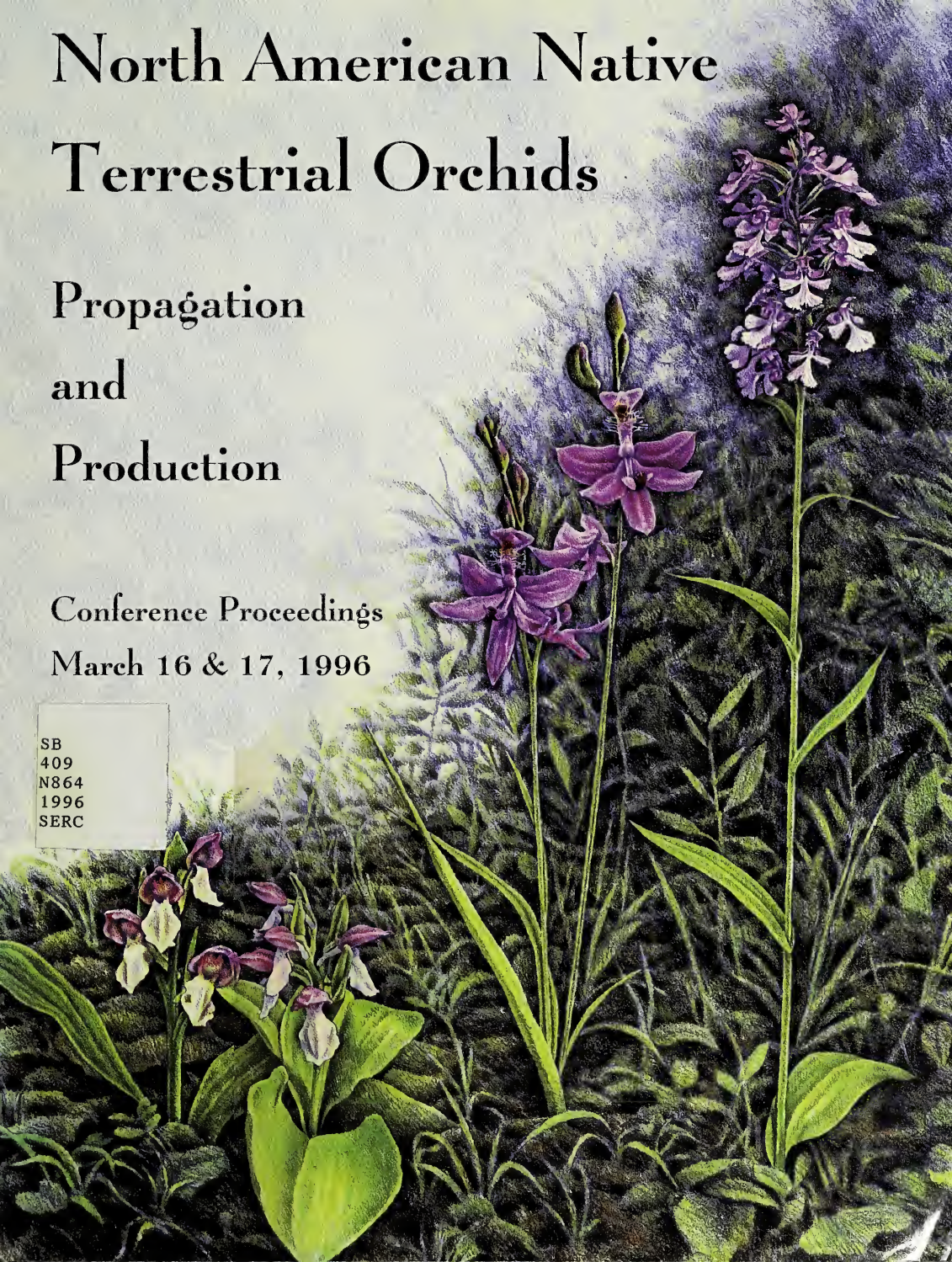
North American Native Terrestrial Orchids

Propagation and Production

Conference Proceedings
March 16 & 17, 1996



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Calypso bulbosa var. *americana,* photo courtesy Paul Martin Brown.

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Edited by

Carol Allen

North American Native Terrestrial Orchid Conference

ISBN 0-9655075-0-5

Published by: The North American Native Terrestrial Orchid Conference
14320 Poplar Hill Road
Germantown, Maryland 20874

Production Services By:

Printed in the U.S.A. on recycled paper.
85% recovered fiber and 15% post-consumer waste.

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About The Supporting Organizations

Friends of the National Arboretum

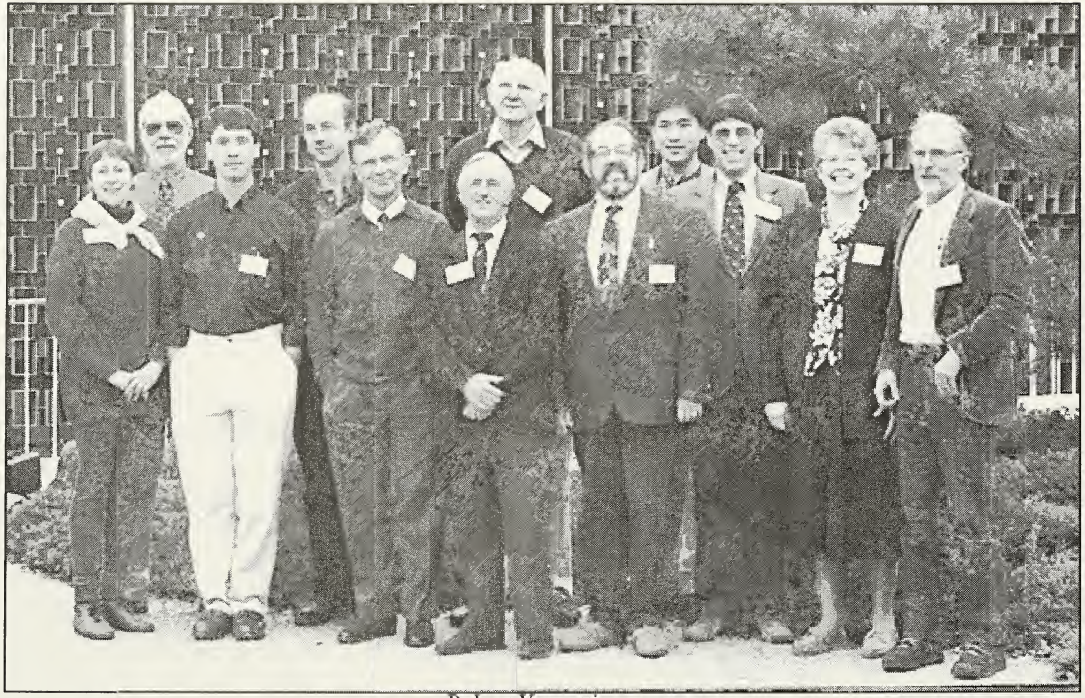
FONA was founded in 1982 as an independent, non-profit organization to enhance, through public and private sector resources, the support of the National Arboretum. Their mission includes increasing accessibility and public involvement in the National Arboretum as a national resource. The Capital Columns Project is one of their more notable efforts.

Maryland Native Plant Society

The Maryland Native Plant Society is a nonprofit organization that uses education, research, and community service to increase the awareness and appreciation of native plants and their habitats; leading to their conservation and restoration. MNPS sponsors monthly meetings, workshops, field trips, a spring field meeting, and a fall conference.

National Capital Orchid Society

The National Capital Orchid Society was founded in 1947 by twenty people. Now approaching its 50th anniversary, it's membership numbers over 400. This reflects a nation wide interest in tropical orchids. Through the annual show, monthly meetings, auctions, and trips; NCOS continues to amaze and educate the public about these rare and endangered species.



Carson Whitlow Svante Malmgren Robert Yannetti Chin-Chang Chu
Lawrence W. Zettler Douglas E. Gill William K. Steele
Paul Martin Brown
Carol Allen Warren Stoutamire Marilyn Light
Allan Anderson

About the Speakers

Allan Anderson has worked in the Botany Department of the University of Guelph since 1966 as a technician and greenhouse manager. He started work on orchid seed germination in 1982 and has since published a number of articles on seed germination and growth in orchids. He received the Canadian Wildflower Society's Conservation Award for his work on orchids in 1988.

Paul Martin Brown is the founder of the North American Native Orchid Alliance and editor of its journal. He regularly publishes new taxa and articles of general native orchid interest, and is the author of *A Field and Study Guide to the Orchids of New England and New York.* He has a new field guide to the orchids of the northeastern United States due for publication in the fall of 1996 by Cornell University Press.

Chin-Chang Chu is a graduate student under the direction of Kenneth W. Mudge, Department of Floriculture and Ornamental Horticulture at Cornell University. Mr. Chu worked with tropical orchid species in his native Taiwan for ten years. His Ph.D. dissertation is on the propagation of several species of *Cypripedium.*

Douglas E. Gill has been a professor in the Department of Zoology at the University of Maryland since 1983. Dr. Gill has worked and taught throughout the world, notably in Costa Rica with the Organization for Tropical Studies. His studies include many diverse biological life forms including frogs and salamanders! He has been studying populations of *Cypripedium acaule* in Maryland, Virginia, and West Virginia since 1977.

Marilyn Light is the Coordinator of Professional Development (Science and Engineering) with the Service for Continuing Education, University of Ottawa. She teaches an orchid micropropagation course at the University and elsewhere in Canada. Ms. Light serves as the Chairman of the Conservation Committee of the Canadian Orchid Congress. She is a regular contributor to the *Orchid Review* of the Royal Horticulture Society, England and *Orchids Australia,* a publication of the Australian Orchid Foundation

Svante Malmgren is a full time practicing surgeon in his native Sweden. He has been growing European terrestrial orchids from seed since 1966. He has done large scale production of *Cypripedium calceolus* and *Orchis militaris* for conservation projects at the Royal Botanic Gardens, Kew.

William K. Steele is a professor of Geology at Eastern Washington University. In 1988, he began experiments in *Cypripedium* germination after a 40 year interest in the genus. His business, Spangle Creek Labs was born in 1990 when he realized he had more seedlings than he had either time or space to care for.

Warren P. Stoutamire is a professor of Biology at the University of Akron where he has taught and done research for 30 years. His interests are in pollination biology and plant evolution. He manages the University greenhouse as well as his own and is primarily interested in the terrestrial orchid species of North America, Australia, and South Africa. He grows and hybridizes *Disa* for fun, not profit.

Carson Whitlow is well known for his work in hybridizing first in 'blue' *Cattleyas*, then in native terrestrials, primarily *Cypripediums* and *Calopogons*. He registered the first artificial *Cypripedium* hybrid in 1987 and the first *Calopogon* hybrid in 1991. He continues to hybridize these genera and is building a small business, Cyp Haven, as a retail outlet for his work. He has published over 30 articles in U.S. and European journals.

Robert A. Yannetti is a retired electronic engineer. He became interested in *Arethusa bulbosa* in the 1970's and began working on the propagation and pot culture of the plant. In the process he has made and registered four intergeneric hybrids among *Arethusa bulbosa, Calopogon tuberosus*, and an allied genus from Japan, *Eleorchis japonica.*

Lawrence W. Zettler is currently with the Department of Biology, Illinois College, Jacksonville, Illinois. He studies the orchid/fungal symbiosis, particularly involving rare and endangered orchid taxa. He is also a biological illustrator, having depicted over 260 insect species in full color for two upcoming books (field guides) covering North American blackflies and dragonflies.

Preface

The second North American Native Terrestrial Orchid Conference was held at the Brandywine Conservancy in 1989. Six years had gone by and the need for another conference was apparent. However, the organizers had moved on to other things. Since my background has me equally planted in both the native plant and the tropical orchid branches of horticulture, it seemed only natural that I take on the challenge of putting together the third of such conferences.

The North American Native Terrestrial Orchid Conference: Propagation and Production was held at the National Arboretum, Washington, D.C. in March of 1996. It was blessed with a powerful group of speakers. The electricity generated by these people was felt by all who attended. My heartfelt thanks goes to these speakers who shared their time and expertise and made the conference such a success. Continuing in the spirit of the two previous conferences, considerable progress was made towards the protection of our native orchids through their artificial propagation.

The supporting organizations; Friends of the National Arboretum, National Capital Orchid Society, and the Maryland Native Plant Society can be proud of their efforts. I wish to thank the members of the above organizations for their support and look forward to the possibility of another such conference.

Carol Allen
October 1, 1996

To obtain copies of the proceedings, join the mailing list, or present a paper at the next conference; contact:

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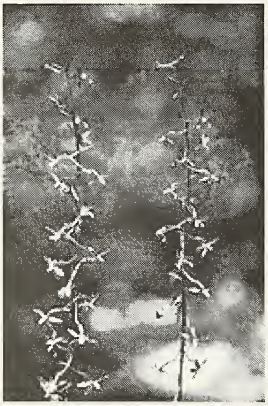
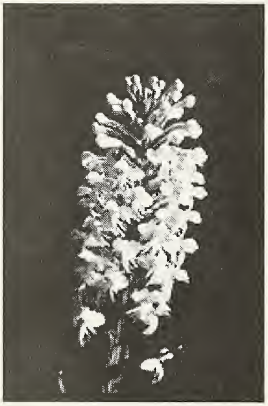


Tipularia discolor

Malaxis diphyllos



Platanthera pallida

Amerorchis rotundifolia

Orchids In North America

Paul Martin Brown
North American Native Orchid Alliance

The diversity of native orchids in North America is one of the greatest in a north temperate climate in the world. We may not have as many species as Europe or Asia but we do have a greater diversity of genera and certainly within those genera of species. From the cold and barren north of Newfoundland and Alaska with large quantities of *Cypripedium* and *Platanthera* to the eastern seaboard and its complement of *Spiranthes* and the spectacular fringed *Platanthera* we find an endless selection of both showy and somewhat esoteric species numbering well over 225.

The northern boreal and barren areas of Newfoundland are home to spectacularly large stands, often in the thousands, of such delights as **round leaved orchis**, *Amerorchis rotundifolia*, **showy lady's-slipper**, *Cypripedium reginae*, **small** and **large purple fringed orchises**, *Platanthera psycodes* and *P. grandiflora* and great swaths of **large yellow lady's-slippers**, *Cypripedium parviflorum* var. *pubescens*, the latter often in the much sought after flat-petalled form (syn. *C. parviflorum* var. *planipetalum* Fern.) The extensive bogs of the north harbor some of the largest colonies of **dragon's-mouth**, *Arethusa bulbosa*, **grass-pink,** *Calopogon tuberosus* var. *tuberosus* and **rose pogonia**, *Pogonia ophioglossoides* var. *ophioglossoides* to be found in the world. On the far side of the continent Alaska shares some of the same species with Newfoundland such as *Amerorchis*, but also has its own specialties such as **Fischer's orchis**, *Dactylorhiza aristata*, **spotted lady's-slipper**, *Cypripedium guttatum* and **yellow spotted lady'-slipper**, *C. yatabeanum*, **two-leaved adder's-mouth**, *Malaxis diphyllos* and **Chamisso's orchis**, *Platanthera tipuloides* var. *behringiana*.

