorous acid (which possesses the disinfection power) requires understanding of the three equations below.

$NaOCl \Leftrightarrow Na^{+} + OCl^{-}$
(Equation 6.1)

$Ca(OCl)_2 \Leftrightarrow Ca^{+2} + 2(OCl^{-})$
(Equation 6.2)

$HOCl \Leftrightarrow H^{+} + OCl^{-}$ (pKa = 7.53)
(Equation 6.3)

Sodium hypochlorite, or commercial bleach, is prepared by bubbling chlorine gas through a solution of sodium hydroxide, forming sodium and hypochlorite ions in solution (Equation 6.1). In this manner, chlorine is stabilized as the hypochlorite anion by the high pH of the liquid. Solutions of calcium hypochlorite (Equation 6.2) are also alkaline. The "saturated calcium hypochlorite" solution used traditionally for flasking orchid seed is very alkaline. The true "killing power" chlorine comes from hypochlorous acid, or the HOCl in Equation 6.3. HOCl is approximately 80 times more effective at disinfection than OCl^{-}. The pKa of the dissociation is 7.53; at a pH above 7.53, there is more hypochlorite ion. Below this pH, there is more hypochlorous acid. At a pH of 7.53, the two are in equal concentrations at equilibrium (Cadena, web URL). Thus, the efficacy of disinfection using hypochlorites may be increased dramatically by carefully lowering the pH. Further, species having been reported as being sensitive to chlorine may be revisited using techniques involving lower concentrations of hypochlorite at a lower pH. It is quite possible that sensitivity demonstrated by some species in the past may be due not to chlorine but to the high pH.

The hypochlorite ion may also be prepared in a solution of DCCA to water, or dichlorine monoxide. DCCA (dichloroisocyanuric acid) is discussed below. Dichlorine monoxide is too difficult to work with for discussion here.

It has been reported that some seed, such as that of *Phalaenopsis*, may be particularly sensitive to sodium hypochlorite, and either dilute calcium hypochlorite or alternative solutions should be used if you experience difficulty in sowing a particular type of seed. Of course, some growers report success with whatever they use, and some report no success at all when using diluted bleach solutions. As noted above, it may not be the chlorine sensitivity so much as the seed is sensitive to the extremely high pH of "classic" saturated calcium hypochlorite solutions. Alternative hypochlorite disinfection techniques should be sought, such as the following by Fred Bergman.

Bergman (1996b) utilizes a technique by which seed is disinfected with a dilute calcium hypochlorite solution. Relying upon the principles behind Equation 6.3, he developed techniques by which seeds are disinfected at a lower pH, increasing the activity of the chlorine.

A stock solution of calcium hypochlorite with 2000 ppm of available chlorine is prepared by using the concentration of available chlorine (AC) as a guideline. Divide 2.0 (for 2,000 ppm) by the decimal equivalent of available chlorine, which is generally given in percentage. For new calcium hypochlorite, this will be 65% (0.65). Its potency is reduced as it loses chlorine to the environment, so only new, pure calcium hypochlorite is this high. Thus 3.08 gm of calcium hypochlorite (2.0 divided by 0.65 = 3.08) dissolved in a liter of water provides us with a 2000 ppm concentration of available chlorine.

This stock solution should be stirred and then filtered and stored in an amber bottle, or similar vessel protected from light with aluminum foil, and refrigerated; under such conditions, it should be suitable for "at least a year."

This stock solution can then be diluted with water 8:1, and its pH adjusted with white vinegar until it is between pH 5 and 6. Add detergent, and use while the solution is still fresh. The concentration of AC (250 ppm) is unstable due to the pH adjustment; it is recommended that you use it within an hour.

The seed is added to 3-5 ml of this solution, and agitated for 10-15 minutes. The entire solution is then added to the flask, disinfectant and all, and the flask rocked back and forth to dispense the seed across the entire surface. After sitting for a minute or two, the flask is re-opened, and the excess solution drained out, leaving behind most of the seed.

It is worth noting that there has been relatively little work done in terms of modification or standardization of the pH at which orchid seed is disinfected. Given that the activity of chlorine is dependent upon pH, the use of a buffer solution in which the seed is disinfected such that the pH could be accurately controlled would seem to be highly desirable in terms of standardizing specific techniques by which orchid seed is disinfected.

Some suggested buffer solutions are given below.

Table 6.1: Buffer Solutions

pH	ml of 0.1 molar NaOH
5.00	22.6
5.10	25.5
5.20	28.8
5.30	31.6
5.40	34.1
5.50	36.6
5.60	38.8
5.70	40.6
5.80	42.3
5.90	43.7

For those buffers listed in Table 6.1, the specified amount of sodium hydroxide (0.1 molar NaOH) solution should be added to 50.0 ml of 0.1 molar potassium hydrogen phthalate; this will yield the desired pH at 25° C.

Table 6.2: Buffer Solutions

pH	ml of 0.1 molar NaOH
5.80	3.6
5.90	4.6
6.00	5.6
6.10	6.8

For those buffers listed in Table 6.2, the specified amount of sodium hydroxide solution should be added to 50.0 ml of 0.1 molar potassium dihydrogen phthalate; this will yield the desired pH at 25° C. Note that the first table requires potassium *hydrogen* phthalate, and the second one relies upon potassium *dihydrogen* phthalate.

Hypochlorous acid is one of several acids collectively known as "hypohalous acids." Addition of bromine or iodine salts in the presence of hypochlorous acid may be used to produce other hypohalous acids. For example, sodium bromide may be added to a sodium hypochlorite solution to produce hypobromic acid. Hypochlorites will oxidize bromide to hypobromite, and either hypochlorite or hypobromite may be used to oxidize iodine to hypoiodite in alkaline solution (Trotman-Dickenson, 1973). At lower pH, the hypohalous acid will predominate. Caution should be used, however, to avoid acidifying strong solutions as toxic gas may be emitted. These techniques are to be used only under lab conditions by knowledgeable individuals using appropriate protective gear and ventilation. Hypohalous acids other than hypochlorous acid have been largely ignored, despite their potential as disinfection agents. Such mixed hypohalous acids might consist, for example, of a solution of hypochlorous and hypobromic acid, prepared by adding small amounts of sodium bromide to a dilute calcium hypochlorite solution at low pH (6.5). Preparation of the hypoiodic acid would occur in a similar manner. Some knowledge of stoichiometry is required to prepare such solutions.

Duration of Exposure with Hypochlorites

Northen reports success with sowing *Cattleya* seed that has soaked in hypochlorite solutions for prolonged durations, some for hours. Others report that *Phalaenopsis* viability drops off sharply with extended exposure times, or high concentrations of hypochlorite, but it is unclear whether this is a function of calcium hypochlorite or sodium hypochlorite. It may be that this is a function of pH, instead of chlorine concentration.

In either event, there are some reports of greatly enhanced germination with some genera and species when prolonged exposure times are used. Reports that some terrestrial species may be soaked in hypochlorite solutions until they blanch are not uncommon, sometimes with exceptional results. Rasmussen (1995) reports that some growers may leave seeds of *Dactylorhiza*, *Gymnadenia* and *Orchis* in solutions of 5% sodium hypochlorite with a wetting agent (1% Tween) for 4 to 6 hours and even longer to reach optimum yields. Symbiotic germination of *Liparis lilifo-*

lia was optimized with a 2-hour pre-soak in 5% calcium hypochlorite, with duration of exposure ranging from 30 minutes to 8 hours. Experimenting with duration may be required in some cases to produce the best results with some terrestrial orchids, as well as the tropical terrestrial species.

Virkon S

Virkon S, a product of Antec International, is new to orchid seed disinfection. The product has been used successfully on food crops in Europe for some period of time to control plant disease and organisms that cause crop spoilage, with "a lack of phytotoxicity in major cereal crops" according to the company's literature.

Those who use Virkon S to disinfect their seed do so with a 0.5% to 2.0% weight solution in water in a manner identical to that used with other hypochlorites. A rinse is said to be optional, but until further work is done to determine whether or not it is phytotoxic to orchids *in vitro*, a rinse is suggested. A 2% solution can be used for heavily contaminated seed. Fournier (1998) suggests using ¾ teaspoon in 500 ml of distilled water for a 1% solution, soaking the seed for 3 to 4 minutes and then using filtration to separate seed from solution, and placing the filter paper directly on the media.

It is biodegradable, so it is not clear how long working solutions of disinfectant can be stored. It is suggested it be stored in clean glass, preferably amber, and kept under refrigeration. The compound itself is reported to have an LD_{50} (lethal dose for 50% of a given group) of 5000 mg/kg in rats–a large quantity would have to be ingested. No LD_{50} for humans is listed, nor is there any carcinogenicity, teratogenicity, or mutagenicity data given.

Virkon S is also used for salvaging infected flasks (see Section 5.1 Dealing With Contamination Problems).

Unlike alcohol or other hypochlorites, it has a pleasant, lemony odor, making it much more pleasant to work with in glove boxes. A pink dye is added (amaranth coloring), whose presence indicates the activity of the solution: when it turns clear, the solution must be replaced. It is evidently unstable at room temperatures in open containers, but solutions may be prepared and stored in the refrigerator in opaque bottles for at least a month. Due to its coloration, it must be stored in a safe place: the mixed solution looks like a fruit drink, which is obviously unacceptable with kids around the house.

DCCA

Dichloroisocyanuric acid (DCCA) has been recommended for use in orchid seed disinfection by Gardner (pers. communication). Although treating seeds with a solution of 300 to 500 ppm DCCA with a soak time of about 20 minutes are recommended, this has lead to high contamination rates for me. It has been noted that this concentration may be useful for disinfection of explants for tissue culture when washing explants for 24 to 48 hours. I have experienced good to excellent results when disinfecting seeds with DCCA at 3.0 to 5.0 grams per liter of distilled water. Unlike sodium and calcium hypochlorite, the pH of strong DCCA solutions is slightly acidic, increasing its activity without having to first change its pH.

Users will have to experiment with concentrations and times to best suit their applications. Preparations of this compound should be refrigerated, and discarded after about a month, or sooner if they show signs of losing efficacy. It is relatively new to tissue culture and other facets of aseptic plant cultures, so experimentation is called for. Gardner suggests that this product demonstrates low toxicity, and may be used for salvaging contaminated flasks.

DCCA is available from chemical supply companies, and its Sigma product number is D-2536. Check the label to determine if it is anhydrous, or the dihydrate. If the latter, you will have to use 16% more by weight to achieve the same strength as the anhydrous compounds. Store the solid as you would calcium hypochlorite, tightly sealed to prevent decomposition or water gain if anhydrous.

Outdated Techniques

In the past, bichloride of mercury ($HgCl_2$, or mercuric chloride) has been used to disinfect seed. This compound is toxic, and its use should be be avoided at all costs. In general, mercury salts have no place in the modern lab. They are toxic, and bioaccumulate in the food chain if disposed improperly. For our purposes, mercury salts have no advantage, and cause many more problems than they are worth.

Potassium permanganate has also been used for disinfection of seed (Sanders, 1951). A solution of 4 grams per liter of water has been used to disinfect seed. Potassium permanganate is a strong oxidizer, and stains surfaces and skin a brilliant purple color. It, too, has more drawbacks than advantages. It has been used in the past to disinfect skin as a dilute solution, and is relatively safe. However, it is less effective than hypochlorites. It is also incompatible with many other chemicals, and is a storage hazard. Other than for use with the McEwen flask (see Section 4.4, Variations on a Theme), it has little use in orchid seed germination.