If the myriad of confusing **ladies'-tresses**, *Spiranthes* spp. can plague the eastern North American orchid lover than Californians certainly have their work cut out for them with the genus *Piperia*. All known species of *Piperia* occur in California and several of them are endemics. Such rarities as **Pt. Reyes piperia**, *Piperia elegans* subsp. *decurtata* and **Monterey piperia**, *P. yadonii* are known from very limited areas on the central coast of that state.

The prairies and central states offer fewer species than the coastal areas but some of our most memorable scenes can be of such choices as **eastern** and **western prairie fringed orchises,** *Platanthera leucophaea* and *P. praeclara* and **small white lady's-slipper**, *Cypripedium candidum* on the mesic prairies and **great plains ladies'-tresses**, *Spiranthes magnicamporum* growing within the fields of autumn wildflowers. The upper Great Lakes has one of the greatest diversities with nearly all of the eastern species and a good representation of the western ones. The **coralroots**, *Corallorhiza* spp., abound in this region with the spectacular **striped coralroot**, *C. striata*, presenting an unforgettable show in large numbers in the spring and early summer in these northern regions. The most spectacular offering of the southern

central states is the unforgettable **Kentucky lady's-slipper**, *Cypripedium kentuckiense.* The large majestic plants have the largest of all our lady-slipper blossoms in a color range from deep yellow to ivory-white.

The central Atlantic states and the southeast have been rapidly overdeveloped of late, but one can still find impressive stands of **orange fringed orchis**, *Platanthera ciliaris,* **orange crested orchis**, *P. cristata,* **southern white fringed orchis,** *blephariglottis* var. *conspicua* and **spreading pogonias** *Cleistes divaricata* and *C. bifaria* in easily accessible sites. The genus *Spiranthes* certainly raises its head again in the Southeast with more than a dozen species to be carefully identified. As we progress southward to the Gulf Coast and southern Florida we see many of our old friends from the north and a whole variety of new species and the beginning of many of the southern Florida specialties - both terrestrial and epiphytic. Many of these terrestrials are easily overlooked for the flashier epiphytes, but they are every much as beautiful as their northern counterparts. The many **fringed orchids** and **grass pinks,** *Calopogon* spp. are throughout the southeast as well as a number of *Spiranthes*-related genera of which *Sacoila lanceolata,* the ***scarlet ladies'-tresses*** or beakless orchid is one.

Texas and the southwest offer many Mexican species that are just creeping over our borders and cannot be easily seen elsewhere. Several **adder's-mouths**, *Malaxis* spp. and **rein orchises**, *Platanthera* spp. fall into this category as well as the majority of **crested coralroots**, *Hexalectris.*

The broad spine of the Rocky Mountains from Canada to northern Mexico offers one of the greatest diversities of all our North American natives. From the abundance of the **eastern fairy-slipper**, *Calypso bulbosa* var. *americana* in the far north to the wide variety of **rein orchises**, *Platanthera* spp. within the main body of the range to some of the southern specialties such as the aforementioned *Malaxis* and *Platantheras,* the Rockies offer an orchid searches dream - or nightmare. All the species of the genus *Corallorhiza* can be found within the delimitation's of the range, some very rare others very common.

The final area which offers such diversity is my own home site - the northeast. New England and New York present us with more than sixty species, most of which are easily seen and still well preserved despite rampant development in the highly populated area. Such familiar sights as the broad woodlands full of **pink lady's-slipper**, *Cypripedium acaule* and endless roadsides of **nodding** and **yellow ladies'-tresses**, *Spiranthes cernua* and *ochroleuca* are memories that need not fade. One of our rarest orchids in North America, **small whorled pogonia**, *Isotria medeoloides,* has the center of its populations in western Maine and eastern New Hampshire. It cousin, the curiously beautiful **large whorled pogonia**, *Isotria verticillata* is a frequent resident of the southern New England woodlands, often growing large colonies of more than one thousand flowering stems.

Regional rarities certainly do exist in the northeast and most are species which may be more readily seen further south or west. One rarity found only in this locality remains. That is the **pale fringed orchis**, *Platanthera pallida,* and endemic on eastern Long Island. The recent description of this species presents us with an

ongoing study as we try to ascertain answers to several unanswered questions concerning its origins. These are interesting parallels coming to light in several other parts of North America as new species are described and existing species are reexamined.

Isotria medeoloides

Pogonia ophioglossoides

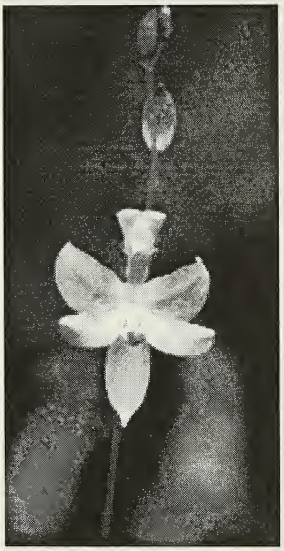
Piperia elegans

Calopogon multiflorus

Corallorhiza striata

Mass Production of *Calopogon tuberosus*

Carson Whitlow
Cyp. Haven
2291 - 28th Street
Adel, Iowa 50003, USA



Calopogon tuberosus is a native American deciduous terrestrial orchid which occurs from south of Florida to Newfoundland, finally a little west of the Mississippi River. Forms are short in its northern extreme, around 6 inches, to quite robust in some cases (nearly three feet) in the southern end of its distribution. There are five species in the genera, which have many similarities, and much of what is presented here is applicable to the other four species.

Calopogons grow from a corm-like structure (enlarged tuberoid is the technical term, I understand). From one of two eyes on the corm, they produce leaves and a flower stem in the spring. As the year passes, the base of the stem enlarges to form a corm for next year. The corm produced the previous year will transfer its energy to the seed pods and new corm, then in the fall die and eventually rot away.

In natural conditions, a sod the size of your hand may contain up to two dozen corms, varying in size from that of a pea to those roughly the size of a dime. Because seed set is often quite high, most of the corms energy goes into seed production, thus leaving little to build next years corm. Likewise, the plants normally bloom late enough in the spring that they are not caught by late frosts.

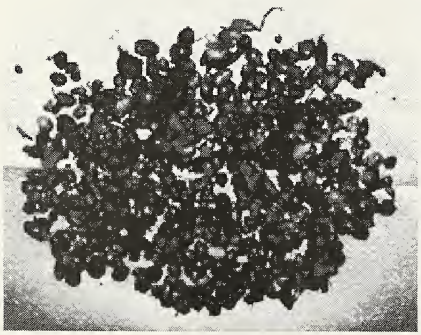
Under cultivation, on the other hand, by removing all seed pods except the very few needed for production, corms can often reach the diameter of a quarter and larger. Flower production is also greater from these large corms, as one would expect. Since plants do not waste their energy in seed production, the secondary eye on the corm often produces a smaller additional corm.

Calopogon tuberosus is a relatively simple species to propagate from seed. It can be seeded on most germinating media used for its tropical epiphytic relatives. It should be seeded thinly on the media and immediately placed in light. The species loves full sun so the flasks can be placed as close as 6 inches from florescent lights with no detrimental results. In natural light, one would want to be concerned with heat buildup in the flask if the light is too intense. In a few weeks, small green protocorms form. They form a great number of hyphae, so they need to be reflasked at a fairly small size. If planted too thickly, they are almost impossible to separate. Reflask from one-quarter to one half inch apart.

Quart milk bottles on their side are good containers to use and allow for easy removal later. They are also sufficiently tall for the plants to grow.

The protocorm will form a small leaf about one half inch long, then literally "sit there" for months. All this time, it is storing energy in its first corm at the base of the leaf. After four months or so, the leaf will start to turn tan and finally die and the small pin head size corm is visible. At this point, the plants have gone dormant.

When the plants are dormant, some may require chilling, others not. They do need the rest. If left alone, many will begin growth a few months later. The unfortunate thing here is that within a few months, you have a flask with plants at various stages of growth, some with new growths, some finishing up their corms, and many in between. This makes for considerable difficulty when removing them from the flask and also for high mortality. Therefore, it is best to put them all on the same cycle.



When most of the plants in the flask have gone dormant, the flasks are chilled in the refrigerator for a week or so, then placed in the "crisper" part of the refrigerator for three months. After that, they are then removed and placed in the light to begin growth. After five or six months, they again go dormant. Usually, this is the time to remove them from the flasks. Many of the corms are

the size of small peas, a good size to handle.

All of the material is removed from the flask, and the seedling corms are separated from the debris. This debris is the remaining media, previous old dead corms, roots and leaves. What you end up with is a small group of corms varying in size from pea-size (some even larger at times) down to pinhead size. After washing, they are dipped in a fungicide and allowed to dry slightly. A small amount of wet sphagnum peat is mixed with the corms, and the seedlings sealed in the bag and placed in the "crisper" of the refrigerator. In the spring, or three months later they are ready to plant out.

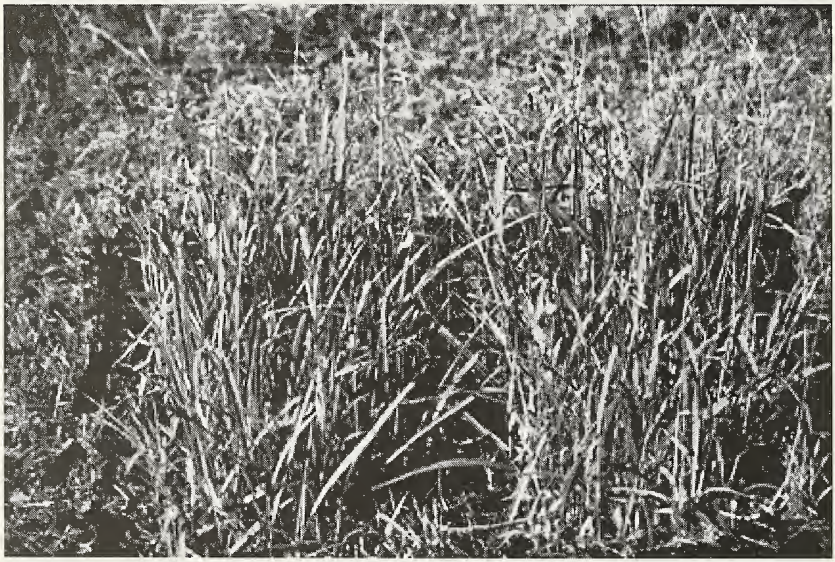
The soil media I use to plant the seedlings in is one half sand, one half sphagnum peat. It is placed in sealed topless boxes about ten inches deep, with a drain about eight inches from the top to drain off excess water. The boxes are placed in full sun. The little corms are usually separated into those which are larger and easier to handle and those which are too small to plant individually. The former are planted an inch or so deep in rows roughly an inch apart, with the space between each corm the size of the corm. For the little ones, an area is dug an inch deep, the little corms mixed with the some of the soil media and spread evenly in the hole, then covered. This is normally done in mid-April in Iowa.

When the seedlings come up, they are like grass (and apparently quite tasty to deer). Occasionally, some of the larger seedlings may even bloom with one or two flowers. The media is kept moist to wet over the entire growing season as the plants make up their new corms. If they have flowered, the flower stems and any seed pods are immediately removed.

After the first frost, all the plants are dug, washed, cleaned of debris, dipped in fungicide, mixed with some wet sphagnum peat and stored in the "crisper" until next spring. In the more southern areas, they may be planted right back in the

containers, if desired, but must be kept wet over winter. They are hardy, but I prefer to store them because it is not as hard on them and I do not have to concern myself with their conditions outside during the winter. Also, I have a better idea of what production I have coming along.

The following spring, they are again planted in similar fashion. More will bloom and the following fall, the process is repeated. It is quite surprising how quickly the little corms gain in size from one year to the next. Usually, most will be mature by the fourth year, however there will still be many smaller corms because of the secondary corms formed even from the smaller plants. These can be culled as you go along.



The corms are really quite tough and easily handled. Often, it is necessary to break off the old roots from the base of the corm. After the cleaning process, they are washed thoroughly and treated with fungicide (optional), then packed and stored. They only need sufficient moisture to keep them plump.

Populations of mature corms can literally be doubled annually by a vegetative propagation technique. This is also applicable for increasing the quantity of selected clonal material. As noted earlier, each corm has two eyes (occasionally more) from which the next years growth will come. To force both eyes, the corm is cut evenly in half, with one eye in each piece. The cut can be sealed and each planted as individual corms. However, to reduce loss through rot, keep the clones together, and to reduce planting time and space requirements, I glue the two cut pieces back together with "Super Glue" and plant them as one corm, giving them a little additional space in the row. The resultant corms, the next fall, are both the same size as the initial corm which was cut in two. The minor loss of flowers which is a result of this procedure is not important since plant propagation is the objective, not flower production.

Meristemming techniques, to my knowledge, have not been done on this

species. However, I think it would definitely be possible without a great deal of difficulty. I would be happy to provide assistance to anyone who would like to undertake this challenge.

While it has been considered very difficult to propagate our hardy terrestrial orchids from seed, I believe a lot of it has been because we did not understand their needs. Over the last decade or so, great strides in production of seedlings has been accomplished. We still have difficulties with some species, but I feel confident that these will yield their secrets in the not too distant future.

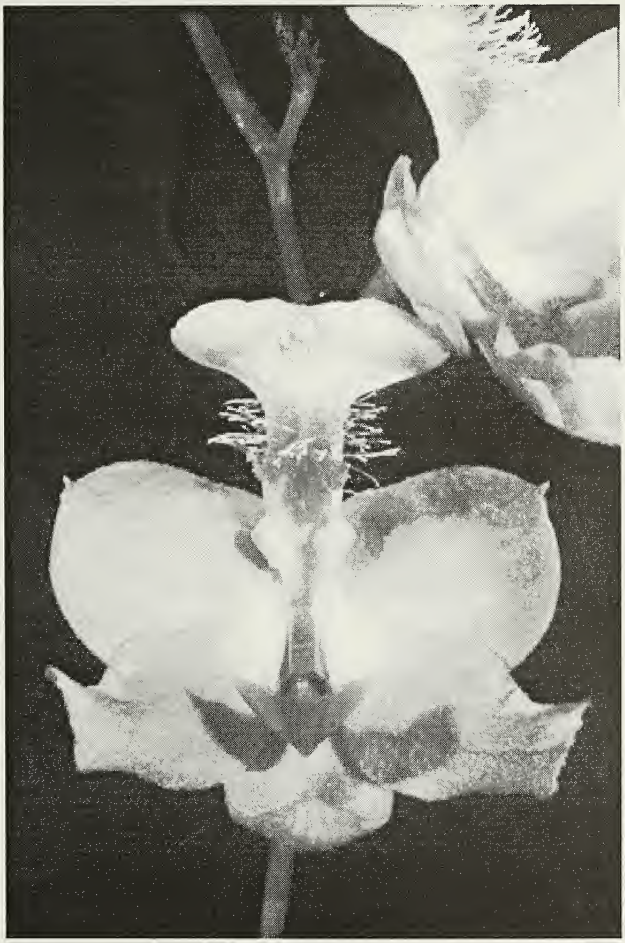


Photo by Robert Yannetti

Fine clone of *Calopogon tuberosus.*

Calopogon tuberosus, near-white strain.