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6.6 Practicing with Inert Compounds

Orchid seed is very hard-earned material. Our plants spend months or years just making the stuff, and then we spend hours of preparation time to sow it. To the best of my knowledge, nobody has suggested a surrogate for orchid seed. This means that we have always had to practice with orchid seed in order to get results. Obviously, it may be desirable to substitute another compound so that we may be able to learn the technique without wasting seed, or teach the technique to others without requiring large volumes of live seed.

It has been found (Hicks, unpublished data) that certain inert compounds of the correct mesh size are suited for this purpose. In particular, aluminum oxide (available as a grinding or polishing compound) in the 80 to 180 mesh range meets these requirements; it is chemically inert, inexpensive, and readily available. The one disadvantage is that it is much more dense than orchid seed, and therefore falls out of solution more rapidly. All the same, the techniques that we have previously covered (disinfecting, washing, inserting into flask) can be simulated with this material. It is also likely that plain sand of an appropriate mesh would do just as well, as would titanium dioxide or any other compound that is insoluble in the different solutions used, is inert enough to resist chemical attack by chlorine, and is fine enough to mimic the size of orchid seed.

You will want to wash it once or twice beforehand, and then re-use the material you have obtained, as there is a large concentration of extremely fine material that will cloud the solution whenever it is used initially.

These different materials may be available from machine shop supply companies, local sandblasting supply stores, and paint companies. The problem will be that you will require, at most, a couple of grams of the material, and most sources will want to supply you with pound quantities.

If so desired, one can practice these procedures on a fake media as well; given how inexpensive orchid seed media is, this may or may not be desirable. Jell-O, bland agar (8 to 10 gm agar cooked in a liter of water) or gelatin will do just fine, but, of course, will not indicate proper technique in the event of contamination from inadequate dis-

infection. The addition of sugar to simulate nutrient agar media will provide for an indicator that you are succeeding at sterilizing the particles, if so desired.

6.7 Alternative Gelling Agents

There have been those that argue that agar, the primary gelling agent in many orchid media, is phytotoxic (i.e., poisonous to plant life). Obviously, given the relative level of success and its record of use for the past few decades, this effect is probably very small, and negligible for most purposes. Nevertheless, for those that desire an alternative to the classics, there is a substitute.

PhytaGel

PhytaGel (also sold as Gelrite or gellan gum) is a gum whose manufacturers proclaim possesses none of the phytotoxic effects of agar. It is used widely, and there seem to be no major problems with it. As with agar, there are differences in opinion with respect to the concentration that should be used, but sources tend to agree that it should be used at around 2.0 gm per liter, or anywhere between 1.5 and 3 gm per liter for media used for orchid seed germination. The compound is sensitive to pH; Novak (pers. communication) suggests the following, from DeMason et al. (1992):

Table 6.1: pH versus concentration of PhytaGel

pH	Concentration (gm/liter)
4	4.92
5	2.93
6	1.36
7	0.64
8	0.50
9	0.40

Clearly, these values are over a broader range of pH than will be used for seed germination, but will serve as rough guidelines for the requirements at hand. Some growers advocate the use of a split between agar and these gelling agents. For example, both agar and PhytaGel could be used, each at 50% of the recommended strength. This composition is also available commercially as AgarGel.

PhytaGel has one apparent drawback in that, unlike agar, it "creeps," and is slightly plastic even after it has set up for a period of time. Therefore, it is probably not acceptable for slants and other media where the surface of the growing media is not kept horizontal.

Simpson (1997) suggests the addition of extra calcium or magnesium in the form of approximately 0.25 gm per liter of either calcium sulfate or magnesium sulfate in order to help replace what has been consumed in the formation of the gel.

Although admittedly "not very scientific," some growers in Canada have used 2.5 gm of PhytaGel per liter of mix using rainwater, and it was solid enough to support *Disa* seedlings at concentrations as low as 1.5 gm per liter (Zytaruk, pers. communication).

It is also worth noting that there have been reports of "vitrification," or seedlings perishing from conditions where they seemingly "turn to glass" with PhytaGel (Bergman, pers. communication). Kyte and Kleyn (1996) describe the tissues becoming translucent or watery, lacking chlorophyll. The cause has been identified as high cytokinin concentrations in conjunction with insufficient agar. Several growers note that this condition may be rectified by decreasing cytokinin, adding more agar, and increasing air exchange. The addition of phloroglucinol has been reported as a remedy or protection against vitrification. With PhytaGel, using half the recommended concentration and adding agar may prevent this condition.

Kyte and Kleyn (1996) note that,according to Stanley (1995), cornstarch has been used as a gelling agent. Gelrite and cornstarch at 0.5 and 50 grams per liter respectively were used to provide a suitable media for cell culture of apples, pears and some berries.

Gelatin has also been used for gelling media for growing orchids *in vitro*.

Although not a gelling agent, another substrate used for culturing plants *in vitro* consists of using sand as the culture media. Silica sand will be less prone to alter the pH, and should be used rather than carbonate sand.

Kappa Carrageenan

McConnell and Tomomitsu (1983) note that kappa carrageenan (a seaweed extract) was capable of replacing agar as a gelling agent. Among *Dendrobium* seedlings, seedlings growing on media with a concentration of 0.8% kappa carrageenan were virtually identical to those grown on agar media at a concentration of 0.8%. Interestingly, plantlet growth on media with a 0.4% concentration of carrageenan was superior to that of agar, but root length was only about half that of the agar control group. They also note that while agar's pH dropped shortly after preparation, media formulated with carrageenan was slightly higher.

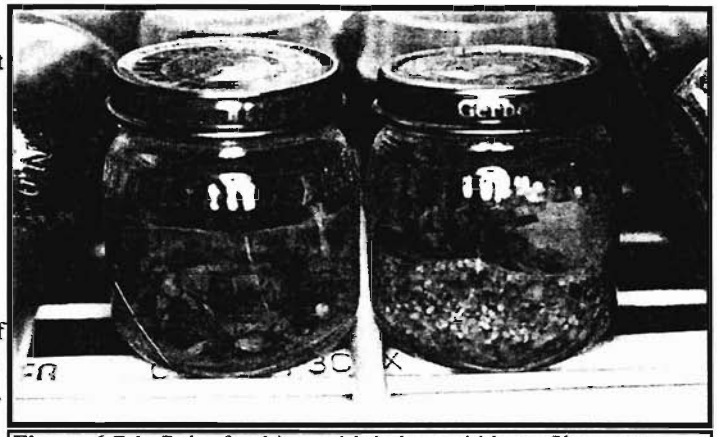

Figure 6.7.1: Baby food jars with baby orchids on filter paper (left) and filter paper on top of vermiculite (right).

Membrane Rafts

The use of polypropylene membrane rafts that replace agar in tissue culture may be of some use to those who germinate orchids from seed, but no clear reasons are capable of being identified at this time. The use of rafts for culturing seedlings is discussed by Adelberg et al. (1992) with regards to tissue cultured plantlets, but there is no reason that seed-grown plantlets could not be maintained in a similar manner.

Capillary Media

The use of glass wool has already been mentioned (Section 3.7) as a substrate for orchid seedling culture. Ernst (1975) notes that glass wool or cotton wool may be used for seedling support, but they are not suited for seed germination. The use of glass wool composed of Pyrex is recommended because other grades may discolor when autoclaved. Interestingly, it was reported that growth in flasks with and without charcoal are similar, and Ernst notes that charcoal settled out in the absence of a gelling agent.

As mentioned above, capillary media may be used for seed germination (Improvised Media, Section 3.7). Seed may be sown on filter paper, which is then used to wick nutrient solution to the seeds; this method may be used with baby food jars (Figure 6.7.1), test tubes, or other vessels in any one of a number of creative ways. For extremely efficient sowing, this may be combined with techniques B or D from Section 4.2, Disinfection of Seed, by which seed is removed from the disinfection solution using a filter. The paper, heavy with seed, may be placed in a flask and permitted to wick nutrient solution until protocorms develop. At this point, they may be replated into larger capillary flasks, or onto gelled media.

Ernst goes on to note that phytotoxic metabolites, such as phenolics, formed in high concentrations by orchids *in vitro* may be diluted in flasks that use glass or cotton wool rather than a gelling agent.

Super Absorbent Polymers

These compounds (also known as "SAPs") have a tremendous capacity for storing water, often hundreds of times their dry weight, and are inexpensive (under a dollar a pound). Moreover, they are capable of surviving autoclaving and absorbing the nutrient mixes commonly used for aseptic culture of orchids. Unfortunately, the only such compounds tested (polyacrylates) are strongly phytotoxic, and quickly kill seedlings transplanted onto these media.

One experiment involved placing seedlings of 4 species (*Peristeria elata*, *Laelia rubescens*, *Dendrobium falcorostrum*, and *Epidendrum nocturnum*) on Phytamax P-1056 at 57.3 grams per liter. One group used polyacrylate SAPs as the gelling agent, the other used agar. After 60 days on SAP media, *D. falcorostrum* and *E. nocturnum* were dead, and *P. elata* and *L. rubescens* had suffered high mortality along with no growth among the survivors. In contrast, all four species were doing well on the agar control, which used agar as the gelling agent (Hicks, unpublished data).

Other types of SAPs, such as polyacrylamides, have not yet been tested.

When mixed at about 18 gm per liter of media, the polyacrylate SAP forms a slurry with the consistency of applesauce. It is not smooth, so even ignoring the substantial problem of phytotoxicity, these compounds will probably never be used for seed germination. Nevertheless, they do present several attractive properties with respect to replate and seedling culture.

First and foremost, the media is soft, and quickly accepts seedling transfers. They may be dropped onto the surface, or pressed lightly into the media with minimal effort, unlike rigid gels. Secondly, since the media swells and shrinks with the availability of liquids, such media would be attractive for particularly slow-growing seedlings as water, sugars, and other components could be added many months after sowing has taken place. As the SAP will incorporate additional liquids with ease, it would allow for media modification many months into the growth process of the seedlings.

Another potential benefit of these polymers is that they are soft, like applesauce, at the water concentrations that would be useful for seed germination. Media may be moved about while sterile, accommodating replated seedlings with ease. Roots would penetrate with ease, allowing for robust root systems. De-flasking would be remarkably simple, facilitated by simply adding an overwhelming amount of water to the flask, stirring, and dumping out the contents.

Obviously, the problem of phytotoxicity must be remedied, and trials are currently underway with similar compounds to determine their suitability.

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6.8 Viability Testing and Seed Analysis

Would that we were able to determine whether seed were capable of germinating prior to sowing it on media, time, effort and materials could be saved by avoiding seed that was not viable.

There are several techniques by which we may determine if orchid seed is or is not viable. However, all have their drawbacks, which will be discussed with each technique.

Microscopic Examination

The easiest and most efficient technique is simple microscopic examination. Obviously, magnification will depend upon the size of the seed of the species at hand, but most orchid seed can best be examined from between 32x and 100x. What you will be looking for is the embryo of the seed (Figure 6.8.1), which is where the genetic material is kept. Without the embryo, the seed is not capable of germinating and therefore useless.

Still, the presence of an embryo does not mean the orchid seed will germinate. There is no guarantee that the embryo is still viable, nor is it a guarantee that it will germinate upon the media you have prepared for it. Some plants will generate the capsule, replete with seed, but the seed will be infertile for one of a variety of reasons. For example, some seed requires (or prefers) special treatment. Cypripediums may require vernalization (a period of time at low temperatures), so presence of the embryos does not necessarily mean we can produce seedlings. Other seed may be too old to germinate, and has lost the ability to sprout due to advanced age.

It is worth noting that sometimes an extended period of time may be required for very old seed to germinate: it is still viable, but it may take several weeks before the seed will swell and sprout. Seed of at least one species (*Zygopetalum mackyi*, two years old) took 3 months to germinate (Hicks, unpublished data).

Seed Types

Dressler (1993) notes that approximately 21 types of orchid seed are generally recognized, with much of this work having been done by B. Ziegler, who has apparently discontinued his research (Dressler, pers. communication).

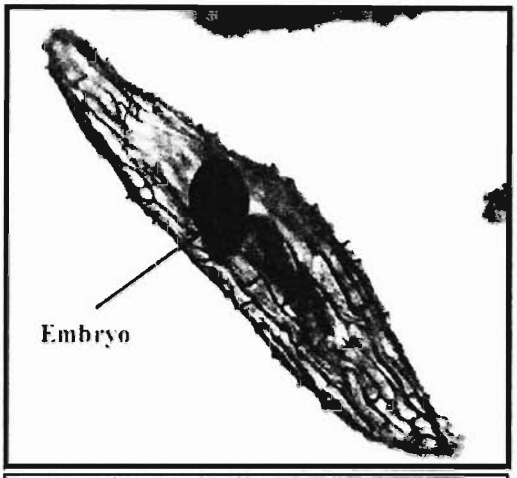

Figure 6.8.1: *Cymbidiella* seed, 40x. Note distinct, opaque embryo.

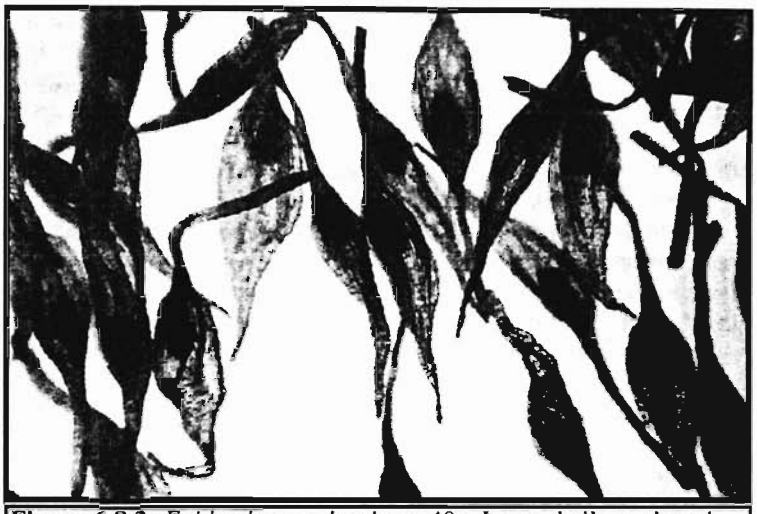

Figure 6.8.2: *Epidendrum calanthum,* 40x. Large, balloon-thread seeds virtually all of which possess embryos.

Figure 6.8.3: *Cattleya aurantiaca,* 100x. "A" seeds lack embryos, and are thin and transparent. "B" seeds possess embryos, and are full and opaque.

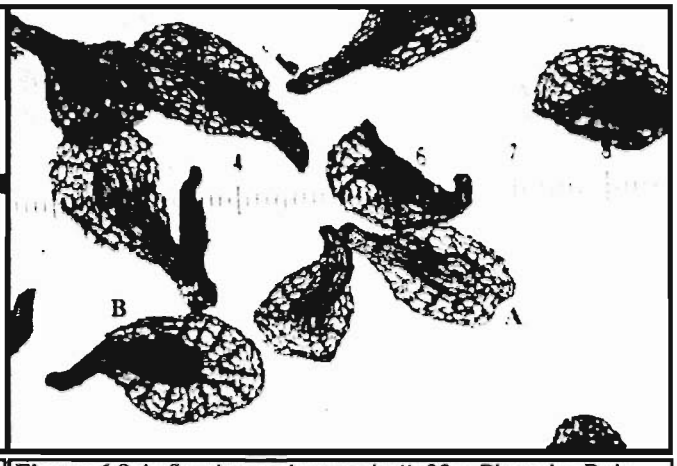

Figure 6.8.4: *Stanhopea hernandezii,* 32x. Photo by Bob Ferry Sr. "A" seed lacks embryo; "B" seed possesses embryo. Note unusual, cage-like structure of seed.

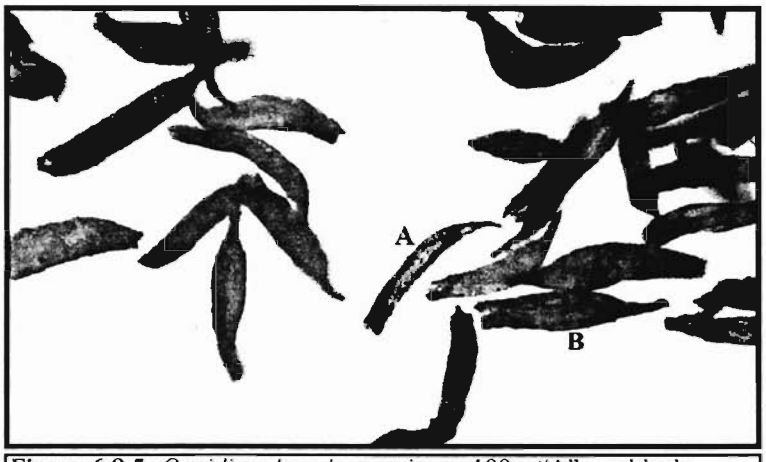

Figure 6.8.5: *Oncidium haephaematicum,* 100x. "A" seed lacks embryo; "B" seed possesses embryo. Note difference in shape and opacity.

Some seeds are much easier to work with than others. "Typical" orchid seeds, such as those of cattleyas, dendrobiums, epidendrums, phalaenopsis, and allied genera, do not present significant challenges with respect to their disinfection (see Section 4.2, Disinfection of Seed). Although small, their proportions are such that they can be readily manipulated using a variety of techniques. The family of orchids is quite large, and we see a corresponding variability in the structure and shape of member seeds. This section will give the briefest possible discussion of some variations, and my own experience with handling them.

Very fine seed, such as that of some oncidiums, vandas, and angraecums, are easy to work with using techniques that do not involve filtration. When using filtration techniques with these species, it is important to use very smooth paper which will allow for recovery of much of the seed. The use of hand tools, such as loops and rods, will miss small seed, so re-suspending them in sterile wash water allows for much better recovery. When dry, seed of some Angraecum species resemble chile powder in both color and texture.

Referred to as "balloon seed," those members of the genus *Stanhopea* often have large seeds with unusual structures (see cover photomicrograph of a *Coryanthes* species). When very dry, making them sink can be difficult. Fortunately, if collected and stored properly, contamination during flasking is still very low despite the inability of detergents and disinfecting solutions to penetrate. As with all seeds, it is important to prevent contamination in the first place, maintaining cleanliness of seed so that rigorous disinfection is not required. Members of the genus *Disa* may act in a similar manner.

As has been mentioned previously, *Vanilla planifloia* produces very large seed. Other members of the genus are reported to be similar in size (Dressler, 1993), as are those of Galeola (Dressler, 1993; Rasmussen, 1995). However, experience has been that the large, hard seeds of *V. planifolia* are difficult to work with. They are hard to recover from fresh or dried fruits, and clump together when in solution. When used with droppers, the tip must be large enough to accommodate the seeds without forming a logjam.

Most peculiar of all are seeds of *Epidendrum secundum*. Categorized as a "ballon-thread seed," they are uncharacteristically large (Figure 6.8.2). Dressler (1993) notes they may be as long as 6.0 mm, but none larger than 4 mm have passed through my own lab. When dry, they are unruly and adhesive to any static-sensitive surface, presenting a remarkable contamination problem. Their size does not correspond to their mass, and they may stay airborne for a surprisingly long period of time. When introduced to disinfection solutions, they clump together and will not separate without manipulation. They are best dealt with by disinfecting, washing once and dumping the entire mass into flask, then teasing them apart with a replate fork until they are distributed as best possible.

Chemical Staining

There are at least two chemical techniques that can be used in order to reliably determine the viability of orchid seed. The first of these involves staining with triphenyltetrazolium chloride (TTC or 2,3,5-TTC). The other involves fluorescein diacetate (FDA). TTC reacts within living cells in the presence of dehydrogenases, forming a red precipitate which may be visible depending upon the properties of the seed when tested.

TTC testing is performed by staining with a 1% solution at pH at 6.0 to 7.0 for 18 to 24 hours, incubating at a temperature of 25° to 30° C. The presence of a red coloration will indicate that the seed is alive, but this must be taken in context: simply because the seed is alive does not mean it will necessarily germinate, depending upon the conditions given. Further, this technique may not work with dormant embryos, or with dark, opaque seeds that prevents the detection of the red color.

The TTC solution is widely recognized to be very sensitive to light, and must be stored refrigerated in amber glass. With any technique used, it is essential to keep the TTC solution in the dark, or a red color will result. If used in conjunction with a hypochlorite pre-treatment, it is essential that the seeds be washed to remove all traces of hypochlorite before testing with TTC. A satisfactory buffer solution is given below (Böhm, pers. communication):

9 gm Na_2HPO_4 dihydrate + 11 gm KH_2PO_4
Add deionized water to make 1 liter of solution.

Another buffer solution suggested in the CRC is:

50 ml 0.1 molar potassium dihydrogen phosphate + 13.9 ml 0.1 molar NaOH
Add distilled water to bring final volume to 100 ml of solution. Final pH should be 6.5 at 25° C.

It has been suggested by Böhm (pers. communication) that terrestrial orchids whose cultivation requires acid soil (pH 3-4) may require a buffer with a correspondingly lower pH, but this claim has not been researched. Further, some species that require light to germinate may be incubated in the presence of light for some days before applying the test to increase the chemical response.

TTC testing is evidently of value to determine whether or not stored pollen is alive as well. To the best of my

knowledge, this experimentation has not been done with orchids, or at least not extensively.

Northen (1990) suggests the following technique for using TTC.

A 1% solution of tetrazolium chloride is produced by adding 1 gm of the compound to 100 ml of deionized water, which is then buffered or adjusted to pH 6-7 (note: although using a buffer as suggested above may be useful, adjusting the pH by titration is also acceptable). The seed is pre-treated by soaking in deionized water beforehand and then added to a small quantity of the TTC solution. The seeds in the solution are then incubated in absolute darkness for 18-24 hours.

If so desired, the seed may then be washed and soaked in a 0.01% solution of malachite green for 30-45 minutes. Then place the seed in glycerin and examine under a microscope. The embryo, if alive, will be red, and the seed coat and other non-living structures will be green. It should be noted that Northen's text specifies 1 ml of malachite green in 1000 ml of deionized water, but this evidently refers to 1 ml of the 10% solution of malachite green, which would provide the 0.01% solution. Singh (1981) indicates that the 0.01% malachite green solution should be filtered before every use.

It is also worth noting that malachite green has been identified as a carcinogen. Despite this, it is still available in preparations for treating fish diseases in home aquaria (although not for fish for human consumption). It is highly recommended that it be used only in the laboratory and not the home lab.