Large Scale Seedling Production of North American *Cypripedium* Species

William K. Steele
Spangle, Washington, USA

In the United states and southern Canada, utilization of land for agriculture, grazing, logging, and urbanization without regard for native vegetation has devastated native orchid populations and their habitat. To compound the problem, collection of the more beautiful orchid taxa by gardeners or for commerce has rendered many species rare, particularly in the vicinity of populous areas. To the extent that native orchids can be grown from seed and offered to the public, incentive for collecting wild plants should be reduced. Under agreeable circumstances, propagated orchids may even be transplanted to suitable outdoor habitat for conservation purposes. The availability of laboratory-grown seedlings permits experimentation with the growth requirements and reintroduction of species without recourse to removal of plants from the wild.

Most species of the genus *Cypripedium* are sufficiently showy to be sought by collectors, and as a consequence, the collecting pressure on these plants is particularly high. My interest in propagation stems from observation of the destruction of natural colonies of these plants over many years. In early efforts, I experimented with growing particular *Cypripedium* species from Washington State for reintroduction in depleted localities, but I soon was producing seedlings in excess of what I could possibly find time for planting and caring for outdoors. Subsequently, I began a small business with my wife for marketing excess seedlings and expanded my efforts at *in vitro* propagation to all *Cypripedium* species for which I could obtain seed. These efforts at propagation are ongoing, and I present here the methods that have yielded the best results so far. The procedures and media recipes have been arrived at through a series of controlled experiments, but the results of the experiments were often assessed by qualitative estimates rather than exact counts or measurements to determine percentage germination, percentage survival, or amounts of growth. For most of the species, the methods have been adequate to produce hundreds of seedlings per year, and for some of the species, thousands of seedlings per year. With a proportional increase in labor, the operation could be scaled up to provide tens of thousands per year, of the more readily propagated species.

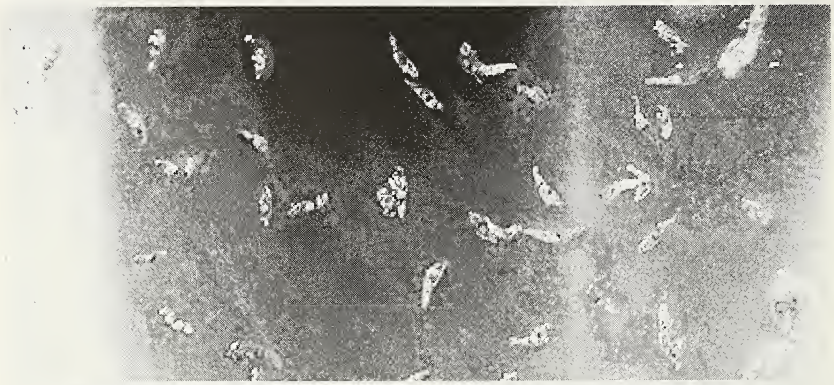
Treatment of Seed

Mature vs. Immature Seed

For restocking wild populations, propagation from seed is preferred over cloning methods as the former promotes genetic diversity. Germination of seeds is carried out using either mature or immature seeds.

Use of mature seeds is generally the preferable approach as mature seeds remain viable for long periods under refrigeration, and mature seeds may be readily shipped in the mails if care is taken to protect them from crushing in mail-handling equipment with plastic bubble-pack in the mailing envelopes (Stoutamire, 1992). After collection, seeds are removed from the capsule and air-dried for several days to prevent infection by fungus during storage in glass vials under refrigeration at 4°C. The two main disadvantages of the use of mature seed

are that the seed must be surface sterilized and that seed-dormancy must be broken.



Ungerminated seeds of *Cypripedium reginae.*

The culture of immature seed, sometimes known as "green pod culture," misleading terminology in several respects (Arditti, 1992), has one major advantage: if seed is harvested before dormancy factors have been introduced by the parent plant, there is no need to find measures to break dormancy; the embryos simply continue to grow when the seed is placed on the culture medium (Light, 1989; Light and MacConaill, 1990; Wagner and Hansel, 1994; Withner, 1953). The disadvantages are that the timing of seed harvest is critical and that the seed must be sown shortly after harvest. As a result, long distance shipment of the seed is usually impractical, and long term storage is impossible.

Because of the significant advantages of using mature seed and disadvantages of immature seed, the bulk of my work has been with mature seed. For species in which germination is markedly better with immature seed and a ready source is available, immature seed has been used, but because most of my efforts have been with mature seed, the rest of this discussion will deal with mature seed except as otherwise noted.

Variables Affecting Germination of Mature Seed

Since beginning work with *Cypripedium* seed in 1988, I have found several factors to have a marked effect on germination of mature seed and early protocorm growth. My initial experiments were done with Knudson's (1946) C germination medium, or its modification labeled "bKCp" by Ballard (1987). In my experiments shortly thereafter, Harvais' (1982) medium gave significantly better results, and I have used it ever since. Limited trials with Murashige and Skoog (1962) medium diluted to one third strength and a commercial medium also indicated the superiority of Harvais' (1982) medium or minor variations of it for *Cypripedium* culture. The present report should not be construed as indicating Harvais' (1982) medium is optimal, for there are many media that I have not tried, some of which are reported as better for *Cypripedium* germination and early growth (De Pauw and Remphrey, 1993). Harvais' (1982) medium or my modifications of it do seem adequate for most American species. Unlike Harvais' original

formulation, my modification lacks exogenous vitamins and contains only mineral nutrients, sugar as a carbon source, one complex additive, and cytokinin for those species for which it is necessary to assure germination. Preparation of the medium from stock solutions is described in Table 1. A detailed account of the general procedures used with application to one species, *Cyp. reginae*, is found in Steele (1995).

During the course of my work, five variables suggested themselves as important determinants of successful germination and early protocorm growth: 1) cold storage of seed ("stratification"), 2) exogenous cytokinin in the medium, 3) bleaching to remove dormancy factors, 4) nitrogen concentration and form, and 5) nature and concentration of undefined constituents. Each of these factors is considered below. Ranges of values for these factors that I presently use for different species are listed in Table 2.

Table 1. This table describes stock solutions for the preparation of Harvais' (1982) medium, in this case with 1400 mg/L ammonium nitrate. Each major element has its own solution, 10 ml of which is added to enough distilled water to make 1 L of medium. The iron solution contains ammonium citrate as a chelating agent. The trace elements all go into the same stock solution, and 1 ml of this solution is added to the medium to give the proper concentrations of these elements. For most *Cypripedium* species, glucose (dextrose) is used in the medium at 20 g/L, and agar is added at the rate of 7 g/L for germination medium and 5 g/L for reflasking medium.

Preparation of Harvais' Medium from Stock Solutions			
Component	Amount per L of Medium	Stock Solution	Vol. of Stock per L of Medium
Macroelements			
Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	400 mg	8.0 g/200 ml	10 ml
Ammonium nitrate, NH_4NO_3	1400 mg	28.0 g/200 ml	do
Potassium phosphate, KH_2PO_4	200 mg	4.0 g/200 ml	do
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	200 mg	4.0 g/200 ml	do
Potassium nitrate, KNO_3	200 mg	4.0 g/200 ml	do
Potassium chloride, KCl	100 mg	2.0 g/200 ml	do
Ammonium citrate plus Ferric ammonium citrate (in same stock solution)	19 mg 25 mg	0.38 g/200 ml 0.50 g/200 ml	do
Microelements + Mn *(all in same stock solution)*			
Boric acid, H_3BO_3	0.5 mg	500 mg/l	1 ml
Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025 mg	25 mg/l	do
Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	0.5 mg	500 mg/l	do
Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.02 mg	20 mg/l	do
Cobalt nitrate, $Co(NO_3)_2 \cdot 6H_2O$	0.025 mg	25 mg/l	do
Potassium iodide, KI	0.1 mg	100 mg/l	do
Manganese sulfate, $MnSO_4 \cdot H_2O$	1.54 mg	1540 mg/l	do

Cold Storage of Seed

Stoutamire (1974) reported that germination of *Cyp. reginae* seed was enhanced after refrigeration on the culture medium at 5-10°C for several months

compared with controls maintained at 20°C. Ballard (1987) carried out extensive experiments to elucidate the conditions for cold storage in this species. He found that two months or more of refrigeration at 5°C greatly enhanced germination.

Table 2. This table gives values of five variables that have produced the best germination of mature seeds and the best early protocorm growth so far. In most cases germination and seedling production have been satisfactory although further refinement can surely be accomplished through additional experimentation. For *Cyp. kentuckiense,* protocorm survival has been poor. Germination of mature *Cyp. montanum* seeds has been minuscule.

Provisional Cultural Parameters for Germination and Early Growth of Cypripedium Species

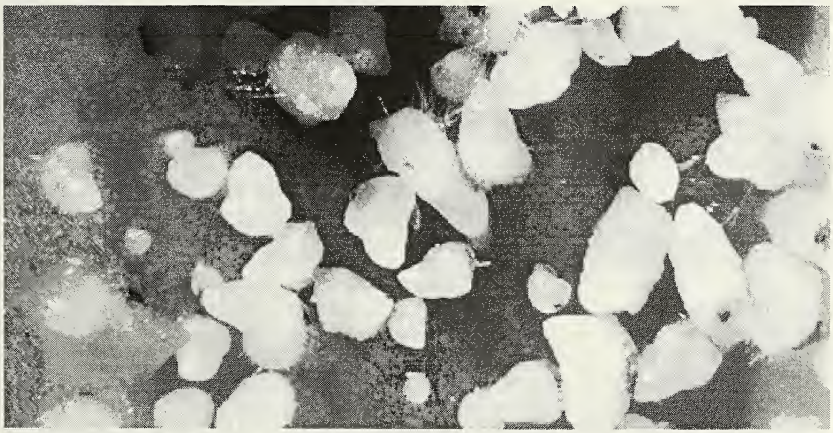
Species	Cold Treatment (<4 °C)	Bleaching Time	Kinetin (1.2 mg/L)	Ammonium nitrate	Raw Potato	Remarks
C. acaule	>3 months	2-6 hrs	No	1000 mg/L	1 cm^3 per 25 mL	Bleaching time variable
C. arietinum	>4 months	2 hrs 35 min	Yes	500 mg/L	0.5-1 cm^3 per 25 mL	Quickly reflask
C. californicum	>8 months	2 hrs 20 min -3 hrs 41 min	No	500 mg/L	4 cm^3 per 200 mL	Germination faster with kinetin
C. candidum	>6 months	>66 min	No	500 mg/L	2-3 cm^3 per 25 mL	A. Anderson (1990) bleaches 2 hrs.
C. fasciculatum	0-6 months	>64 min	No	500 mg/L	2-3 cm^3 per 25 mL	Growth slow
C. guttatum	0-5 months	12-30 min	No	500 mg/L	2 cm^3 per 25 mL	Bleaching time variable
C. kentuckiense	0-6 months	20-40 min	No	500-1000 mg/L	1-2 cm^3 per 25 mL	Poor survival with mature seeds
C. montanum	>9 months	>3 hrs	No	500 mg/L	1-3 cm^3 per 25 mL	Germination <1%
C. parviflorum var. makasin	>4 months	40 min	No	500 mg/L	>0.5 cm^3 per 25 mL	Seems to prefer more potato
C. parviflorum var. pubescens	>3 months	20-60 min	No	500 mg/L	2-3 cm^3 per 25 mL	Bleaching time varies from clone to clone.
C. passerinum	0-3 months	20 min	No	500 mg/L	2 cm^3 per 25 mL	One of the easiest to germinate
C. reginae	3-11 months	35 min	Yes	1.0-1.4 g/L	0.12-0.25 cm^3 per 25 mL	Quickly reflask
C. yatabeanum	0-3 months	23 min	Yes	500 mg/L	0.25-2 cm^3 per 25 mL	Quickly reflask

Germination of seeds of many species of native plants is inhibited until the seeds are stored in moist conditions at near-freezing temperatures, a process known as "stratification," but Ballard (1987) found that *Cyp. reginae* seeds germinated at nearly 100% after dry refrigerated storage for a year. Orchid seeds that have not been surface-sterilized should only be stored after drying, for moist orchid seeds

are readily attacked by fungus. The hyphae of the fungus penetrate the seeds, and the fungal infection cannot be eliminated by subsequent surface-sterilization. Mature seed in my lab is routinely air-dried for several days and then placed in glass vials for storage at temperatures of 1-5°C. Thus most of the seed used in trials leading to the results reported here received refrigeration for varying periods. In some species, however, sowing of unrefrigerated seed was carried out with positive results, and this information is provided in the tables of results.

Exogenous Cytokinin

Harvais (1982) found it necessary to add a cytokinin to the culture medium to achieve rapid germination of *Cyp. reginae*, and he tested three aminopurines for their effectiveness in promoting germination, early growth, and survival. Of these three, he found that kinetin gave the best overall survival and that concentrations of 0.5-1.0 mg/L produced excellent germination. To avoid the labor of the additional step of filter-sterilization that Harvais employed, I simply add 1.2 mg/L kinetin to the medium before autoclaving; the extra 0.2 mg/L seems sufficient to replace any kinetin lost through thermo-degradation. In my experiments, kinetin retards growth of *Cyp. reginae* seedlings once they reach the first root stage, and so at this point they are reflasked to medium containing no kinetin.



Two month old *Cypripedium reginae* protocorms. Note rhizoids.

Kinetin at 1 mg/L appears to stimulate germination of most *Cypripedium* species. For many species, however, kinetin in the germination medium apparently prevents normal shoot differentiation. Protocorms on medium with kinetin often appear to attempt to grow many shoots instead of one or two, or in some cases no shoot at all. For these species, the only recourse seems to be to germinate them on medium without exogenous cytokinin. In Table 2, column four indicates whether kinetin is routinely used in our germination medium.