The second technique uses FDA. Fluorescein diacetate reacts within living cells to release fluorescein, an organic dye that is brilliant, fluorescent green. It is suggested that a 0.5% solution of FDA in absolute acetone be mixed with an equal quantity of disinfected and washed seed in a droplet of water. The fluorescein coloration disappears rapidly, and it is suggested that the seed be examined "within ten minutes" of initial exposure to the FDA solution (Rasmussen, 1995).

Rasmussen suggests that pretreatment involving a two hour soak in hypochlorites permits the best possible conditions for staining of terrestrial orchids, but it is not clear if such pretreatment affects epiphytic species, if at all. Extended exposure to hypochlorite solution is said to decrease the reaction to the stain, and therefore for best results, the technique should be modified for different types of seeds.

Large-scale viability testing may be facilitated through the use of small, multi-well plates such as those used in biochemistry. They are typically polystyrene or polypropylene and available in a variety of shapes, sizes, depths and other characteristics. It is important to use a brand whose depth does not exceed the focal length of your microscope at the power used for examination, otherwise, seeds will need to be removed with a dropper and placed on a slide for examination. Sigma Chemical Company (see Appendix III, Sources) is one of the few companies that provides these plates in anything other than massive numbers, but a package of five or ten seems to be about the minimum. A 96 well plate will do very nicely for analyzing small batches of seed for viability. Although designed to be disposable, they may be used several times.

Photomicrography

For documentation, it may be desirable to photograph your observations. One of the better texts on the subject has been written for the Eastman Kodak company. *Photography Through the Microscope* has gone through nine editions at the time of this writing, and serves as an excellent text for starting point. The McCrone company of Westmont, Illinois (see Appendix III, Sources) supplies this book and many more on microscopy and similar subjects.

Floating Seed

It has been repeated often that floating seed is probably not viable. Thus, when disinfecting a batch of seed, if one notices that the seed does not sink, it is not worth taking the time to flask. This is also discussed in Section 4.2: Disinfection of Seed.

This rule is a poor one. If possible, seed should be examined microscopically for the presence or absence of embryos before flasking in order to save time and effort because it is more reliable than the "sink or swim" rule-of-thumb for floating seed. Even so, microscopic examination is nothing more than a guideline, and seed that has appeared to be barren has occasionally surprised me by germinating very well.

All the same, there are some species that will display tendencies to float because they are very difficult to wet, and therefore retain air inside of the seed. *Gongora* and *Stanhopea* seed, for example, look like a balloon within a balloon–the embryo looks lost inside a greater cage, the structure of which is reminiscent of a buckeyball.

It has been suggested that the use of detergents or surfactants (the same used to wet seed when disinfecting it) be used in order to sink as much seed as possible. Another suggestion has been that pre-treating seeds with a sucrose or other sugar soak, such as those suggested in Section 5.1, Dealing with Contamination Problems, will saturate and sink a higher proportion of seed that is otherwise difficult to wet. As noted in Section 4.2, Disinfection of Seed, vacu-

um treatment of seed may be used to help sink seed that would otherwise float.

It is also worth mentioning that chaff that is mixed in with seed may also float. This waste may be skimmed off the top, or the seed removed from underneath, to minimize contamination.

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Appendix I: Where to Start

The purpose of this text is not to bewilder. There has been a great deal of information presented up to this point, with little or no practical information for the beginner. There are, to me, few things more frustrating than being seated before a powerful analytical instrument, replete with racks of books on how to use it–and none with anything that actually tells you how to *use* it. If you, too, are easily bewildered by what other, more experienced growers regard as simplicity itself, feel at home. Take your time. Read.

Most of all, don't be frustrated by apparent failures. What only a few flaskers will admit is that they, too, have had (and still do have) infected flasks. With experience comes technique and confidence, and combined with proper tools and conditions, germination of orchid seed via the asymbiotic technique with minimal contamination can be the result.

There are also some other articles on the subject. Marilyn Light wrote an excellent series of articles for the American Orchid Society, and they are featured in the August, September, and October 1990 issues of the *Bulletin*. They are literally flower-to-flower articles from pollination to compot and seedling culture.

For most neophytes, the greatest problem will be with appropriating seed. Many growers may be willing to offer you some, but please–don't just try any old "found-a-capsule-in-the-greenhouse." One day, you will reap what +-you sow–and if it's garbage, then it's several years later that you will discover this.

If you wish to experiment, some growers may be kind enough to send you some seeds for free, or for a nominal price. Ask around your orchid society, and see if anyone else who does flasking will part with a little. The Orchid Seedbank Project (see Appendix III: Sources) stocks orchid species seed at a reasonable price.

Several companies support home growers and flaskers. Fungi Perfecti of Olympia, Washington serves to provide mushroom cultivators with products that overlap with flasking (HEPA filters, autoclaves, pressure cookers, culture vessels, etc.) PhytoTechnology Labs of Shawnee Mission, Kansas sells media, components, and equipment to home growers, and companies such as Morel Chemical, G&B, Gallup and Stibling, and others deal to small and large growers alike (see Appendix III, Sources).

Media

Companies like Sigma will ship plant culture media to residences, provided you can satisfy their requirements. Most will not ship chemicals unrelated to plant culture to your home. Others are specifically in the business to deliver media and components to the home lab. All these vendors will be found in Appendix III: Sources.

Flasking Area

Several different options have been presented. Most people can find a used aquarium or other suitable enclosure, or make one out of a box as described in Section 6.3 on making your own glove box. Other options, such as Bergman's technique of working under towels, or Tompson's technique of working under glass, also work, but you may experience a higher infection rate than with better equipment.

A typical "go" at flasking

As discussed above, there are many ways to get from seed to seedlings. To help cut down on confusion, here is a step-by-step account of a typical flasking session.

1) WRAP ALL ARTICLES THAT NEED TO BE STERILIZED

Aluminum foil can be used for many things in flasking, and one of its best uses is to keep things sterile. Razor blades, Pasteur pipets, replate forks, forceps, rinse water, and other glass or metal items are best sterilized in aluminum foil. Wrap individual items in thin foil; Pasteur pipets can be folded in as part of a long, thin piece of aluminum foil, which is then folded at both ends and along the sides. Individual bottles or vials of wash water for seed should be filled, then capped with a piece of aluminum foil, then "clustered." A group of seven (a hexagon with one filling the center) flasks of equal size works well.

Take the individual articles, and then use heavy freezer aluminum foil to wrap these. Everything should be fully and completely wrapped with the edges of the foil folded over. These bundles can be placed in shallow, heat-resistant trays, placed inside of upright canning jars, or otherwise held up and out of the boiling water below any way you see fit.

2) PREPARE THE BOTTLES

Having washed and assembled your flasks, inspect them for chips. Boil stoppers for 10-15 minutes or longer.

Punch and fill the lids with wads of cotton, or cover with band-aids, if using Mason jars. Prepare a small pile of aluminum foil squares with which to cover the lids.

3) MIX AND PREPARE THE MEDIA

Pour the media into individual flasks and screw the lids on lightly, cover with aluminum foil, and place these containers into the autoclave or pressure cooker. Place any articles from #1, above, that will also need to be sterilized. If necessary, use supports made from wads of aluminum foil to keep them off the bottom and out of the water. If your pressure cooker or autoclave tends to leak, the seal may be dry. Lubricate with an agent recommended by the manufacturer. The use of water-soluble compounds, such as lecithin (found in health food stores) is suggested. Oil may leave residue on flasks, but olive oil works fine.

4) AUTOCLAVE THE CONTENTS

Be certain lids are on loosely or properly vented prior to autoclaving flasks so they don't burst from pressure differences. Let stand until they are safe to handle. As we have said before, hastening the cooling process of a pressure cooker and/or autoclave is a dangerous procedure that should never be applied.

5) REMOVE THE CONTENTS

As discussed above, if you are going to use the flasks soon, place the pressure cooker in or at the flasking cabinet or glove box. The contents will be sterile until you open it, and you can open it when it is cool enough to touch. Remove the flasks with a sterile glove, and put them in the position in which you wish the agar to solidify.

If you are not going to use them immediately, flasks may be removed when they are cool enough to pick up. Open the pressure cooker and remove the flasks, tighten the lids, and then place them in position to solidify. The exterior of these flasks can be chemically sterilized when you are ready to use them later.

6) PREPARE THE SOWING AREA

Sterilize the glove box with alcohol or bleach solution. Do this in advance of performing any work. Remove flasks from the pressure cooker, and place them inside the glove box or laminar flow cabinet. If you have flasks that are not sterile, put them in the glove box and spray them with or dip them in a sterilizing solution (10% bleach, saturated calcium hypochlorite, etc.).

Seeds

Once you have prepared the glove box, the media, and everything else, you can prepare to deal with the seeds. "Sterilant" refers to isopropyl or ethyl alcohol, or simply a bleach solution for sterilizing surfaces, NOT seed; use a regular spray bottle, filled with the compound of choice. "Disinfection solution" refers to the hypochlorite or other disinfectant used on seed.

Wetting agent may be any commercially available wetting agent (Tween 20, for example) or a solution of dish soap, detergent, etc. One solution that has worked well for us is two drops of antibacterial dishwashing detergent in about 200 ml of water. Shake before use. Add two drops of this wetting agent to the seed as described in step 2, below.

1) If so desired, pre-soak seed in a sugar solution the night before using a technique listed in Section 5.1, Dealing With Contamination Problems.

2) Place a small quantity of seeds (about the size of a grain of rice, or less with small seed) into a test tube with 5-7 milliliters of disinfection solution (let's say saturated calcium hypochlorite for now) and a wetting agent. Put on the stopper. Start a timer.

3) Agitate the seeds intermittently. This does not have to be done inside the glove box; however, give yourself 5 minutes or more to sterilize the exterior of the test tube before you move it inside your sterile area.

Several times during the agitation, remove the stopper, rotate it around, spray it with disinfection solution and replace it to assure that all the sides of the plug are sufficiently wetted.

4) When you approach the end of your disinfection time (we'll say 12 minutes total time, so after 7 minutes), put the tube inside your flasking cabinet and spray it with sterilant solution. Shake once more, then put on gloves and stick your gloved hands through the ports on the flasking cabinet. Spray your gloves with sterilant.

5) Continue to agitate tubes as your gloves and tube are sterilized.

Let the seeds settle for a minute or two before decanting the disinfection solution. Let the seeds accumulate, either on the bottom or on the top.

WASHING

1) Once the time is up, remove the disinfection solution with a sterile Pasteur pipet.

The Pasteur pipet bulb should have been sprayed with sterilant; the option is to plug your pipets with cotton prior to autoclaving. Open the foil protecting the pipets, having sprayed the outside of the foil with sterilant if necessary. Select one foil-wrapped pipet, and put it aside. Close the foil to the rest, and set them aside.
Rip the end off of the fat side of the packet, where the bulb fits. Put on the bulb, and set this on a support to keep the end up and off any wet or contaminated surfaces, with the remainder of the pipet still sheathed in foil. Pick up the test tube with one hand, and remove the stopper.

Remove the pipet from its sheath, and use it with the bulb to remove the solution from the seeds or (if easier) the seeds from the solution. Eject the waste solution into a waste container. If removing seeds from solution, you can either dump out the disinfection solution from the tube, or eject the seeds into an empty, sterile tube for washing.