In the case of *Cyp. californicum*, adequate germination was obtained either with relatively short bleaching of 30 to 60 minutes and kinetin in the medium, or with much longer bleaching of from 2 hours 20 minutes to 3 hours 41 minutes with subsequent sowing on medium without kinetin. This result suggests that longer

bleaching may be a substitute for the use of kinetin in the medium in overcoming dormancy, and a similar effect should be sought in a number of other species. Future experiments with *Cyp. arietinum, Cyp. reginae,* and *Cyp. yatabeanum* may show that longer bleaching time alone may be sufficient to promote germination without the need for exogenous cytokinin in the medium.

While little is known about the mechanism of dormancy in *Cypripedium* seeds, abscisic acid (ABA), a natural dormancy-inducing substance in the buds of woody plants, has been detected in seeds of the European terrestrial orchids *Epipactis helleborine* and *Dactylorhiza maculata* (Van der Kinderen, 1987). Cytokinins including kinetin have been shown to exert a permissive role in overcoming dormancy induced by application of ABA to buds and seeds of various plants (Khan, 1971). ABA-cytokinin antagonism is thus a plausible mechanism for dormancy and its release by exogenous cytokinin in asymbiotic culture of *Cypripedium* seeds.

Bleaching to Remove Germination Inhibitors

In experiments in the germination of northern terrestrial orchids, Lindén (1980) noted that in disinfection of seeds of *Dactylorchis maculata, Gymnadenia conopsea,* and *Orchis morio,* germination increased strikingly with immersion time in 2% NaOCl solution up to a certain point and then fell off with additional bleaching. He speculated that the bleaching process leached or destroyed germination-inhibiting substances. Harvais (1980) noted that bleaching *Cyp. reginae* seeds to the point at which they sank improved germination but attributed the effect to the removal of suberin in the seed coats and the escape of air from between seed coats and embryos, in either case improving diffusion into the embryos. Anderson (1990) reported that *Cyp. acaule* seeds accidentally left in the sterilizing solution too long germinated better than those sterilized for the intended time. This result prompted him to perform experiments to determine the optimum bleaching time for this species as well as for *Cyp. candidum* and *Cyp. calceolus* (now *Cyp. parviflorum*).

Following Anderson (1990), we routinely subject seeds to vacuum treatment that removes air from the interior of the seed before bleaching. Microscopic observation during bleaching clearly shows that the bleach solution enters the seed coat, probably through the opening at the suspensor end, for bubbles often form in the space between the embryo and seed coat. Furthermore, in species with dark embryos, the surface of the embryo is observed to lighten at the same time as does the seed coat indicating the action of bleach interior to the seed coat. This observation suggests that the hydrophobic nature of the seed coat is not an important mechanism of dormancy as some authors have suggested. Moreover, even after minimal bleaching, the embryos of some species are observed to swell after sowing, indicating diffusion into the embryo, but no subsequent germination occurs. In these same species, increased bleaching time results in this initial swelling being followed by germination, i.e., rupture of the seed coat by the embryo. From these observations, I infer that the mechanism by which bleaching improves germination is in accord with Lindén (1980), viz., that the bleach leaches or oxidizes one or more dormancy-inducing compounds on the surface of the embryo.

Many orchidists use $Ca(OCl)_2$ solution for surface sterilization because this

compound is more gentle to orchid tissue than NaOCl. Calcium hypochlorite also seems to be more gentle to pathogens, unfortunately, and so longer sterilization times are required. Another concern is that $Ca(OCl)_2$ loses strength with time, even when stored as a solid. For these reasons, I have routinely used NaOCl for bleaching, and all the work described here is based on bleaching with NaOCl. The bleaching times given in Table 2 are for 0.5% NaOCl solution. The bleaching solution can be prepared from commercially available household bleach containing 5% NaOCl, but care should be taken to use bleaching solution freshly prepared from the commercial product. To insure consistency of results, I monitor the oxidation-reduction potential (ORP) of the bleaching solution with an ORP meter. The ORP is approximately 750 mV.

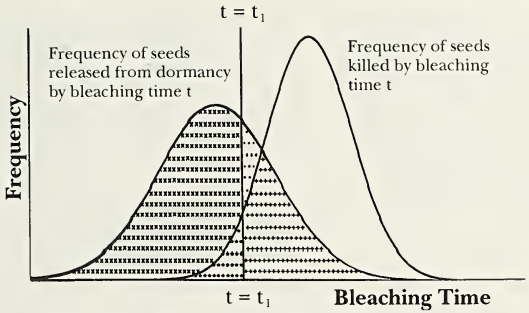


Figure 1. Bleaching Model. The curve on the left is the frequency distribution for release from dormancy by bleaching. The curve on the right is mortality from bleaching. The area shaded with x's represents seeds germinating after bleaching for time $t = t_1$. The area shaded with *'s represents seeds killed by the bleaching. The area shaded with •'s indicates seeds that would germinate with longer bleaching, and the area shaded with +'s contains seeds impossible to germinate because bleaching time for release from dormancy exceeds lethal bleaching time. The optimal bleaching time corresponds to the intersection of the two curves. Longer bleaching kills seeds that would otherwise germinate.

While the exact mechanism by which bleaching improves germination is not known, a crude model can still be proposed to describe the response of a population of seeds to the process. I assume that the frequency distribution for the release of seeds from dormancy by bleaching is normal in character as is the frequency distribution for the death of the seeds. These distributions are represented by the left and right curves, respectively, in Figure 1. The area under the left curve is the population of seeds released from dormancy by bleaching; the area under the right curve is the population of seeds killed by bleaching, and the area common to the two curves represents seeds impossible to germinate because they are killed by the length of bleaching required for germination. The particular shape and position for each of these curves will vary from one species to another, and indeed the curves may not even be normal, but Figure 1 at least gives a qualitative depiction of the relationship between the alternative effects of bleaching, viz., germination and mortality. Easily germinated species can be seen to be those for which the two curves are widely separated and have little overlap.

In contrast, if the two curves have much area in common, the seeds cannot be expected to have high germination. The object in bleaching experiments is to find the bleaching time t_1 that maximizes the number of seeds germinating.

Attention should be focused on the point that the effectiveness of bleaching in NaOCl in breaking dormancy was an accidental discovery made during bleaching for surface sterilization. It seems highly likely that a deliberate search might find chemical agents more effective for removing germination-inhibiting substances from the embryo sac while being less destructive to embryo tissues.

Nitrogen Concentration and Form

Early experiments with *Cyp. reginae* and *Cyp. parviflorum* on Ballard's (1987) bKCp modification of Knudson's C and on Harvais' (1982) media soon showed superior growth of both species on the latter medium. Subsequently, it was found that *Cyp. reginae* protocorms grew faster and produced larger diameter roots with bright yellow meristems on Harvais' original version of the medium with 1400 g/L ammonium nitrate, whereas *Cyp. parviflorum* grew faster on Harvais medium with only 500 mg/L ammonium nitrate. Both species do very well with ammonium nitrate as the primary nitrogen source, and so most of my work with other *Cypripedium* species has been with the Harvais' mineral base but with different concentrations of ammonium nitrate. Table 2 gives the ammonium nitrate concentration that I have found most satisfactory for each species.

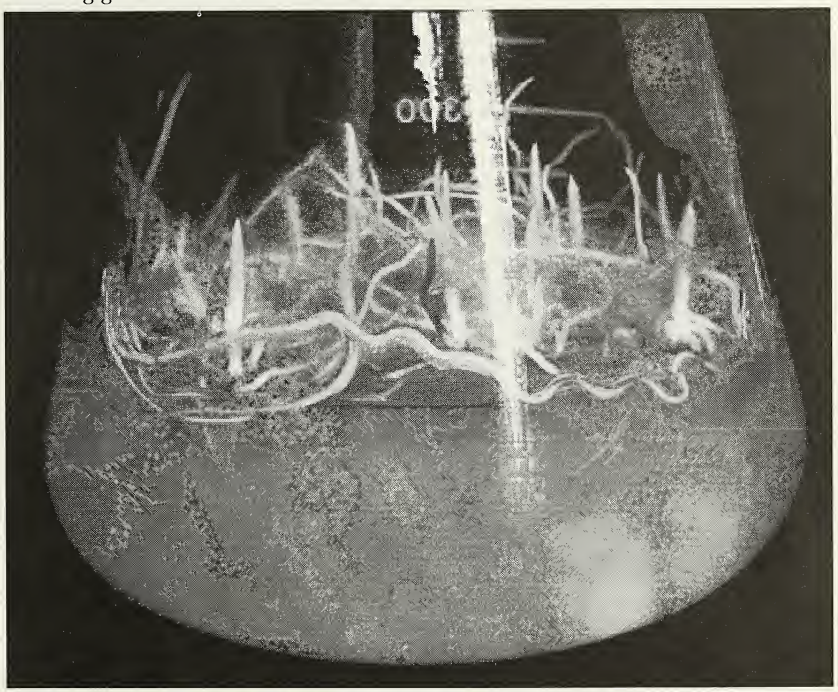
A number of workers (DePauw and Remphrey, 1992; Henrich and others, 1981; Malmgren, 1992, 1993; Van Waes and Debergh, 1986; Wagner and Hansel, 1994) have reported successful germination and growth of *Cypripedium* seed on media with amino acids as the nitrogen source. Because Harvais' (1982) medium has been satisfactory for most species, I have been slow to try amino acid media, but within a few weeks prior to preparation of this paper, experiments were begun comparing Harvais medium with 500 mg/L ammonium nitrate to Harvais medium identical except for the replacement of the 500 mg/L ammonium nitrate with 500 mg/L casein hydrolyzate, a mixture of amino acids. Currently these experiments have not progressed to the point that results can be reported.

Undefined Constituents

In addition to the major and minor minerals, growth regulators, and sugar that are important constituents of most orchid germination media, growers and tissue culturists usually find a need to add some form of complex organic ingredient as well. Coconut milk, banana, or potato are often used in this regard. Apparently these materials contain one or more growth factors not yet identified or recognized as valuable to germination and protocorm growth. While observers may consider use of such undefined substances as less than scientific, plant culturists should take note that medical researchers and others working with animal cell cultures have usually found the addition of such undefined substances as fetal bovine serum necessary for their work. In both plant and animal cultures, the need for an undefined complex additive indicates an incomplete knowledge of cultural requirements.

Having experimented with banana, fresh and canned coconut milk, and potato, I now exclusively use the latter because it gives the most consistent results.

Banana and coconut both stimulate germination of *Cypripedium* seed and protocorm growth, but the results were not consistent from one experiment to another, apparently because a given volume of either banana or coconut milk may contain more or less of the effective growth stimulants depending on the stage of ripeness and moisture content of the fruit. In contrast, use of a given volume of potato tissue from a particular variety of potato (russet) seems to give consistent results. To facilitate measurement, I usually use various fractions or multiples of 1-cm cubes of raw potato cut to size with a knife and added to the medium in a culture vessel before autoclaving. Harvais (1982) used an elaborate cooking procedure to produce potato-dextrose-agar, but I have adopted my simpler method as a time-saving expedient although it probably does an inferior job of releasing growth factors into the medium.



Cypripedium parviflorum var. *pubescens* on Harvais' medium. Note potato cubes at lower right.

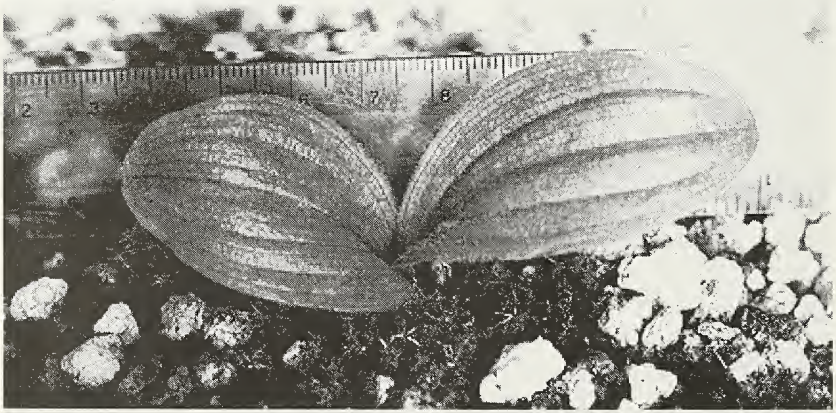
The requirement for potato for seed germination and early growth seems to vary considerably among species, and Table 2 indicates amounts that have produced successful germination and early growth in most species. In *Cyp. reginae*, a greater amount of potato is needed for germination if the kinetin is omitted suggesting that a cytokinin may be one of the important growth factors in potato, but complete omission of potato from medium with optimal kinetin results in poor germination and very slow early protocorm growth.

Notes on Requirements for Particular Species

Table 2 lists values of cultural variables that have produced the best results for me so far. These are not necessarily optimal conditions, and for two species, *Cyp. kentuckiense* and *Cyp. montanum*, results with mature seeds have not been satisfactory. For the latter species in particular, germination has been abysmal. Comments pertinent to particular species follow.

Cyp. acaule

Cypripedium acaule generally germinates well in excess of 20% from mature seeds if they are given the proper bleaching. The bleaching requirement seems to vary considerably from one plant population to another or perhaps even from one clone to another. I have had the best germination with a bleaching time of two hours in 0.5% NaOCl, but Anderson (1990) reported the best germination with six hours of bleaching. Germination is slow and requires at least two months. Although the plant usually grows in highly acidic soil in the wild, the plantlets seem to have no difficulty in medium with pH as high has 5.6 and may very well tolerate higher values.



Cypripedium acaule, second summer out of flask.

Cyp. arietinum

Bleaching time is critical for mature seeds of this species. Bleaching for 1 hour 30 minutes was apparently insufficient, and there was no germination in seeds bleached 2 hours 55 minutes. With bleaching of 2 hours 35 minutes, germination is usually in excess of 60% on medium containing kinetin. To assure that seedlings develop normally, they must be promptly reflasked to medium without exogenous cytokinin as the protocorms enter the first-root stage.

Cyp. californicum

Germination can be stimulated after very little refrigeration if kinetin is added to the medium, but higher survival and better development of seedlings are obtained with several months of refrigeration, long bleaching times, and medium without kinetin. This species seems to prefer less potato in the medium. Experiments with this species suggest that the requirement for exogenous cytokinin for germination can be circumvented by the use of long bleaching times.

Cyp. candidum

This species germinates very well on medium containing kinetin, but does not develop normal seedlings unless they are promptly reflasked to kinetin-free medium. Somewhat lower germination but better development of protocorms is obtained without kinetin. I have found germination improves with increasing bleaching time up to the maximum used so far: 66 minutes. Anderson (1990) found the best germination with two hours bleaching. *Cyp. candidum* does better with plenty of potato, even with kinetin in the medium.

Cyp. fasciculatum

No refrigeration is necessary for germination of mature seed of *Cyp. fasciculatum.* Long bleaching is important. In experiments, germination increased to a maximum of roughly 50% for a bleaching time of 64 minutes, but germination might be better with still longer bleaching. This species needs relatively large amounts of potato in the medium, and even so, the first germination requires two months. Compared with other *Cypripedium* species, the protocorm bodies grow to enormous size, often over a centimeter in length, before entering the first root stage. The large protocorm body is retained in the seedlings, and shoots are disproportionately large relative to the roots compared with other species. These seedlings seem to develop normally after planting out. Determination of the normal proportions of natural protocorms and seedlings would require digging them from the ground in wild colonies. A major difficulty encountered with this species has been that seedlings develop at greatly different rates, so that at any one time only a small fraction of the seedlings are ready to remove from the flask.

Cyp. guttatum

This species germinates very readily with no requirement for refrigeration or an exogenous cytokinin in the medium. The duration of bleaching for optimum germination varies from one population to another up to a maximum of about 30 minutes. Germination is usually 90% or more of the seeds with normal-appearing embryos.

Cyp. kentuckiense

Unfortunately, this desirable species is one of the more difficult to grow from mature seed. Germination is usually good, but early protocorm growth is

exceedingly slow on the media that I have tried. Protocorm mortality is high, and there is also high mortality after seedlings are reflasked. Addition of kinetin to the germination medium improves survival of small protocorms but causes abnormal shoot development in the seedlings. In two experiments, protocorm survival and growth were enhanced by using glucose at the rate of 30 g/L in the medium instead of the usual 20 g/L. I have had to rely on immature seeds to obtain most of my seedlings.

Cyp. montanum

Seeds of this extremely beautiful plant are by far the most difficult to germinate. Even after bleaching times of three hours and four hours, germination has been roughly 0.1%. Not enough seeds have germinated to permit determination of the requirement for potato. Immature seeds germinate only slightly better, and these seeds should be sown at less than six weeks after pollination. In nature, seed capsules often dehisce only 10 weeks after anthesis. Such rapid seed production is probably necessary because the soil usually becomes very dry during summer in the habitat of the plant. Protocorms from immature seeds grew very well on Harvais' medium with 500 mg/L ammonium nitrate and 1 cm^3 potato per 25 ml of medium. Mature seeds of *Cyp. montanum* will probably require some radical departure from my usual methods, perhaps an amino acid nitrogen source or some other treatment than bleaching to break dormancy.

Cypripedium montanum, Spokane County, Washington, June 4, 1993.

Cyp. parviflorum var. *makasin*

This subspecies germinates at roughly 20% with the treatment listed. Protocorms from immature seeds do very well with 1 cm^3 potato per 25 ml of medium.

Cyp. parviflorum var. *parviflorum*

This subspecies seems to germinate very well with the same conditions as for subspecies *pubescens*, viz., bleaching for 60 minutes and 2 - 3 cm^3 raw potato per 25 ml of medium. Germination usually begins within a month of sowing.

Cyp. parviflorum var. *pubescens*

Cyp. pubescens seed from some plants often germinates very well with the conditions given, whereas normal-appearing seeds from other clones fail to germinate or germinate very sparsely. Again, bleaching for 60 minutes and including 2 - 3 cm^3 potato per 25 ml of germination medium are recommended.

Cyp. passerinum

This is one of the easiest species to germinate. No refrigeration of the seed or exogenous cytokinin in the medium is required. Two cm^3 of potato per 25 ml of germination medium is optimal. Percentage germination is usually high. Germination begins within a month of sowing.

Cypripedium parviflorum awaiting planting outside.

Cyp. reginae

Cyp. reginae is one of our largest and most spectacular native orchids. Germination is excellent, often over 90% with kinetin and a relatively small amount of potato, no more than 0.25 cm^3 per 25 ml. Germination is usually apparent a month after sowing. Protocorm growth becomes very slow if the

protocorms are not reflasked to kinetin-free medium as they begin the first-root stage, usually six to nine weeks after sowing. The seed will germinate and the seedlings will grow on medium with 500 mg/L ammonium nitrate, but seedlings grown on medium with 1000 - 1400 mg/L are much more robust.

Cyp. yatabeanum.

This species requires exogenous kinetin in the medium for good germination, but the protocorms must be reflasked to kinetin-free medium as soon as they enter the first-root stage.

Reflasking

Protocorms are usually removed from the germination medium as they enter or shortly after they enter the first-root stage. They are transferred to kinetin-free medium in 500 ml Erlenmeyer flasks. Regardless of the amount of potato required for germination, the various species all seem to do well on medium containing potato at the concentration of 4 cm^3 per 200 ml of medium. Again, the potato is provided as cubes cut from the raw tuber and added to the medium before autoclaving. The flasks of seedlings are incubated in the dark at temperatures from 20 to 25°C as are the germination cultures.

When the seedlings develop shoots 1-3 cm in length or if the root meristems turn from the bright yellow color they have during growth to brown indicating dormancy, the seedlings are removed from the medium with forceps, rinsed free of agar, sealed in freezer bags with the roots of the seedlings in a little water, and placed in a refrigerator for at least three months for vernalization.

Conclusion

The methods presented are probably not optimal, but for most of the species listed Harvais' (1982) medium is adequate to produce large numbers of healthy seedlings suitable for planting out. Two exceptions are *Cyp. kentuckiense* and *Cyp. montanum.* More work is required to develop a medium that promotes rapid growth for young protocorms of the former. So far, mature seed of *Cyp. montanum* has resisted germinating under conditions adequate for the other species. More innovative methods seem required. Even for those species now produced in large numbers, improved methods and media will no doubt be developed to grow seedlings with less effort. For most of these species, however, the major propagation problem now is growing the seedlings on to flowering-size adults economically.

Community pot of *Cypripedium reginae* planted out March 1990, photographed June 1991.

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Arethusa bulbosa
Life Cycle, Propagation and Production

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Introduction

Fig. 1. *Arethusa bulbosa.*

Arethusa bulbosa (figure 1) is an orchid of our northern latitudes. Its range in southern Canada extends from Saskatchewan to Newfoundland, with the southeastern edge of the Boreal Forest Region being the northern limit. The southern limit of the plant lies within our northeastern states where its range extends from Minnesota and Wisconsin through the central and lake states to Pennsylvania, New Jersey, New York and north through New England. It has also been reported from the mountains of North Carolina.

Along its southern boundary the *Arethusa* is not doing well. From state listings of rare and endangered vascular plants and other sources we have the plant as:

Extirpated from Delaware, Maryland, Virginia and Illinois.
Endangered in North Carolina.
Declining and vulnerable in Connecticut, New Hampshire, and Vermont.
Disjunct in Maine.

In New Jersey however the plant is not considered to be endangered or rare, and while not common it is not difficult to locate. I monitor two populations of *Arethusa* in the Pine Barrens of southern New Jersey.

The number of plants in bloom in a given location will vary greatly over time. In a waning population the impression is that the plant is failing but if the habitat has not been disturbed the population will begin to increase in a few years. Figure 2 shows the number of blooming plants, from 1984 to 1995, at two very similar sites separated by less than two miles. The areas are well defined and the counts made were exhaustive. The population represented by the filled bars declined from a high of 127 blooming plants in 1984 to a low of 25 plants in bloom in 1990. It then recovered to a high of 174 blooming plants by 1993. The population represented by the open bars reached its peak when the other fell to its lowest count. In both areas a high level of seed production was maintained by hand pollination during the periods shown. No satisfactory explanation has been put forward to account for the variance and we are left with Case's (1989) observation that "The apparent environment may not be what the orchid is responding to at all".

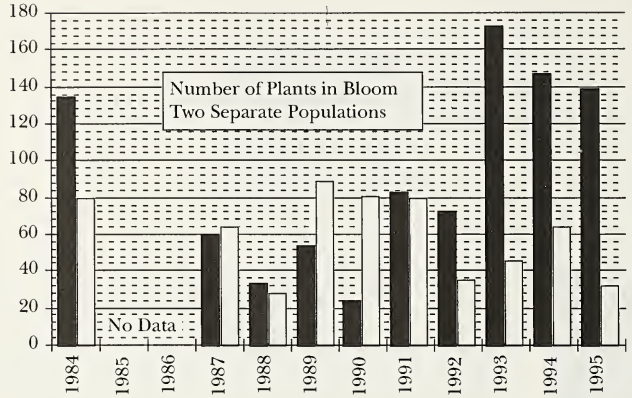

Figure 2. Comparison of the number of plants in bloom in two separate populations.

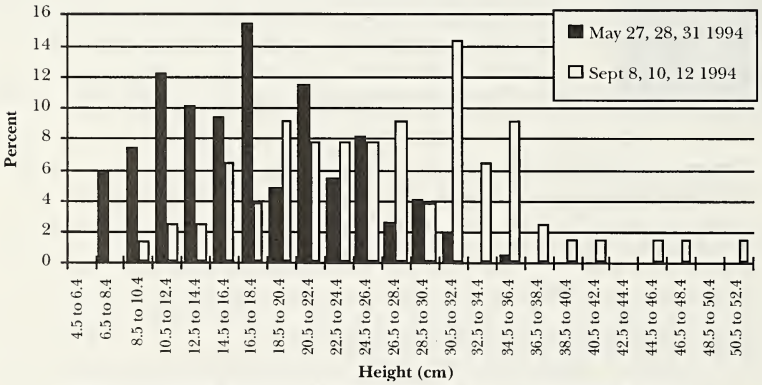

Figure 4. Comparison of plant height at anthesis and maturity (See Endnote).

Morphology

Before taking up the life cycle of *Arethusa bulbosa* it may be best to look at the morphology of a typical mature plant (figure 3) so that we may have a frame of reference for discussing its development. The plant is generally described in the literature as being 6 to 40 cm tall and since the descriptions are usually of a plant in bloom, the range in plant height is assumed to be at that time. The plants of southern New Jersey fit comfortably within this range. Figure 4 compares the distribution of plant height at anthesis and at maturity for a single population of *Arethusa bulbosa.* The bars show the percentage of plants in each height class. The filled bars are the height of the plants measured at blooming and the open bars are the height of the plants measured at maturity. The average height of a plant in bloom was 17.5 cm, with a range of 6.5 to 35.0 cm. When fully mature the plants will have increased to an average height of 26.5 cm and will range from 9.5 to 48.5 cm. The tallest plant that I have measured was 61.0 cm when mature.

Fig. 3. Typical Arethusa bulbosa plants.

The plant winters over by expanding the cells of two of its internodes into a corm (figure 5) for the storage of starch. The axillary bud of the upper internode being the primary eye, and the bud of the lower internode the secondary eye. The primary eye of a mature corm is fully formed with sheath, flower, and root primordium when the plant becomes dormant at the end of summer. This enables the plant to bloom within 35 to 45 days after breaking dormancy the following spring. The secondary eye is usually not developed.

When new growth begins, the shoot and roots develop at the same time. The plant normally has three roots which form below the first node of the shoot. The center root grows down into the peat while the two lateral roots extend horizontally through the sphagnum at or just below the boundary between the living and the dead stems and branches.

The shoot will usually consist of seven nodes and seven internodes which are shown schematically in figure 6. The first and second internodes are compressed so that the outer and middle sheaths appear to arise together. The outer sheath covers the compressed second internode, the lower portion of the middle sheath and the third internode. It seldom reaches to the fourth internode. The middle sheath arises from the third node. It covers the third, fourth and fifth internodes and the lower part of the sixth internode, which is the flower stem. The inter sheath arises from the fourth node. It will cover the fourth and fifth internodes, which will develop into the new corm, and the lower part of the sixth internode, reaching above the middle sheath. These three sheaths do not develop leaf blades. The fourth sheath will bear a leaf. It arises from the fifth node, covering the fifth internode and extends upward, clasping the sixth internode. The leaf blade is delayed in its development until after the flower has bloomed, then it will unclasp the internode and elongate, reaching up to or above the ovary.

Fig. 5. Blooming size corm showing the primary eye.

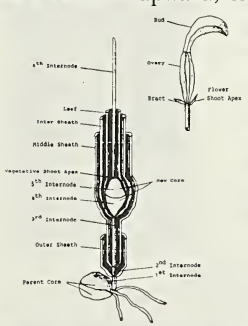

Fig. 6. Schematic diagram of a mature plant.

If the plant will not produce a flower the vegetative apex will be the top of the fifth internode. When the plant is to bloom a sixth and seventh internode are formed and the flower develops from the auxiliary bud of the seventh internode. Both the sheath arising from the seventh node and the seventh internode are reduced to bract like structures. Should the plant carry a second flower then a fully developed seventh internode develops and a reduced eighth internode is formed. The second flower then develops from the auxiliary bud of the eighth internode. The concentric sheaths and the clasping leaf protect and support the soft intercalary meristems of the

internodes during their period of growth.

Life Cycle

The usual place to begin the description of a plant's life cycle is with its seed. The seed of *Arethusa bulbosa* is small, even for an orchid, the embryo within the testa measuring about 0.15 mm long and 0.1 mm in diameter.

We will assume that the seed of our plant is mature, dormant and has been delivered to a suitable habitat. For *Arethusa* the most suitable habitat would be a bed of sphagnum moss, which is a fairly stable environment in terms of plant secession. But, while stable it is not at all static. The moss has a number of physical characteristics (Clymo 1970) to which *Arethusa* has had to adapt:

Its growth is predominantly apical and indeterminate, so that its axis of growth is also an axis of time.
It grows from 3 to 8 cm a year and can continue to grow at temperatures as low as 2°C.
The capitula density is fairly constant for most species.
The depth of the living moss below the capitulum can range from 2 to 6 cm.
More than 90% of the incident light is absorbed in the top 2 cm of the moss.
Over time the dead branches and stems are compacted into peat.

The seed finds itself resting on the dense moss. It is October and the rain will wash it in among the developing branches. As the moss continues to grow and the lower dead portions of the plants are compacted, the seed effectively sinks into the moss so that at the onset of winter it will be below the surface, at a reduced light level, and wet. With the coming of spring the moss renews its growth and the seed continues to sink until it is in near or complete darkness. Having been chilled for some four or five months and washed by rain and melting snow the seed is now ready to germinate.

Figure 7. (Left) Developing embryos 90 days after germination.
Figure 8. (Center) Hyphae of a mycorrhizal fungus coiled in a digestion cell.
Figure 9. (Right) Developing first corms.

The description given here is based on observation of *Arethusa bulbosa* seed germinating in flasks and the subsequent protocorm development. The first sign of germination is the swelling of the seed due to the physical absorption of water. With the increased volume, aerobic metabolic pathways are activated and the lipids, stored in the embryo, are converted into starch. The expanding embryo sheds its seed coat, becoming spheroid in shape, and begins to develop rhizoids.