2) Wash

Using the same technique for the wash bottles as you did with the pipet, select a vial of sterile distilled water. Using a sterile pipet, add a small volume of this water, and agitate gently. As you did with the previous step, let the seeds settle, and remove the wash water with the pipet. Repeat if desired; no foam should remain after the first wash, and certainly not after the second. If there is, you used far too much wetting agent.

SOWING

1) Open the flask.

Select a flask. Remove the foil lid, and place it somewhere dry, with the clean inside layer facing up. If using flasks, wash the neck of the flask with sterilant, and loosen the stopper. If using Mason jars, unscrew the outer ring and hang it somewhere dry and sterile. Gently break the seal after washing the neck with sterilant.

If so desired, the flask may be balanced on a wad of clean (preferably sterile) aluminum foil or a specially-constructed stand to keep the mouth at the correct angle.

2) Draw up the seed.

Draw a small quantity of seeds (200-500) into a pipet. If necessary, add a little sterile distilled water to suspend the seed, and gently flick it to keep them suspended, then quickly draw them up with the pipet. Set the test tube aside.

3) Put the seed in flask.

Using one hand, gently open the mouth of the flask. Taking care not to leave the flask open for too long, insert the tip of the pipet, and gently place several drops of suspended seed in the center of the media. Re-cap the flask, and set aside the pipet. Return ring and aluminum foil, as required.

4) Repeat with the next flask.

Easy Starts

Some orchids are easier to germinate from seed than others.

For best results when starting out, the new grower would be well advised to stick to easy-to-germinate genera. It is suggested that encyclias and epidendrums are generally very easy to start from seed, as are many cattleyas and cattleya-types, such as brassavolas and laelias. Many oncidiums are easy to grow from seed, particularly the equitant species. If possible, examining the seed under a microscope at about 100x magnification can demonstrate the presence or absence of embryos, possibly saving time and effort.

Although not trying to discourage new growers from trying, *Phalaenopsis* are reported to be chlorine-sensitive, so if possible, calcium hypochlorite or embryo culture (green capsule technique) should be used when trying to germinate this genus (they may simply be pH sensitive, so acidified hypochlorite solutions, discussed in "Hypochlorites," Section 6.5, New Directions in Seed Disinfection, may be of use). Staying away from terrestrial species, at least to start, is recommended because paphiopedilums, phragmipediums, selenipediums and many cypripediums can be difficult to start from seed.

Appendix II: Improvised Home Flasking

The title of this section is, perhaps, misleading. After all, relatively few products either used or suggested for use in home flasking have their roots, if you will, firmly planted in the tradition of orchid seed germination.

Still, improvised is improvised, and products even further derived from direct orchid seed germination have been used successfully to produce results roughly equivalent to those more directly involved in such endeavors. The information given here is recommended for those that wish to perform flasking under relatively primitive conditions, be they in the home lab, or conserving species in developing countries as recommended by Behar (1998).

The following information is the product of work by David MacDonald (pers. communication) of Florida, and he reports success with genera such as cattleyas, encyclias, brassavolas, oncidiums, bulbophyllums, and dendrobiums, as well as with *Phalaenopsis* via seed and stem props, and replate for each of these. It is suggested that those interested in this technique try to work with other genera as well. His mix uses banana; although some have suggested that coconut milk is better for the germination of seeds, and that banana inhibits germination, his experience is such that he has had bad luck with substituting coconut for the banana.

The mix itself consists of two quarts of distilled or RO water, 4 ripe bananas, ¾ ounce of agar from a Chinese food store, 2 tablespoons of powdered activated charcoal, ½ teaspoon of Peter's 20-20-20 fertilizer with minor elements, and ½ teaspoon of "Super Thrive". The activated charcoal is aquarium-grade activated charcoal reduced to a powder in a blender, and is very messy to prepare. Fortunately, powdered activated charcoal for plant tissue culture can also be purchased from PhytoTechnology or Sigma Chemical Company.

The agar (approximate weight- ½ of a 1.5 ounce packet of stringy agar agar) is added to 6 cups of water in a large stainless steel stew pot, and put on the stove on "high." The bananas, having been frozen and thawed to produce a mushy consistency, are added to 2 cups of water along with the fertilizer and Super Thrive. This mixture is put into a clean blender, and reduced to a homogenous liquid. Add this liquid to the heated agar and water, stirring constantly to keep the mix from burning. When all the strings have dissolved, add the charcoal powder slowly to prevent from dusting your entire kitchen black with the powder.

A set of 24 pint Mason jars are used for flasks. Into each, ⅓ cup of the above mix is placed in each jar. The lids may be fitted with cotton for venting. Another technique suggested by MacDonald involves drilling a ⅛" diameter hole, and plugging the hole with fabric by forcing a square of suitable material ("fleece," made of recycled soda bottles, is recommended). The material is held in place with clear nail polish, which is also used to coat the exposed metal edges produced when the lid is punched to prevent corrosion. Lids are seated with the bands threaded on, then backed off a half turn to allow for pressure equalization, and the containers are autoclaved.

Each batch is cooked at 15 pounds for 15 minutes for the first lot, and 20 minutes for subsequent lots that could not be fit into the pressure cooker for the first run; he strongly recommends against cooling pressure cookers with cold water, as this may cause flasks to boil over.

Once the flasks have been prepared, MacDonald suggests flasking in the following manner.

Flasking is to be performed on a cheap plastic table cloth, to prevent damage to the work surface underneath. Flasking is performed in a Styrofoam cooler, tipped on its side; a cloth soaked in 5-10% bleach solution is hung over the open side of the container, with the end hanging over the opening about halfway down. Sources of air circulation, such as fans and open windows, should be turned off or closed. Wear gloves.

Green capsule is performed by soaking the unopened capsule in 5% bleach solution (10 cups of water + ½ cup of Clorox), and scrubbing it with an old toothbrush or nail brush. Stop scrubbing and let it soak for 5 minutes, and then scrub again. When finished with this step, loosen the lids on several (3 or so) flasks, but do not open them. The interior of the box should have been sprayed with the bleach solution, and the gloves should be occasionally disinfected with the same solution. Remove foil caps, if present, and rings if using Mason jars, but leave the lids on.

At this point, fold down the towel so you can barely see the work at hand. Spray gloves and the interior of the box with the 5% bleach again, then the capsule. Place the capsule in the bottom of a small, sterile dish, and cut crosswise in half with a sterile blade. Use a sterile wire or other tool to remove a small gob of seed, using the other hand to open and then re-close the jar after you place the seed in the jar. Repeat with the other jars. MacDonald suggests spraying the inside of the lid before replacing it, but if one is very quick and does not hesitate, this will not be necessary. Regularly spraying the jars and the inside of the box with the bleach solution while working is suggested.

With dry seed, ½ teaspoon of dry seed is mixed with 1 teaspoon of 5% bleach. Stir for about two minutes, mixing to remove any air bubbles (a surfactant might help with this). Open a jar, and place about ⅓ of the seed in the jar with a sterile tool (teaspoon, etc.), and spread it around as best as possible. This step is accompanied by a spray of bleach solution onto the media and the seeds, and the interior of the lid. Repeat until all the seeds are used.

Bear in mind that such a large quantity of seeds may result in tremendous numbers of seedlings, and may not be required for some growers. Therefore, much smaller numbers of seeds may suffice.

For replating, MacDonald suggests using the same mix he uses for germination. The apparatus remains the

same: a cooler on its side, sterilized with bleach solution. A teaspoon and a pair of forceps are sterilized in the same solution.

Once the working area is prepared, the spoon is used to retrieve a number of seedlings from the mother flask, and these seedlings are then placed in a small glass dish (MacDonald suggests a custard dish), sterilized in advance. Wet the seedlings with a squirt or two of bleach solution, and then spray the inside of the box again. Open up a planting jar, add about 25 seedlings from the custard dish, and use the forceps to spread them evenly across the surface. They are to be resting on the surface so there is no need to push them into the media. Spray the lid, and re-seal the vessel. Spray your gloves and the inside of the box again, and repeat until you require more seedlings from the mother flask.

The last stage involved in the process is taking seedlings from flasks, and putting them into community pots (compots). The technique described by MacDonald involves opening the flasks in an aseptic environment (the aforementioned cooler), removing the plants and placing them in a 5% bleach solution, even when they are to be placed on non-sterile media as we would grow any other orchids.

After suspending the seedlings in bleach solution, they are to be removed and placed in a fungicide solution of your choice. Fill several 4-6" (10 to 15 cm) pots with dried *Sphagnum* moss, "baby" bark or "mud" mix, which consists of peat, baby bark, charcoal, and perlite. Use a crochet hook to tease the plants apart and sow into individual holes in the media of your choice. Mist the plants with clean water, making sure they are seated well and do not fall over when water is introduced. Cover the pot with a plastic bag, and place in an area that is brightly lit, but does not receive direct sunlight. Examine the seedlings weekly to make sure they are moist and not rotting.

After two weeks, the bag may be removed, allowing the seedlings to "harden." This works well for areas that are very humid, such as Florida, but growers may need to modify this technique to increase survival. Poking small holes in the bag, followed by progressively larger holes, may be required for those of us that grow in drier areas, or without the benefit of a greenhouse. When the seedlings have progressed to this stage, the level of light may be increased, along with regular watering and fertilizing schedules to enhance growth. Always remember that orchids prefer weak solutions of fertilizer, and very dilute solutions ($^1/_{10}$th label strength at the most) for young seedlings.

References:

Behar, M. 1998. Orchid Growing from Seed Made Easy. *American Orchid Society Orchids*, 67: 1040-1042.
McDonald, D. Telephone conversations, 1998 and 1999.

Appendix III: Sources

AMERICAN SCIENCE AND SURPLUS
Source of all kinds of surplus equipment. They carry small motors and sometimes glassware of value to the home laboratory.
American Science and Surplus
3605 Howard St.
Skokie, IL 60076
(847) 982-0870
FAX: (800) 934-0722

ANTEC INTERNATIONAL
Supplier of Virkon S disinfectant for flasking. Most commonly available through veterinary supply companies.
Durvet, Inc.
PO Box 279
100 SE Magellan Drive
Blue Springs, MO 64014
(800) 821-5770
(816) 229-9101

CYROBIO SYSTEM
Supplier of cryobiological storage systems, including IMV straws used for storing orchid seed in liquid nitrogen.
IMV International Corporation
6870 Shingle Creek Parkway - Suite 100
Minneapolis, Minnesota 55430
(800) DIAL IMV
FAX: (612) 560-8125

DAIGGER AND COMPANY, INC.
Source of lab supplies: pH papers and meters, glassware, pipets, sterilizing trays, Büchner funnels, desiccants, stoppers, graduated cylinders, filtration supplies, Pasteur pipets, and so forth.
A. Daigger & Company, Inc.
199 Carpenter Avenue
Wheeling, Illinois 60090
(800) 621-7193
FAX: (800) 320-7200

DRI-DEK
Rugged plastic matting suitable for lining the bottom of glove boxes in order to keep your work above pools of disinfectant. Versatile and chemical-resistant.
Dri-Dek Corporation
Kendall International Centre
2706 Horseshoe Drive South
Naples, FL 34104-6100
(800) 348-2398
dri-dek@kictr.com

EM SCIENTIFIC
Source for pH papers with broad as well as specific ranges, with resolution to 0.2 to 0.3 pH units, as well as t.h.e. Desiccant.
EM Scientific
480 Democrat Rd.
Gibbstown, NJ 08027
(609) 354-9200
(800) 222-0342

FUNGI PERFECTI
Caters primarily to mushroom cultivators, but the equipment overlaps strongly with asymbiotic technique. HEPA filters, culture vessels, autoclaves, Bacti-Cinerator sterilizers, and pressure cookers, etc.
Fungi Perfecti
PO Box 7634
Olympia, WA 98507
(360) 426-9292
(800) 780-9126
mycomedia@aol.com
http://www.fungi.com

G&B ORCHID LABORATORY
Source for orchid seed germination media, as well as small quantities of chemicals for flasking.
G&B Orchid Lab
2426 Cherimoya Dr.
Vista, CA 92084
(760) 727-2611
(760) 727-9466
(888) 727-2760
FAX: (760) 727-0017
http://www.orchidsource.com

GALLUP & STIBLING
Source of orchid seed germination and replate media.
Gallup & Stibling
645 Stoddard Lane
Santa Barbara, CA 93108

I^2R (INSTRUMENTS FOR RESEARCH AND INDUSTRY)
PO Box 159D
Cheltenham, PA 19012
(215) 379-3333
FAX: (215) 663-8847

KITCHEN CULTURE KITS, INC.
Home plant tissue culture kits for TC in home or classroom. Manuals, supplies, technical support, updates, web resources, and chemicals including PPM.
Kitchen Culture Kits, Inc.
Carol M. Stiff, Ph.D.
818 Ford Street
Moscow, ID 83843
(208) 882-8131
FAX: (208) 882-5320 (not a dedicated line; call ahead)
http://www.home.turbonet.com/kitchenculture
kck@turbonet.com

MCCRONE ACCESSORIES AND COMPONENTS
Microscopes, microscopy equipment, imaging supplies and books.
McCrone Accessories and Components
850 Pasquinelli Drive
Westmont, Illinois 60559-5539
630-887-8100
800-622-8122
http://www.mccrone.com

MCMASTER-CARR SUPPLY COMPANY
Deals in everything from raw materials to industrial tools. Includes sheet rubber, specialty screws, hinges and other hardware. Incredible catalog.
McMaster-Carr Supply Company
PO Box 54960
Los Angeles, CA 90054-0960
(562) 692-5911
FAX: (562) 695-2323
la.sales@mcmaster.com

MARKSON LABSALES, INC.
Source for Whatman filter paper, sterilizing filters, filtration setups, Pasteur glass pipets, pH test papers, and many other miscellaneous lab products.
Markson LabSales, Inc.
PO Box 1359
Hillsboro, OR 97123-9981
(800) 942-8626
FAX: (800) 858-2243
Whatman@teleport.com

MOREL CHEMICAL
Source of orchid seed germination media.
Morel Chemical
PO Box 1167
Englewood, FL 34295-1167
(941) 474-5759

NORTH AMERICAN NATIVE ORCHID CONFERENCE
Supplies the NANTOC proceedings, as well as the CD that may accompany it. Available as either the text or the CD for $25 each, or $45 for both.
NANTOC Proceedings
14320 Poplar Hill Road
Germantown, MD 20874

ORCHID SEEDBANK PROJECT
Deals in dry species seed of orchids. Provides for research and dissemination of the orchid seed, and the propagation of the Orchidaceae. Kicks butt, takes names.
Orchid Seedbank Project
PO Box 1873
Socorro, NM 87801

PERMATITE INDUSTRIAL
Manufactures "Color Guard" plastic tool dip; industrial grade material available through local distributors only.
Permatite Industrial
Newington, CT 06111

PHYTOTECHNOLOGY LABORATORIES, LLC
Sells media, chemicals, reagents, babyfood bottles and lids, glass bead sterilizers, and other equipment and supplies for plant cell culture including PPM.
PhytoTechnology Laboratories, LLC
PO Box 13481
Shawnee Mission, KS 66282
(888) 749-8682
FAX: (888) 449-8682
http://www.phytotechlab.com

SCIENTIFIC GLASS COMPANY, INC.
Sells the "Rain Crystal" water purifier. Also manufactures custom glassware.
Scientific Glass Company
113 Phoenix N.W./PO Box 85125
Albuquerque, NM 87125

SIGMA CHEMICAL COMPANY
International company that sells most of the commonly available media for orchid seed germination. Chemicals (including nutrient agar media) can only be sold to businesses, educational facilities, etc. Labware, such as culture tubes and vessels, transfer loops and polyethylene pipets, etc. are available to the individual, as are plant culture media.
Sigma Chemical Company
PO Box 14508
St. Louis, MO 63178
(800) 325-3010
FAX: (800) 325-5052
http://www.sigma-aldrich.com

SMALL PARTS INC.
Deals in small quantities of products. Tubing, metal stock, sheet rubber, small machine parts, specialty tools, forceps, nuts, bolts, screws, glue needles, and syringes.
Small Parts Inc.
13980 N.W. 58th Court
PO Box 4650
Miami Lakes, FL 33014-0650
(305) 557-7955
FAX: (800) 423-9009

UNITED DESICCANTS
Desiccants. $100 minimum order. Sells silica gel, molecular sieves, and activated desiccating clays.
United Desiccants
1227 South Twelfth Street
Louisville, KY 40210-1570
(502) 634-6800
FAX: (502) 634-7727

VWR SCIENTIFIC
Science and supply company: lab furniture to test tubes, including microspatulas, pH meters, pH papers, etc.
(800) 932-5000
Supplies through distribution network. Local distributors around the United States and Canada.

Conversions

Length

1 inch (US) = 2.540 cm
1 foot (US) = 30.48 cm
1 mile (US) = 1,609.3 meters

Volume

1 dram (US liquid) = 0.125 ounces (US liquid)
1 tablespoon (US measuring) = 14.79 ml
1 teaspoon (US measuring) = 4.93 ml

1 pint (US liquid) = 473.2 ml
1 pint (US liquid) = 2 cups (US measuring)
1 pint (US liquid) = 16 ounces (US liquid)

1 quart (US liquid) = 946.35 ml
1 quart (US liquid) = 4 cups (US measuring)
1 quart (US liquid) = 32 ounces (US liquid)

1 gallon (US liquid) = 3.78541 liters
1 gallon (US liquid) = 4 quarts (US liquid)
1 gallon (US liquid) = 8 pints (US liquid)
1 gallon (US liquid) = 16 cups (US measuring)
1 gallon (US liquid) = 128 ounces (US liquid)

1 hogshead = 52.4585 gallons (British)
1 hogshead = 63 gallons (US)

Mass

1 ounce (US avoirdupois) = 28.349 grams
1 pound (US avoirdupois) = 453.592 grams
1 carat (metric) = 200 milligrams

Temperature

Celsius to Fahrenheit: Multiply by (9/5), and add 32.
Fahrenheit to Celsius: Subtract 32, then multiply by (5/9).

Other

1 cubic foot of water = 7.48 gallons
1 cubic foot of water = 62.462 pounds (39 degrees F)
1 cubic centimeter (cc) = 1 milliliter (ml)

Component (All weights expressed in mg unless otherwise noted)	Gamborg	Knudson	Modified Knudson C (Arditti, 1982)	M&S Medium	M&S Salts	Lloyd & McCown	Orchid Mutliplication Media (P-6793)	Orchid Maintenance Medium (P-6668)	Phytamax (P-1056)	Malmgren's Formulation	Mellard's version of Malmgren's terrestrial mix
Ammonium nitrate		500		1650	1650	400	825	825	825		
Ammonium sulfate	134	500	500								
Boric acid	3		0.056	6.2	6.2	6.2	3.1	3.1	3.1		
Calcium chloride, anhydrous	113.24			332.2	332.2	72.5	166	166	166		
Calcium nitrate		347.2	694.8			386					
Cobalt chloride • $6H_2O$	0.025			0.025	0.025		0.0125	0.0125	0.0125		
Cupric sulfate • $5H_2O$	0.025		0.0625	0.025	0.025	0.25	0.0125	0.0125	0.0125		
EDTA, disodium salt	37.3			37.26	37.26	37.3	37.3	37.3	37.3		
Ferrous sulfate • $7H_2O$	27.8	25	25	27.8	27.8	27.8	27.85	27.85	27.85		
Magnesium sulfate	112.09	122.125	122.09	180.7	180.7	180.7	90.35	90.35	90.35	50 to 100	98
Manganese sulfate • H_2O	10	5.682	5.682	16.9	16.9	22.3	8.45	8.45	8.45		
Molybdic acid, sodium salt, • $2H_2O$	0.25		0.016	0.25	0.25	0.25	0.125	0.125	0.125		
Potassium chloride		250									
Potassium iodide	0.75			0.83	0.83		0.415	0.415	0.415		
Potassium nitrate	2500			1900	1900		950	950	950		
Potassium phosphate monobasic		250	250	170	170	170	85	85	85	50 to 100	
Potassium sulfate						990					
Sodium phosphate monobasic	130.5										
Zinc sulfate • $7H_2O$	2		0.331	8.6	8.6	8.6	5.3	5.3	5.3		
Activated charcoal			2000					2000	2000	500	500
6-benylaminopurine							2				
Glycine, free base				2							
MES (buffer, free acid)							1000	1000	1000		
myo-inositol				100			100	100	100		
alpha-napthaleneacetic acid (NAA)							0.5				
Nicotinic acid (free acid)				0.5			0.5	1	1		
Peptone							2000	2000	2000		
Pyroxidine (Vitamin B_6)				0.5			0.5	1	1		
Sucrose		20,000	20,000				20,000	20,000	20,000	10 to 20 g.	10,000
Thiamine (Vitamin B_1)				0.1			1	10	10		
Banana powder, 50% maltodextrin									30,000		
Ripe banana			100 to 150 g.								
Phytagel gelling agent											2500
Nitsch & Nitsch (Sigma N-0390)											100
Calcium tri-orthophosphate ($Ca_3(PO_4)_2$)										50 to 100	200
RPMI 1640 amino acids (Sigma N-7131)											5 ml
Pineapple juice										10 to 25 ml	20 ml
Agar-agar			12 to 15 g.							6 g.	
Vamin (amino acids)										5 ml solution	
Soluvit (vitamins)										0.1 to 0.3 amp	
Kinetin										*	
Total weight for 1 liter of mix, grams	3.1	22		4.4	4.3	2.3	25.3	27.3	57.3		

* 2 to 5 mg for "some" cypripediums

Mother Flask #	Genus and species	Replated onto:	Sterilization type	Contamination?	Notes

Flask #	Genus and species	Date sown	Sterilization type	Contamination?	Sugar hatch?	Notes

The following information on laminar flow hood construction is reprinted in its entirety with permission.

LAMINAR FLOW HOOD CONSTRUCTION

G.W. Forister and D.W. Burger
Department of Environmental Horticulture
University of California, Davis

The laminar flow hood is an important piece of equipment in any plant tissue culture laboratory. It provides a nearly sterile environment in which to work. Laminar flow hoods function by passing room air through a HEPA (high efficiency particulate) filter that removes 99.99% of all airborne materials (dust, spores, mycelia) and delivers the filtered air into an enclosure open on one end.

The following description details how we made two laminar flow hoods connected to one blower by dryer vent hose. We started with a conceptual idea obtained from a paper published by Meyer in 1986 (HortScience 21(4):1064-1065). We describe here our construction details for the fan housing and laminar flow hoods along with a cost analysis. Our laminar flow hoods have proven (based on open petri plate contamination tests) to provide a sterile environment in which to conduct plant tissue culture work.

FAN HOUSING - Construction Details

The construction could be assembled using the following instructions in sequential order.