As growth continues the embryo elongates becoming upright and stalk-like (figure 7). Development to this stage was probably carried out using only the seed's stored nutrients. Having germinated in the dark the seed can not make use of photosynthesis so when its limited reserve of energy is exhausted another supply has to be found. At this time the plant must establish a mycorrhizal association for its source of complex carbohydrates. The rhizoids, which were the first structures developed, provide access for the hyphae of the fungus which are broken down in digestion cells (figure 8) and the released metabolites are absorbed by the plant. If the transfer of nutrients is adequate development will continue and the first corm (figure 9) will form at the top of the stalk-like structure. At the end of the first summer the corm becomes dormant, the stalk disintegrates and the mycorrhizal association is ended.

After 4 to 6 months at a temperature of 2 to 4 C dormancy is broken and the second period of growth begins with the onset of spring. The corm finds itself in the same situation as the seed after germination. Its stored resources are not sufficient for sustained development and it is not in a position to carry out photosynthesis. Therefore soon after the beginning of new growth the plant must again establish a mycorrhizal association.

During the second period of growth the corm will be receding ftom the surface of the moss at the rate of the moss' growth. To keep from being buried deeper with each season the plant must fix the position at which the new corm will develop relative to the surface of the moss. This is accomplished by controlling the growth of the third internode and to a lesser degree that of the fourth and fifth internodes. The length of the internodes is determine by the amount of light received by the apical meristem, the light being conducted down through the elongating middle and inter sheaths. If no light is detected the growth of the third internode will stop after it reaches a length of a few centimeters. But if the corm is high in the moss, light will be detected earlier in the plant's development and the third internode will be short. With the position of the new corm fixed and a mycorrhizal association reestablished the plant begins to store its reserve of energy, as starch, in the tissue of the fourth and fifth internodes. At the end of the second summer the new corm will be approximately 4 cm below the surface of the moss and about 0.5 cm in diameter.

Figure 10. Plants grown under lights for the third growth period.

Figure 11. Plants grown in the dark for the third growth period.

There are no reports on the number of years a plant, in the field, takes to pass from a germinating seed to flowering. When grown in flask it generally takes four or five growth periods, with the earliest being three periods. So our plant will need a few more years to reach blooming size. It is also not known if the plant

normally develops an aerial shoot in the third year or if it remains within the moss and then produces its first leaf in the fourth year. When a flask of vernalized two year corms is placed under lights for the third growth period the leaf sheaths elongate, developing narrow blades which are more an extension of the sheath then a leaf. The new corms which develop in such a flask will be normal in form and size (figure 10). However, if a sister flask of like corms is held in the dark for the third period the corms (figure 11) will also develop normally in size and shape. The difference being that the leaf sheaths do not elongate or attempt to develop a leaf blade. Corm development in the dark and in the light is about the same. The lack of a significant difference in corm development indicates that the plants under lights did not need to or could not take advantage of the opportunity to carry out photosynthesis in the third growth period. The plant probably does not produce an aerial shoot in the third year, but spends the third growth period, as the second, buried in the moss in association with a mycorrhizal fungus. The period ends with the corm going dormant with the onset of winter.

It is in the fourth year that the plant will produce an aerial shoot and the new corm that develops will set a flower primordium. Bolan and Scott (1991) counted the number of *Arethusa* plants at three different sites in Newfoundland and found that out of a total of 280 plants, only 85 were in bloom at the time the tally was made. This would indicate that the plant produces an aerial shoot before reaching blooming size. However another explanation is that a plant, having bloomed, could not produce a new corm of sufficient size to bloom again in the following year and could only send up a leaf. The 195 plants found in leaf probably represent a mix of those that had not reached blooming size and those which had bloomed before but whose corms were not then of blooming size. Except for the development of a leaf, the fourth year is spent as was the second and third years. At the end of the growth period the new corm will be about one centimeter in diameter and will have a distinctive primary eye that indicates the development of a flower primordium.

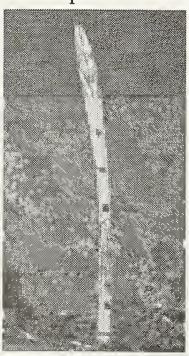

Figure 12. Developing shoot marked for rate of growth measurements.

Winter passes and dormancy is once again broken in the spring of the fifth year. This is the year in which our plant will bloom. The shoot elongates, but this time a flower primordium is also developing and a sixth and seventh internode will be formed. The three sheaths protecting and supporting the shoot penetrate the moss and when the proper light level is sensed the growth of the outer sheath and the third internode stops, fixing the location of the fourth and fifth internodes within the moss.

The middle and inter sheaths will continue to lengthen until they are at the surface, or a centimeter or so above, the moss. Here the middle sheath stops growing and is breached by the shoot now made up of the inter and leaf sheaths and the developing flower bud. The shoot continues to lengthen for another four or five centimeters and then the inter sheath stops growing and is breached by the flower bud. The sixth internode and the clasping leaf sheath continue to elongate as the flower bud develops. At this point the rate of growth of the sixth internode is greater than that of the leaf sheath which will only reach a few centimeters above the inter sheath until after the flower blooms. The sixth

internode and the ovary continue to lengthen until the flower is at an average height of 17 cm when it blooms. The sheaths and internodes grow primarily at their base due to the location of the intercalary meristems. The most active internodes are the third and sixth, while the fourth and fifth determine the shape of the new corm. The growth of the individual sheaths and internodes can be tracked by marking a developing shoot at intervals (figure 12) and plotting the increase in length over time. Figure 13 is a graph of the rate of growth of the middle, inter, and leaf sheaths, and the sixth internode from the time the shoot appeared above the surface of the substrate until maturity.

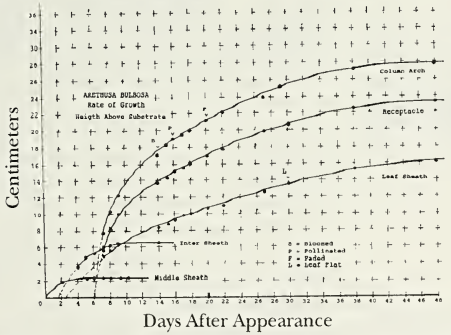

Fig. 13. Comparison of rate of growth of the middle, inter and leaf sheaths, and the 6th internode.

There are four external factors which have an effect on the height of the plant at anthesis:

Time of breaking dormancy.
Temperature during the period of growth.
Availability of nutrients.
Day length.

The time of breaking dormancy sets the beginning of the growth period, while the temperature and the availability of resources will determine the overall rate of growth. Day length sets the rate of flower development, which is essentially independent of the rate of growth of the internodes.

Of the four factors day length has the most dramatic effect. When a mature, vernalized corm is removed from a cold, dark condition and immediately exposed to a long photo period (16 hours) it will respond by blooming on a very short sixth internode. Figure 14 shows a plant which has received such a treatment. The flower bloomed just as the bud breached the intersheath. Plants in the field set their bloom period in response to the lengthening day.

Fig. 14. Plant responding to a long photoperiod immediately after vernalization.

Normally a plant will bloom around 40 days from breaking dormancy and carry a single flower some 4.0 cm across, depending on the attitude of the lateral sepals, with the lip being 1.0 to 1.5 cm wide at its broadest measurement. In southern New Jersey the plant blooms as early as May 15th, reaches its peak

around the 28th, and the last flowers are in bloom as late as June 9th. Overall about a three week period. Depending on the weather an individual flower will be in bloom for a week or so but will fade sooner if it has been polinated.

With our plant in full bloom and its flower raised it is time to attract a pollinator. There is only one reported in the literature. It is the queen bumble bee, Bumbus. The queens are larger than the male workers and because of their size they are the effective pollinators. Having lived through the winter the queen bumble bees are prominent in the bogs during the early spring. The mechanics of the pollination of *Arethusa* has been described by Thein and Marks (1972) and will not be covered here.

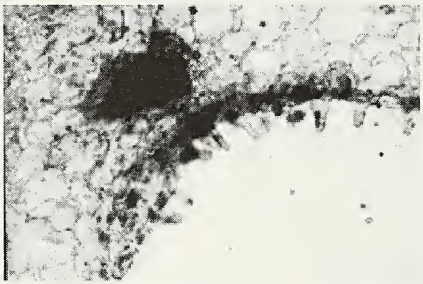

Fig. 15. Transverse section through the nectary showing papillose cells.

Most authors state that the flower is pollinated by deception, with no reward offered. Thein and Marks (1972) however, indicate that the flower does provide small amounts of nectar to its pollinator. Looking at the question of pollination by deception we find that the flower of *Arethusa bulbosa* has a well developed nectary concealed within its ovary. And that the nectary walls are lined with papillose cells that are highly colored in those plants which produce yellow pigment. Figure 15 is a transverse section through the nectary showing the papillose cells. In heavily pigmented plants the yellow coloration of the lining of the nectary is visible externally through the throat of the flower. Plants usually signal the availability of nectar not only with floral color but with floral fragrance as well. In the case of *Arethusa* the occurance of floral fragrance has not been consistently reported. The earliest reference to the flower's fragrance that I have been able to locate is the brief description given by Lindley (1830), "Flowers purple, sweet scented". Of twenty five descriptions of the flower written from 1893 to 1989 only five make reference to its fragrance. In the populations which I monitor 15 to 20% of the plants will be fragrant to some degree.

A nectary and scent producing glands are a substantial investment for a plant to make without a purpose. While it is not possible to determine in which direction the plant is evolving it is most likely that it has recently, in terms of evolutionary time, changed or is changing from a reward system of pollination to one of deceit. The floral scent and nectary being remnants of the old system. This could also be the case for the markings on the lip, which may have been continuous at one time, serving as guidelines to the nectary when a reward was offered but now are breaking up due to a loss of function (Himrich 1975). It may be that there are populations, or plants within a population, which still produce some nectar since there are still fragrant plants.

Our blooming plant, having been visited by Bumbus, is now pollinated. Up to this time there has been little development in the ovary other than the accumulation of a small amount of starch in the tissue adjacent to the traces and under the placenta. During the first 30 days after pollination the accumulation of starch increases and by the 36th day it is being converted into lipids in the

Figure 16. Transverse section through a rib and adjoining walls of a mature capsule.

developing embryos. Some 10 days later, the embryos are well formed and their suspensors are beginning to disintegrate. At this stage the developing seed is viable and can be sown to flask.

By day 55 the seed is fully formed, the suspensors have completely disintegrated, and the embryos are separated from the parent plant except for what metabolites may be available through diffusion. During the next month or so the seed will mature until by day 100 the testae will be suberized and the embryos will be going dormant.

In late September the plant is dormant, as is the seed in the drying capsule, and the task at hand is the distribution of the seed. Our plant takes advantage of three factors in the effective dispersal of seed by the wind:

First, its seed is very small and light, with an average terminal sink rate of 20 cm per second.

Second, after the flower has faded the 6th internode continues to lengthen until, at maturity, the average height of a capsule is 26 cm. This increase raises the point of release of the seed an average of 50% of the plants height when in bloom. By delaying the increase in height until after the flower has faded, loading on the stem during anthesis was minimized thereby reducing the risk of buckling.

Third, the capsule is held vertically and the sutures open from the top downward over a period of weeks. In addition to this timed release, the seed must also pass through or over a comb like structure that lies across the opening. The comb is part of the capsule rib, which separates from the wall as the suture opens. Figure 16 is a transverse section through a mature capsule showing the rib and comb structure. This barrier ensures that the turblence of the atmosphere, when the seed is shed, will be sufficient to raise the seed above the capsule height thereby increasing the range of dispersal.

With the dehiscence of the capsule and the release of the seed the cycle is completed.

Propagation

The medium used for both germinating and carrying the plants to maturity is Knudson's C (1946), with a few slight modifications. The formulation of the medium is given in table I. The phosphate and potassium requirements of the original formula are satisfied by a phosphate buffer (Arditti 1982), while the need for iron and manganese is provided by Murashige and Skoog's micronutrient salt base solution (1962). Niacin and thiamine are also added, as is 2 grams of

vegetable charcoal. This is the basic medium used for germinating the seed. Once germination has occurred and the protocorms are developing they must be transferred onto the same medium to which 60 grams of fresh banana and 100 ml of coconut water have been added. Protocorm development is greatly enhanced by these additives, but do not use them in the medium when sowing the seed because they will inhibit germination. This medium satisfies the needs of *Arethusa*, and has been used to bring achlorophyllous plants into bloom.

Table I. MODIFIED KNUDSON C MEDIUM (Knudson 1946)

Calcium Nitrate $Ca(NO_3)_2 \cdot 4H_2O$	1 g
Magnesium Sulfate $MgSO_4 \cdot 7H_2O$	250 mg
Ammonium Sulfate $(NH_4)_2 \cdot SO_4$	500 mg
Phosphate Buffer (Arditti 1982)	18 ml
Murashige and Skoog Micronutrient Salt Base (100X Solution) (Murashige and Skoog 1962)	10 ml
Niacin	2 mg
Thiamine	2 mg
Sucrose	20 g
Vegetable Charcoal	2 g
Agar	8 g
Water, Distilled	to 1 liter
pH to be adjusted to 5.3 if required	
When reflasking include:	
Banana	60 g
Coconut Water	100 ml

Babyfood jars, closed with Magenta Corporation's B-caps, serve as flasks for germinating the seed and reflasking protocorms in their early stages of development. The medium is cast flat in the flasks. After the second or third growth period or when the corms are larger than 0.5 cm in diameter they are reflasked into quart Mason jars which are closed with polyethylene film, held in place with rubber bands. All flasks are placed in individual polyethylene bags. This helps reduce the contamination rate in those closed with B-caps. In jars that are closed with polyethylene film, the bags provide a second line of defense in case the film should split.

Usually the seed is sown from green capsules which are at least 60 days old. However, if you have a capsule in the field and you wish to reduce the risk of it being damaged or lost, the seed can be sown from it as early as the 45th day.The sterilization of the capsule surface and the sowing of the seed is done following standard procedures, with a little extra attention paid to the nectray, which should be opened and scraped clean. After the seed is sown approximately 2 to 3 ml of sterile distilled water is added to the small babyfood jar. Some of the seed will float but most will adhere to the medium surface and be submerged. The amount of water added is important. It must be sufficient to allow for evaporation during the five to six month stratification of the seed. At the end of the period the surface of the medium should still be wet but with little free water. If too much water remains, the flask can be tilted slightly to expose the surface of the medium

and the seed.

The sowing of dried seed is also fairly standard. The desired amount is placed in a 5 cc vacutainer with distilled water and a very small amount of Tween, just what will adhere to a toothpick. The vial is then evacuated and agitated until the seed settles. The wetting solution is drawn off and a 5% Clorox solution is added. The vial is again agitated until the seed begins to lose its color. The length of time in the sterlizing solution will vary with different seed lots but most seed will be bleached in 10 to 15 minutes. At this time the vial is sterilized externally with the Clorox solution and passed into a clean box where the sterilizing solution is drawn off and the seed rinsed three times in sterile distilled water. The seed is then sown. As when sowing seed from a green capsule 2 to 3 ml of sterile distilled water is added to each flask. After the flasks are sown they are wiped with 70% alcohol, marked and placed into individual polyethylene bags.

The seed is stratified by storing the flasks at 10°C, in total darkness, for 30 days. The flasks are then transferred to a refrigerator where they are held at 3 to 4°C for 4 to 5 months. After the cold period they are again placed at 10°C, in the dark, for another 30 days. This completes the stratification process. During this time the flasks will have lost water through evaporation. How important is this temperature treatment? It is not an absolute requirement. The treatment at 10°C can be omitted. What is necessary is a minimum of 4 months in the dark at 3 to 4°C. However, if facilities and time are available I strongly suggest that the seed be treated at 10°C both before and after the cold period.

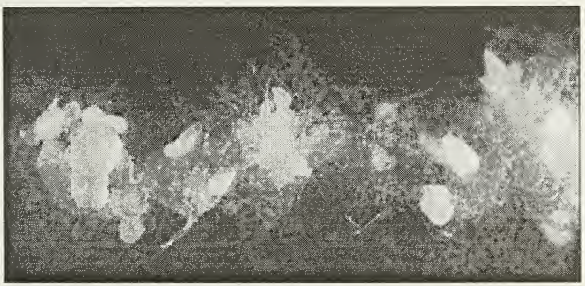

Figure 17. Protocorms ready for reflasking.

With the stratification of the seed completed the flasks are placed at 20 to 25°C still in complete darkness. In a few months, as the flasks continue to dry, the seed will germinate. When the protocorms have developed rhizoids and begin to elongate (figure 17) they should be reflasked onto the medium containing banana and coconut water. The time to reflask is not critical but if done to soon development may stop because of the additives. It is best to error by being a bit late rather than early. If the quantity of seed in the flask is such that the rhizoids become entangled, the entire mat of protocorms can be lifted, transferred to the new flask and pressed into the medium. After reflasking the flasks are returned to total darkness at 20 to 25°C. The original flasks can be irrigated with a little sterile distilled water and retained to see if there will be additional germination. The newly reflasked protocorms will continue to develop for 4 to 6 months then go dormant. Dormancy can be forced by placing the flasks at 10°C about 4 months after reflasking but the corms will be better developed if they go dormant on their

own.

When dormant the corms will be spheroid, and about 0.3 to 0.4 cm in diameter. The sheaths will have dried and the stalks will be dead. This is the time to reflask. The corms are removed from their flasks under sterile conditions and cleaned of all dead material. They are small at this stage so too much should not be made of the cleaning process during this reflasking. The corms are then placed in the new flasks being sure that they are all in contact with the medium. The orientation of the corms is not important at this time but in later reflaskings orientation should be maintained. A small baby food jar can hold 20 to 30 corms at this stage of development. The reflasked corms now must be vernalized. They can be placed directly into a refrigerator at 3 to 4°C or they can first be stored for a month or so at 10°C then transferred to the lower temperature. The corms are kept in the dark at all times. The vernalization period at 3 to 4°C should be at least 5 months. The flasks can be held at this temperature for 6 months or longer, if necessary, but they should be inspected periodically for the beginning of new growth after the 5th month. Four degrees Celsius is not cold enough to hold all corms dormant indefinitely.

After the corms are vernalized, the flasks, are placed at 20 to 25°C, still in complete darkness, to begin their second growth period. The developing shoots, in the absence of light, will elongate for two or three centimeters setting the location of the developing corms above the medium. The period of growth is 5 to 6 months after which the new corms become dormant and the supporting shoot will disintegrate. Again it is time to reflask. The procedure is the same as after the first growth period except the range in corm size will be greater. Due to the high concentration of nutrients in the medium and a starting pH of approximately 5.3 the secondary eye of most corms will also produce a new corm. These should be treated the same as the primary corms. The production of secondary corms allows a plant line to be maintained in flask while blooming sized primary corms are harvested. One plant line was maintained in flask for 12 years in this manner. The corms should be reflasked according to size. After reflasking the corms are ready to be vernalized once more.

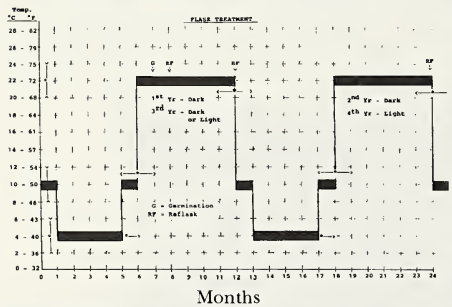

Figure 18. Generalized 4 year schedule for the germination of seed and corm development in flask.

After completion of the 2nd vernalization the corms can continue to be grown in complete darkness for the 3rd growth period or they can be placed under lights with a 16 hour photoperiod. The largest corms are generally placed under lights while the smaller corms spend the 3rd period in the dark. By the end of the 3rd period the range of corm sizes will be even greater than at the end of the 2nd period. When reflasking, corms larger than a centimeter in diameter can be held out, placed in containers and vernalized along with the reflasked smaller corms.

After the 3rd vernalization all the flasks should be placed under lights for the fourth growth period where the leaf sheaths will develop a blade and the larger

corms will set a flower primordium in preparation for blooming in the fifth period. Figure 18 shows a generalized schedule for the treatment of corms in flask. As plants mature they will bloom from a smaller corm so that after the fifth year most corms will bloom when held in flask.

Before leaving the subject of propagation something must be done with the larger corms removed when reflasking. These are washed in distilled water and the roots, stem and sheaths are removed. They are then soaked for a few minutes in a broad spectrun fungicide and packed in damp coarse vermiculite in plastic deli-pac containers of various sizes. The larger cups can hold 5 to 10 blooming size corms. The storage containers are treated in the same manner as the flasks. After being vernalized they are placed at 10°C until growth is just beginning. Do not allow the growth to proceed too far as the developing shoots and roots are easily damaged when being potted.

Production

The area used for the study of *Arethusa bulbosa* was not designed for the production of large numbers of corms. Rather it was set up so that plants, in all stages of development, could be grown throughout the year and to allow the plants to be left unattended for periods up to three weeks. The plants are grown in pots, under fluorescent lamps, in 2 by 4 ft wooden boxes which are lined with plastic sheeting and filled with either #2 sandblasting silica sand or fine gravel. The small boxes allow the treatment of groups of plants to be varied. When the plants are attended, nutrient solutions and water are applied from the top of the pot and allowed to drain through the beds into containers. Four to six inch azalea clay pots, which have their bottoms knocked out and replaced with nylon netting, are used. The netting is held in place by friction between the pot wall and a layer of coarse sand. The potting medium is equal parts of sphagnum peat, coarse sand, coarse perlite, coarse vermiculite and fine charcoal. The medium only has to hold up for 6 months or so as the corms are removed at the end of each growing period and placed in storage. The pots are mulched with a layer of coarse vermiculite which is covered with a layer of chopped pine needles. Coarser needles, such as those of the Pitch Pine, are best as they do not mat as readily as finer needles. They are cut into lengths of 2 to 3 cm with a pair of tin shears. The mulch serves a number of purposes:

It allows the pots to be watered from above without compacting the potting medium or washing it out of the pot.
It keeps algae from growing on the surface of the potting medium.
It provides a place for the new corm to develop.

When the plants are left unattended the pots are placed in the sand up to their rims. The individual boxes are set up as either a flood and drain hydroponic system or as a constant level, gravity fed system. The plants respond equally well to either system. Properly set up the surface of the sand will remain dry.

The fluorescent lamps are 8 ft coolwhite, super high output, 150 ma tubes in industrial 2 tube fixtures. There are two fixtures over each 4 by 8 ft bench. These are suspended by a pully system that allows the plant to lamp distance, for each fixture, to be easily adjusted. A 16 hr photoperiod is provided by a timer.

Adjacent to the growing area there are racks for flasks kept under fluorescent lamps and an area where the flasking is done. In addition there is an unheated basement where flasks and corms can be vernalized during the winter months and a small insulated cellar that is kept at approximately 10°C throughout the year. Two household refrigerators, used to stratify seed and vernalize corms, round out the facility.

After observing the plant in the field and growing it under varying conditions in both flasks and pots, I have reached the conclusion that *Arethusa bulbosa* is an obligate mycotrophic plant. One that never completely outgrows its need to be associated with a mycorrhizal fungus. It can not photosynthesize sufficient carbohydrates to produce a new corm of adequate size to maintain itself over an extended period of time. A large, mature corm removed from flask has sufficient stored resources to complete a cycle of blooming, setting a capsule and producing seed if its general requirements for growth are met. But it can not produce and store sufficient resources to repeat the cycle continuously. Each year the new corm produced will be smaller that its parent. Such a plant may bloom for 3 or more years before the new corm will be too small to bloom again.

Figure 19. Group of Arethusa *bulbosa* plants from the same cross.

In trying to get around the problem of diminishing corm size the conditions within the flask were looked at in detail. First, the flasks are positioned close to the lamps so that the plants, in flask, receive a relatively higher level of light than those in pots. Second, the medium in the flasks has a conductivity greater than 2000 microsiemens. This is much higher than usually suggested for native terrestrial orchids. Third, the pH of the medium, when prepared, is adjusted to 5.3, and as the plants develop the pH drops to levels as low as 3.0. During this time the major and minor elements, with the exception of iron, become less available to the plants. The early rapid growth that takes place in flask, when a corm renews its growth after being vernalized, occurs when the pH of the medium is at its highest. Then as the pH drops the plants mature and eventually go dormant.

Considering these three factors; light level, nutrient concentration and pH, the conditions in pot culture were adjusted to provide:

As high a light level as possible under fluorescent lamps.
Nutrient solutions with a conductivity between 1200 and 1400 microsiemens.
Solutions and water adjusted to a pH between 5.0 and 6.0.

At present the nutrient solutions used are alternated between a balanced (7-7-7) and a high phosphate (5-50-17) plant food, both with trace minerals. The solutions are applied every 3 to 4 days depending on the humidly, and after each second or third feeding the pots are flushed with rain or tap water. The pots are never allowed to become dry. These adjustments have greatly improved the development of corms but the growth in pot culture still does not match that which occurs in flask.

There is another approach to the commerical production of *Arethusa bulbosa* corms that should be considered. The corms could be carried to blooming size in flask and then marketed without ever having been potted. If production were to be scheduled on a seasonal basis, in a northern temperate region, stratification of seed and the subsequent development and vernalization of corms could be accomplished in cold frames while periods of growth requiring light could be provided for in a lath or greenhouse. Energy costs would be minimal. Refrigeration would only be required if dormancy was to be extended so as to increase the time when corms could be shipped. Skilled labor would be required only for flasking and there would be no day-to-day plant maintenance. Corms not marketed would be reflasked and carried over to the following year. Once a system is set up and a library of the plant's various color forms is established, normal in flask development of secondary corms would provide a near continuous supply of clones. The general concept was tried as part of a study that require a large sample of a particular cross, all in bloom at the same time (figure 19). The corms were maintained in flask until most were blooming size then, after being vernalized, all were potted at the same time.

Enough is known about the life cycle and propagation of *Arethusa bulbosa* to bring the plant into cultivation. The question now is: Can a market be made for the plant at a price that is economically feasible? If native orchids are to be brought into commercial trade then it is vitally important that the demand be satisfied at a cost to the consumer that will make commerical collecting of field plants uneconomical. The cultivated plant offered must be superior in every way to a field collected plant, including cost, and it must be readily available in numbers to meet the demand. To do otherwise will only increase the pressure on wild populations.

End Note

For figure 4:

May 27, 28, 31 1994			September 8,10, 12 1994		
n	=	148	n	=	77
$\bar{x}$	=	17.5 cm	$\bar{x}$	=	26.5 cm
sx	=	6.3	sx	=	8.2
σx	=	6.3	σx	=	8.2
Range	=	6.5 to 35.0 cm	Range	=	9.5 to 48.5 cm

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Symbiotic Seed Germination of Terrestrial Orchids in North America During the Last Decade - A Progress Report

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Introduction

It is generally accepted that the Orchidaceae is the largest plant family in the world with at least 12,000 species (Dressler, 1981), but some investigators (*e.g.*, Cronquist, 1981) speculate that this number may actually exceed 35,000. The vast majority of orchids reside in the tropics where the destruction of rain forests and the associated loss of biodiversity continues at a rate of 35 hectares per minute (Moore *et al*). In temperate regions, loss of habitat also threatens a large number of taxa with extinction. Recently, the Center for Plant Conservation concluded that at least 213 of the 20,000 plant species native to the United States have become extinct, and another 680 taxa will likely follow by the year 2000 (Wilson, 1992). Of the 160+ terrestrial orchid taxa native to the United States and Canada, it is suspected that fewer than two dozen species have been reliably propagated from seed using either symbiotic and asymbiotic means. Understandably, most of the effort has been directed at the showy, attractive species with the greatest horticultural potential (*i.e.*, *Cypripedium*, *Arethusa*, *Calopogon*, *Platanthera*). In contrast, many of the "less attractive" taxa (*i.e.*, *Malaxis*, *Tipularia*) have been largely ignored. Unless all of our native terrestrial orchids receive immediate and increased attention, we can expect the number of rare and endangered taxa to significantly rise during the next century, accompanied by near-certain extinctions. At best, the survival/propagation of North American terrestrial orchids may hinge upon successful foreign conservation programs, assuming there is interest and funding.

The vulnerability of our terrestrial orchids is attributed to their dependence on naturally-occurring, symbiotic fungi to initiate seed germination and seedling development. It is this close symbiotic association that has discouraged and/or prevented a large number of terrestrial orchid species from being artificially propagated from seed. Although some taxa (*e.g.*, *Spiranthes*) have been propagated from seed on artificial media alone (asymbiotic germination), seedling survivability on soil is often minimal compared with seedlings that harbor symbiotic fungi within their root systems. The use of fungi to germinate orchid seed *in vitro* (symbiotic seed germination) has shown great promise in North America (*e.g.*, Smreciu and Currah, 1989; Zettler and McInnis, 1992, 1993) and abroad (*e.g.*, Clements *et al.*, 1986; Dixon, 1987; Stewart, 1993), but is considered more complicated and problematic due various factors (*e.g.*, isolation and testing of suitable fungi). In addition, the use of two organisms (orchid seed and fungi) instead of just one (seed) often deters many, especially those who have little or no mycological experience.

While symbiotic seed germination should not be viewed as a "cure-all" for propagating North America's orchids, it will almost certainly be necessary to implement the technique for numerous taxa, especially those that consistently resist asymbiotic methods. This report summarizes the North American efforts during this decade to investigate the orchid/fungal symbiosis as it relates to seed propagation. In addition, an attempt is made to emphasize significant developments by researchers in North American that may be applied to related conservation projects.