1. The 3/4" thick 4'× 8' sheet of plywood should be cut into the pieces as diagramed in the Parts Layout sheet.

2. Cut all rabbets to 3/4" wide and 1/2" deep.

3. Cut out air passages in the END and END inside pieces.

4. Glue together and attach with screws or nails the SIDEs, ENDs and bottom. Careful attention to accuracy will make a perfectly square open top box.

5. Make the Fan hood (Fig. 1) with #2 pine. There are two views of it. One separate and one attached to the fan. It should fit tightly inside the large box already constructed. The bottom of the fan hood is attached to the fan outlet with #10 3/4" screws. When the fan and attached fan hood are set in the box with the fan holes facing the filters, the top of the fan hood should be aligned to the bottom of the

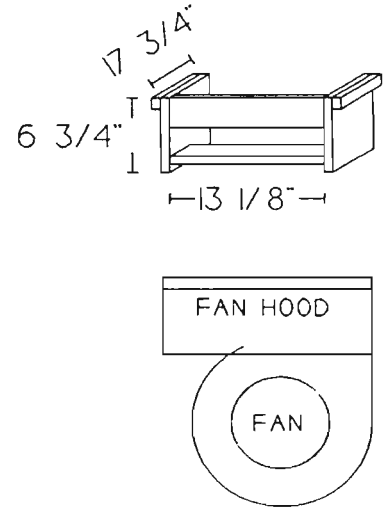


Figure 1: Fan Hood.

rabbet of the sides. Center the fan hood in the box and attach cleats on each side of the fan at the bottom of the box (Fig. 2). The fan or fan hood need not be attached to the fan housing box. Notice the strips of pine on the side of the fan hood. They are there for support of the filter lids and are not covered up by the "Top Center" piece with holes for air passage.

6. The "Top Center" has at least six 4" diameter holes in it. The hose attachments are made from 4" aluminum irrigation pipe 3" long. Tabs can be cut, bent out and a hole drilled for a 3/4" #10 screw. The hose attachments are then inserted into the "Top Center" with screws. This piece is then attached to the top of the "Fan Hood" with 2" screws. No glue was used in case later we want to replace the top with another unit with more outlets. This top piece could be made like a box and more hoses attached since the motor is capable of much more work than is allowed by only six holes.

7. The filter lids are made as a loose fit over the filters and a handle attached. No screws are necessary to attach the lids in place. Access is needed to change the filters.

8. The next step (see Figure 2) is to attach the inside end to the spacer with 2" inch screws with the fence wire held between them. The inside ends should fit loosely inside the box allowing for paint and ease of removal, if needed. The spacers are attached to the inside ends with enough room between them to let a filter (16"x20"x2") slide between them. This construction is then attached to the end piece with screws and no glue.

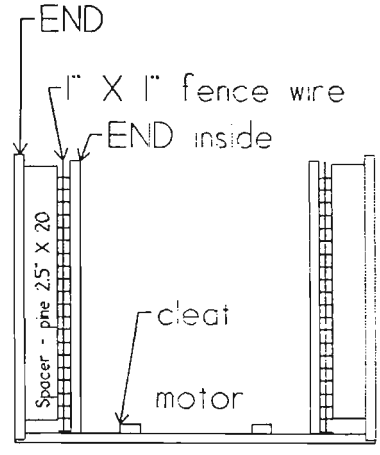


Figure 2: Side View.

9. At this time the space for the switch can be located between the fan and the "END inside" piece that holds the filter in place. The switch did not have a long enough shaft to work without taking the switch apart and reversing the plate with the screw holes. Then if a hole was made that was large enough the switch would be recessed into the 3/4" plywood and the shaft will stick out far enough to attach a face plate to cover the hole. Then the switch knob can be attached.

10. A 1" hole is cut in the box below the switch position at least 2-1/4" from the bottom of the side. A grommet to protect the wire (14 gage) can be made from conduit end parts attached to a steel conduit coupler cut to the 3/4" thickness of the wood. Two 3/4" washers sandwiched the coupler and kept in place.

LAMINAR FLOW HOOD - Construction Details

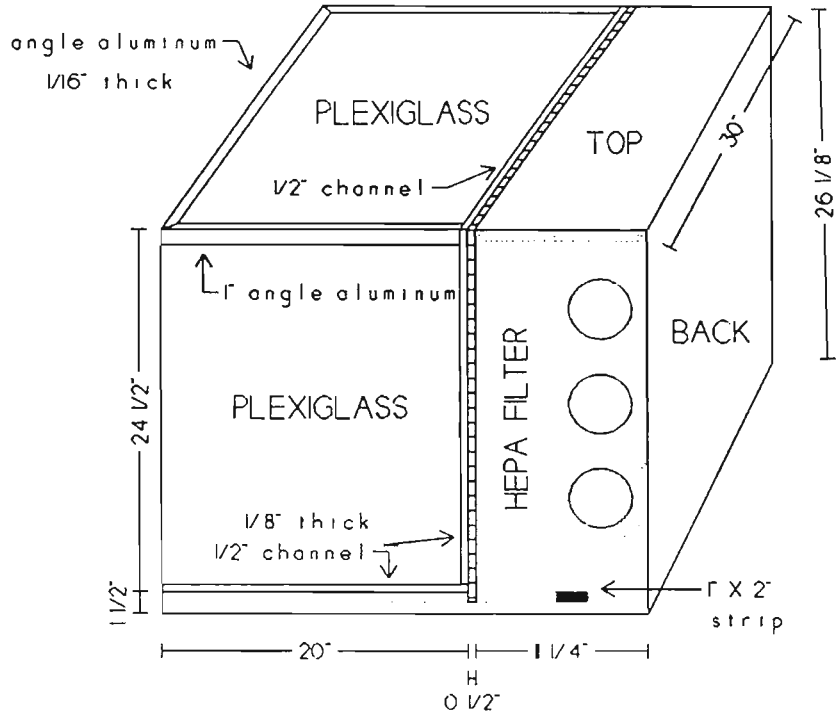


Figure 3: Side View of Hood.

1. The bottom and table area was made from 2 layers of 3/4" plywood (one 20"x31-3/4" and one 31-3/4"x31-3/4") glued and screwed together. The table area (2 layers thick) was laminated.

2. The HEPA filter was set back from the table the width of the egg crate white panel. The spacer blocks attached to the base with screws held the filter in place. The egg crate panel was held in place to the HEPA filter by machine screws through the 1/2" channel - plastic grid - foam insulation - into the aluminum filter frame.

3. The enclosure around the filter was made from 1"x12" pine dadoed to receive the filter edges to make a better seal and to hold the filter. The enclosure was screwed into the 3/4" base.

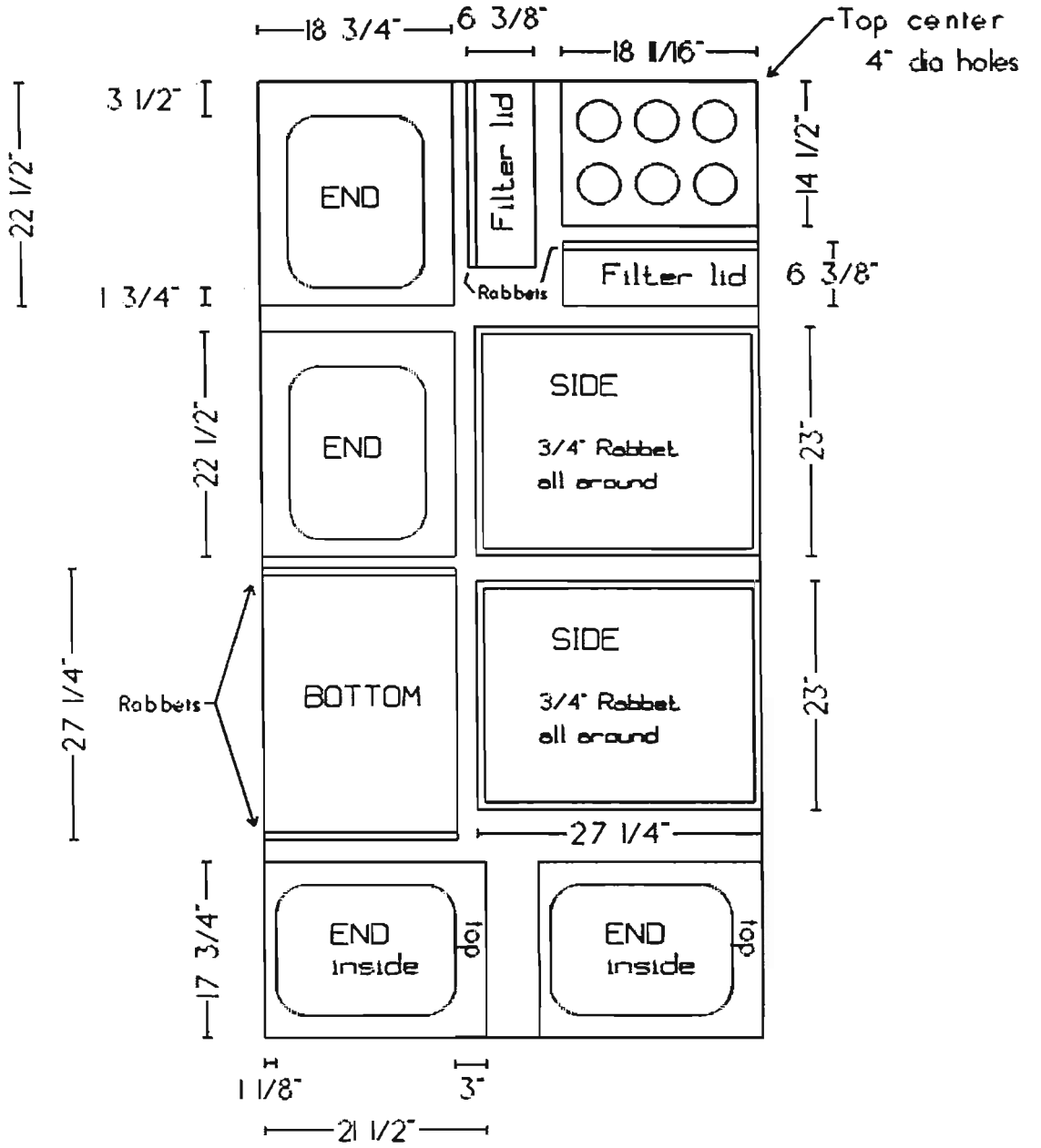
4. The back of the filter enclosure was covered with 1/4" plywood.

5. The 1" angle aluminum (1/16" thick) frame for the top of the plexiglass hood was made from one continuous piece of angle aluminum. The 90 degree bends were made after a triangle was cut out of the top side of the piece at the corners. The ends fit inside the 1/2" channel which secured them.

PARTS LAYOUT
FAN HOUSING

Aug. 15. 1991

Rabbets are 3/4" wide and 1/2" deep



COST ANALYSIS FOR BUILDING TWO LAMINAR FLOW HOODS (ONE FAN)

Hood	
HEPA Filter (2), 24"x30"x6" (under Filters-Air & Gas - Yellow Pages)	220.00
Plywood, 4'x8'x3/4" A/B Int	34.00
Plywood, 4'x8'x1/4" Shop	10.00
1"x12"x16', #2 Common Pine	17.00
Formica laminate, white, 4'x4'	24.00
Laminating adhesive	6.00
Clear Plexiglass, 4'x8'x1/4"	110.00
Egg Crate White Lighting Panel, 2'x4', two of them	24.00
Aluminum channel, 1/2"x1/2"x16'	25.00
Paint	15.00
Angle aluminum 1"x1", 1/16" thick, 2 pieces 6'	10.00
Miscellaneous supplies	30.00
TOTAL (Hood)	525.00
Fan Housing	
Direct-drive blower, Dayton #5C094	185.00
Rotary switch*	40.00
Plywood, ABX, 3/4"	30.00
Molding, pine	18.00
Pre-filter**, 16"x20"x2", case of 6	24.00
Dryer vent hose and clamps	35.00
Miscellaneous supplies, screws, glue, paint, etc.	46.00
TOTAL (Fan Housing)	378.00
GRAND TOTAL	**903.00**

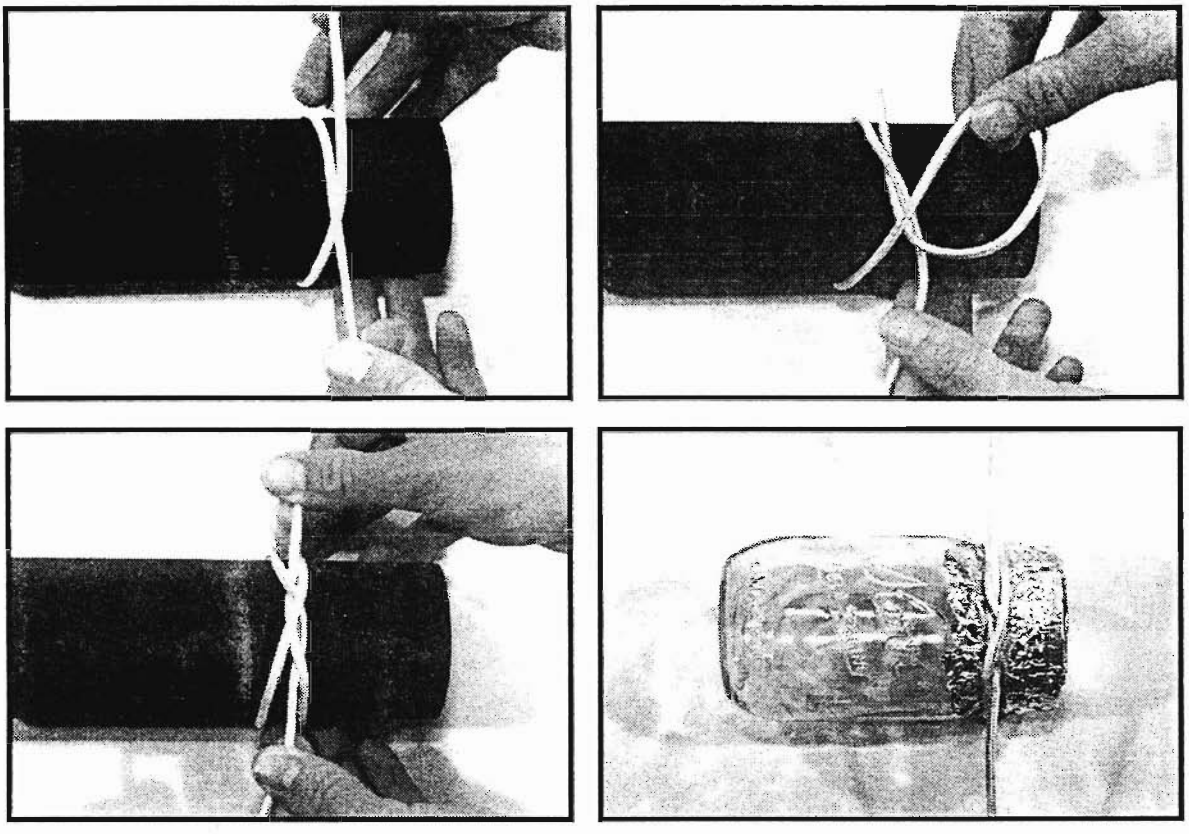
* Switch - ELECTROSWITCH part #21301A
series 21, 1 deck, 4-hole, 1-7 throw
From - ELECTRIC SWITCHES INC.
National 800-421-8855
CA. except (213);800-252-4640
(213)-660-1310

** Filter - Extended Surface Air Filter
Mfg. - DAYTON
Supplied by -
Granger Industrial and Commercial Equipment and Supplies.

Appendix IV: Miscellaneous

The constrictor knot, as described in Chapter V, may be useful to those who wish to secure aluminum foil or plastic caps. The knot has the excellent property of being able to be tied loose, and then drawn down to any desired tension up to the breaking point of the cord used. To demonstrate its strength, as well as its safety and security, I suggest tying it around the circumference of a soda can, and then pulling as hard as you can. Perhaps the can will dent where the actual knot is, but collapsing the can requires both physical strength and strong rope.

This knot may be used to cap a flask once flasking or replating is finished; as a result, there will be a snug fit between the glass and foil (or plastic), reducing the chances of contamination. I suggest the use of waxed dental floss, which is both strong and "sticky" enough to keep it tied. Snug it down with a length of floss, and go on to the next one.



References:

Ashley, Clifford W. 1944. The Ashley book of knots. Doubleday press. New York.

Additional Reading

Micropropagation:

Arditti, J., and Ernst, R. 1992. Micropropagation of orchids. John Wiley and Sons, Inc., New York.
Kyte, L. and Kleyn, J. 1996. Plants from test tubes, 3rd edition. Timber Press, Portland, Oregon.

Conservation:

Quinn, D. 1992. Ishmael. Bantam/Turner Books, New York.

Trademarks

The following trademarks are accurate to the best of my knowledge. For further information, please contact the manufacturer.

3M Health Care: Nexcare™ Active™ Strips
Antec International Ltd.: Virkon®S
Becton Dickinson Company: Vacutainer®
Bestfoods: Karo®
The Clorox Company: Clorox
Colage-Palmolive Company: Palmolive™
Corning, Inc.: Fleaker®, Pyrex®
Discwasher, Inc.: Zerostat®
Dri-Dek Corporation: Dri-Dek®
General Electric Co.: Lexan®
Kimble: Kimax®
Maril Products Inc.: Physan
Nalge Nunc International: Nalgene®
Parkway Research Corporation: Consan Triple Action 20 (Consan 20)®
Plant Cell Technologies, Inc.: PPM™
Prism Technologies: Mityvac®
Sanford: Sharpie®
Scientific Glass Company, Inc.: Rain Crystal™
Sherwood Medical Industries: Bacti-Cinerator™
Sigma-Aldrich Co.: AgarGel™, PhytaGel™, PhytaMax™
Stern's Miracle-Gro Products, Inc.: Miracle-Gro®
Vitamin Institute: SUPERthrive™
W.A. Hammond DRIERITE Company: Drierite™

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About the Author

Born and raised in a library on the outskirts of Pittsburgh, Pennsylvania, Aaron J. Hicks was raising orchids by the age of 12. He first successfully germinated orchid seed via asymbiotic technique at age 15 after reading up on the subject, and currently serves as the director, chief of research, head of shipping and receiving, operations manager, and head bottle-washer for the Orchid Seedbank Project, located in Chandler, Arizona.

In his copious free time, he serves as a chemist at Pacific Scientific, in Chandler. Caving and technical ropework are also favored pastimes.

In previous incarnations, he has been involved in raptor rehabilitation; a locksmith; the R&D, shipping, receiving, and technical section of a dry-charge battery company; a lifeguard and lifeguard instructor, a firefighter, rescue instructor, and an Emergency Medical Technician and crew chief. His favorite stunt kite is the Top of the Line North Shore "Radical."

The author, shown here not researching rupiculous laelias in New Mexico. Photo by Carsten Brandt.

About the Orchid Seedbank Project

The Orchid Seedbank Project (OSP) is a conservation-oriented organization providing orchid seed to researchers, conservationists, hobbyists, commercial interests, and others through a process involving collection of seed from donors, cataloging and listing seed that is available, then processing and distributing the seed. It exists largely as a last-ditch effort to encourage the conservation of orchids, and deals exclusively in orchid species–no hybrids are stocked or sold. We perform research, and disseminate information freely to assist growers in the cultivation and seed propagation of orchids. We collect formulations, techniques, germination information, and other data, and encourage growers to send us this type of information to assist others in their research.

How it Works

A lot of seed goes to waste every year. A LOT of seed dies a horrible, miserable death in the back of refrigerators all over the world. Orchids produce more seed than any one individual can use in most cases, and is either disposed of or lies in a desiccated refrigerated container until it is no longer desirable or viable. The OSP serves as a sort of "clearinghouse" between those that have seed, and those that want seed. Donors send the OSP excess seed, and receive in turn "credits" for free seed, proportional to the amount of seed donated.

The OSP performs research in the realm of long-term seed storage, and tries to collect data with respect to the longevity of specific genera. We collect and disseminate information on orchid seed germination, and post it on the Internet. We also collect capsule maturation times, and send the data to be added to other databases of capsule times, most notably the Bakers, authors of the Orchid Species Culture series, who have their own Internet web site for such information.

Other projects include the compilation of other orchidaceous wisdom–the kind that is hard-earned, and has to be repeated each time someone new attempts to master it. This, too, is added to the orchid information that is stored and disseminated throughout the world on the Internet, and can be accessed at the URLs below.

Due to the rapidly changing nature of the Internet, and that some of these websites are undoubtedly ephemeral and will be gone by the time you read this, we suggest using "Excite" or other web search engines that may be in use in the future to find these web sites, using key words like "orchid," "capsule database" and specific names as mentioned in this section.

The Orchid Seedbank Project will maintain webspace for as long as it exists. Due to the volatile nature of the Internet, it is recommended that a web search be performed in order to find out its current location and e-mail address.

ftp://ftp.nmt.edu/pub/orchids (The accumulated wisdom, as gleaned from the Internet)
http://www.teleport.com/~cmbaker/PODS/index.html (The Baker's web pages for capsule times)

The ability of the OSP to perform conservation and research depends upon your generosity in terms of sending us high-quality, live plant material. Contributions of equipment, consumables and your financial support are greatly appreciated, and permit us to increase the quality and capacity of our facilities. With our new expansion into the realm of cryogenic preservation, we can use all the help we can get in this expensive undertaking.

Chapter 1: THE ORCHID SEED

Chapter 2: A BRIEF HISTORY OF ORCHIDS FROM SEED

Chapter 3: THE BASICS: orchid seed production, harvesting, storage; laboratory basics, media, sterile sowing areas.

Chapter 4: TECHNIQUE: preparing the sowing area, sterilizing and sowing dry seed, green capsule technique.

Chapter 5: POST-SOWING: dealing with contamination, caring for sown flasks, replate, de-flasking and seedling care.

Chapter 6: ADVANCED TECHNIQUES: media components, making your own glove box, alternative gelling agents, seed testing and analysis.

Asymbiotic Technique of Orchid Seed Germination covers every step in growing orchids from seed, from pollination to de-flasking your seedlings. From the beginner to the established grower, this is a complete text on how to grow your own orchids from seed in the home or in the lab, using readily available tools and components. With important, up to-date information on how to grow orchids from seed, this manual provides the essential knowledge of laboratory practices involving all phases of growing your own plants from seed.

Cover illustration: scanning electron microscope image of a *Stanhopea* seed.
Graciously provided by Julian Guerdon, Rocky Mountain Seed [illegible]