The Canadian Contribution

The first modern attempt in North America to germinate temperate terrestrial orchid seeds *in vitro* using fungi was initiated in Alberta, Canada by R. S. Currah, A. Smreciu, and S. Hambleton in the mid-1980s (Currah *et al.,* 1988). Three species, *Goodyera oblongifolia* Raf., *G. repens* (L.) R. Br., and *Platanthera hyperborea* (L.) Lindl., germinated and survived transflasking, but seeds of *Calypso bulbosa* (L.) Oakes, *Corallorhiza maculata* (Raf.) Raf., *Cypripedium passerinum* Richardson, and *C. calceolus* L. failed to germinate (Currah *et al.,* 1986). Subsequent germination attempts involving 10 North American taxa (and 10 European species) were carried out in 1987-88 using two types of media (Fast's synthetic medium, Warcup's cellulose agar) and six fungal strains, the results of which were published in the inaugural issue of *Lindleyana* (Smreciu and Currah, 1989). Various stages of embryonic development were reported for 17 of the 20 orchid taxa tested, and at least one fungus (*Ceratobasidium cereale*) was singled out as being most effective at promoting germination; however, the same fungus tended to become pathogenic in the North American orchids (Smreciu and Currah, 1989).

In conjunction with seed germination trials, Currah's group was also the first to pioneer the identification (taxonomy) of fungal endophytes of north temperate orchids, especially those that were isolated for use in on-going symbiotic experiments. This was (and still is) considered a bold undertaking due to the tendency of most orchid endophytes to remain sterile in pure culture. Initially, fungal strains that produced distinctive characteristics as anamorphs (*i.e.,* monilioid cell morphology) were often assigned to the ubiquitous form-genus *Rhizoctonia.* Using this approach, several fungal taxa were identified that originated form mycorrhizal roots of North American orchids, including at least one new species [*Rhizoctonia anaticula* Currah from *C. bulbosa, Platanthera dilitata* (Pursh) Lindl., and *P. obtusata* (Banks ex Pursh) Lindl. cited in Currah *et al.,* 1987]. Only rarely did pure cultures produce a perfect state (teleomorph). Nevertheless, Currah (1987) was able to describe a new species of *Thanatephorus* (*T. pennatus* Currah) from the roots of *C. bulbosa* after it produced basidiospores following 6-8 weeks of incubation at room temperature under natural illumination.

With the advent of R. T. Moore's revolutionary publication (1987) which recognized new anamorphic genera once included in the genus *Rhizoctonia,* Currah subsequently adopted this new treatment for the naming of endophytes from North American orchids (*e.g.,* Currah *et al.,* 1990; Currah and Zelmer, 1992). Despite the usefulness and authenticity of Moore's (1987) revision, which based the new genera on conservative characteristics (septal ultrastructure, nuclear condition of vegetative hyphae, correspondence with known teleomorphs),

Currah remains one of the few authorities to have implemented this taxonomic treatment on a large scale (*e.g.*, Currah *et al.*, 1990; Currah and Sherburne, 1992; Currah and Zelmer, 1992; Richardson *et al.* 1993). Since then, Currah and Zelmer (1992) constructed a dichotomous key to the genera of mycorrhizal fungi associated with orchids, and described a new species (*Epulorhiza albertaensis* Currah and Zelmer from *Calypso, Coeloglossum* and *Platanthera*) in the process. Presently, these and other symbiotic isolates are maintained at the University of Alberta Microfungus Collection and Herbarium (UAMH) and are available to other investigators. *Epulorhiza* currently remains the most common and distinctive anamorphic genus associated with the mycorrhizas of terrestrial orchids (Currah and Zelmer, 1992). Should other investigators follow Currah's example by carefully identifying other anamorphic species, ecological questions related to the orchid/fungus symbiosis (*e.g.*, fungal specificity) could be resolved.

The pioneering work with the orchid/fungal symbiosis was not limited to Alberta. Allan Anderson at the University of Guelph, Ontario continued the Canadian effort by comparing the growth and survivability of *Spiranthes magnicamporum* Sheviak seedlings following *in vitro* symbiotic and asymbiotic seed germination (Anderson, 1991). Using a fungus identified as *Epulorhiza repens* (Bernard) Moore, isolated from *S. magnicamporum* (UAMH 6565), he reported that the orchid could be successfully propagated from seed to flowering in 23 months. He also noted that seedlings infected with the fungal endophyte were better suited for soil transfer and long-term survival compared with seedlings that lacked the fungus (Anderson, 1991).

The American Contribution

Clemson University (1989-1994)

Although the Canadian effort to propagate temperate terrestrial orchids from seed was well underway since the mid-1980s, interest in symbiotic seed germination in the United States was slower to develop. In 1990, the first modern account of symbiotic seed germination in this country of a native terrestrial orchid [*Platanthera integrilabia* (Correll) Luer] was published (Zettler *et al*, 1990). Oddly, this study transpired under improbable circumstances as a graduate project in a mycology course at Clemson University (SC) (Zettler, 1994a). In the December, 1990 issue of *Lindleyana* which also featured Arditti's speculative article regarding the orchid/fungal symbiosis (Arditti *et al.*, 1990), Zettler and Fairey (1990) emphasized the need for the implementation of symbiotic techniques to propagate our native orchids from seed, particularly rare and endangered species. A similar plea was made during the North American Native Terrestrial Orchid Conference in Chadds Ford, PA in March, 1989 which culminated in published proceedings the following year. Shortly thereafter, efforts at Clemson were directed at broadening *in vitro* symbiotic seed germination to include 10 orchid taxa native the southern Appalachians (Table 1). As a result, five of the 10 species [*Platanthera clavellata* (Michaux) Luer, *P. cristata* (Michaux) Lindl., *P. integrilabia*, *Spiranthes cernua* (L.) Rich., *S. odorata* (Nutt.) Lindl. were propagated from seed onto greenhouse soil under septic conditions (Table 1). However, three orchids, including the federally-listed *Isotria medeoloides* (Pursh) Raf. resisted all attempts at germination (Table 1). Due to the pending endangerment of *P. integrilabia* (a C2

candidate for federal protection), additional experiments were carried out with that species that were directed at improving seed germination, protocorm development, and seedling survivability. Following promising reports (Rasmussen *et al.*, 1990; Rasmussen and Rasmussen, 1991) which suggested that light had a stimulatory effect on germination for a European species [*Dactylorhiza majalis* (Rchb.f.) Hunt & Summerh.], a similar experiment was attempted with *P. integrilabia*. Subsequently, it was determined that seed germination was also stimulated by light when embryos were initially exposed to a 16 hour white light (irradiance = 55.8 ($mol/m^2/s^{-1}$) photoperiod for 7 days at the time of sowing/fungal inoculation, followed by darkness (Zettler and McInnis, 1994). A similar stimulatory effect was also observed for a second *Platanthera* species (*P. clavellata*; A. R. House, L. W. Zettler, T. M. McInnis, unpub. data). Because this phenomenon was also observed for the European orchid, *D. majalis* (Rasmussen and Rasmussen, 1991), it would appear that light usage to stimulate the germination of other temperate terrestrial orchids is a distinct possibility, and deserves further investigation.

Smithsonian Environmental Research Center (SERC)

While symbiotic seed germination continued at Clemson, the arrival of visiting scientist Hanne Rasmussen from the University of Copenhagen (Denmark) into this country greatly contributed to our understanding of the orchid/fungal symbiosis. Working in conjunction with Dennis Whigham at the Smithsonian Environmental Research Center (SERC) in Edgewater, Maryland, they attempted to study the early life histories of five terrestrial orchids and the dynamics of the mycorrhiza in the natural habitat (Rasmussen and Whigham, 1992). By devising an ingenious method that enabled the dust-like seeds of terrestrial orchids to be sown in soil, infected by fungi, and subsequently retrieved for study (see Rasmussen and Whigham, 1993), they were able to investigate this problematic aspect of orchid biology with some promising results. [*Note*: a nearly identical technique was published over a year later in *Lindleyana* by van der Kinderen (1995)]. For example, seeds of *Goodyera pubescens* (Willd.) R.Br., *Galearis spectabilis* (L.) Raf. and *Corallorhiza odontorhiza* (Willd.) Nutt. were observed in the field, with the latter species yielding seedlings nearly 16 mm in length, 54 weeks after sowing (Rasmussen and Whigham, 1993). They also observed seedlings of *Tipularia discolor* (Pursh) Nutt. in nature for the first time, and noted that the species' seeds colonize and germinate on decaying logs on the forest floor (Rasmussen and Whigham, 1992; H. Rasmussen, pers. com.).

SERC-Inspired Research

At least two other researchers in this country soon adopted Rasmussen and Whigham's field germination technique. Ann Antlfinger (University of Nebraska at Omaha) and her co-workers implemented field sowings using seeds of *S. cernua* at two sites near Lincoln, Nebraska. Two months after sowing in soil, they reported numerous swollen seeds but few protocorms of that species, and concluded that longer burial times may be necessary for substantial germination (A. Antlifinger, pers. com.). Field sowings of *I. medeoloides* were attempted by L.

Table 1. Summary of the initial Clemson effort to propagate terrestrial orchids of the southern Appalachians.

[*Note:* seeds of all taxa were sown on oat meal agar (2.5 g/L rolled oats, 7.0 g/L agar) and incubated initially in darkness following fungal inoculation using the method outlined in Dixon (1987)].

Orchid Species	Most Effective Fungus	% Germination@	Soil Establishment#	Reference
Corallorhiza odontorhiza	—	0	0	L. W. Zettler, T. M. McInnis (unpublished)
Isotria medeoloides	—	0	0	L. W. Zettler, T. M. McInnis (unpublished)
Goodyera pubescens	Pcil-154 (UAMH 7633) isolated from *P. ciliaris*	> 72	0	Zettler and McInnis (1993)
Platanthera ciliaris	Pcil-154 & Pcil-127 isolated from *P. ciliaris*	ca. 50	0	L. W. Zettler, T. M. McInnis (unpublished)
P. clavellata	Pcla-115 (*Epulorhiza* sp. nov.) isolated from *P. clavellata*	ca. 34	4 %	L. W. Zettler, T. M. McInnis (unpublished)
P. cristata	Pcst-163 (*Epulorhiza* sp. nov.) isolated from *P. cristata*	ca. 39	8 %	L. W. Zettler, T. M. McInnis (unpublished)
P. integrilabia	Pi-89 (*Epulorhiza* sp. nov.) isolated from *P. integrilabia*	15-73	20 %	Zettler and McInnis (1992)
Spiranthes cernua var. *cernua*	Pcil-154 (UAMH 7633)	7-71	50 %	Zettler and McInnis (1993)
S. odorata *	Sodr-223 isolated from *S. odorata*	> 65	5 %	Zettler *et al.* (1995)
Tipularia discolor	—	0	0	Zettler and McInnis (unpublished)

* seeds of *S. odorata* were polyembryonic; embryos were scored individually
@ germination was defined as the production of one or more rhizoids and/or the rupture of the testa by the embryo
# numbers reflect the maximum % of seed that yielded soil-established seedlings for any treatment

Zettler, B. May and T. McInnis (unpub. data) at a natural population in Oconee County, South Carolina; however, seeds of that species failed to germinate after one year in soil. In another case, Zettler (1994b) observed protocorms of *P. integrilabia* in packets placed in natural areas that lacked the species but otherwise appeared suitable for its habitation. Interestingly, *P. integrilabia* protocorms obtained through field sowings yielded fungi that appeared identical to those acquired from adult plants (Zettler, 1994b), and were also effective at promoting *in vitro* seed germination of *P. integrilabia* (L. Zettler, A. House, unpub. data). As Rasmussen and Whigham (1993) pointed out, the field sowing technique may be thought of as a "fungal baiting" method because the fungi obtained from protocorms would likely be those directly responsible for initiating the germination process. This could enable researchers to germinate the problematic species that do not seem to harbor long-lived mycorrhizae as adults, or often yield endophytes that are incapable of promoting seed germination. The idea of "fungal baiting" is especially appealing when applied to the slipper orchids (*Cypripedium* spp.), which have yet to be propagated through *in vitro* symbiotic seed germination despite several independent attempts (*e.g.*, Smreciu and Currah, 1989; Stoutamire, 1991; Zettler and McInnis, unpub. data). Like *in vitro* symbiotic seed germination itself, however, the use of field sowings as a "cure all" for the propagation of temperate terrestrial orchids should be view with guarded optimism.

Recent and Current Work

After the establishment of symbiotic techniques in Canada and the United States, interest in the orchid/fungal symbiosis began to materialize. Tonya McKinley, under the direction of N. Dwight Camper (Clemson University), investigated seed germination of *Goodyera repens* var. *ophioides* Fernald under varying environmental conditions. Two fungal isolates [*Ceratorhiza goodyera-repentis* (Costantin & Dufour) Moore = UAMH 6440, and TN29-Pcil-154 = UAMH 7633, an *Epulorhiza* sp.) were reported to form a symbiosis with *G. repens* seeds; however, the former fungus (UAMH 6440) was considered "weakly symbiotic" (McKinley, 1995). Of additional interest was her conclusion that light enhanced seed germination and protocorm root formation in the absence of symbiotic fungi. Under different light regimes, she found that red and fluorescent light enhanced the percent germination, but far red light treatments decreased percent seed germination to levels below that of continuous darkness, implying that seed germination is mediated by phytochrome (McKinley, 1995).

At the University of Georgia, graduate student David Handaly is investigating the effect of nitrogen and phosphorus nutrition on symbiotically-germinated seedlings of *Spiranthes vernalis* Engelm. & Gray. In 1995, he reported that fungal isolate TN29-Pcil-154 (UAMH 7633) promoted seed germination and seedling development of *S. vernalis in vitro* (D. Handaly, pers. com.). Ann Antlfinger also used the same fungus (UAMH 7633) to germinate seeds of *Spiranthes* (*S. cernua*) *in vitro*, but it was considered inferior to an isolate recovered from *S. magnicamporum* (*E. repens*, UAMH 6565) by Allan Anderson (A. Antlfinger, pers. com.). Tom Stich, under the direction of Simon Dabydeen (Frostburg State University), used a different strain of *E. repens* (UAMH 5430) to germinate the seeds of *Platanthera ciliaris* (L.) Lindl. (T. Stich, pers. com.). After exposing seeds to white light at the

time of sowing (as outlined in Zettler and McInnis, 1994), *E. repens* promoted a maximum percent germination of 57% (T. Stich, pers. com.). Using a different fungal isolate recovered from the roots of *Platanthera grandiflora* (Bigelow) Lindl. in Maryland, the percent germination was raised to 74.8 % (T. Stich, pers. com.). Stich and Dabydeen also reported seed germination of *P. grandiflora* using light pre-treatment and the latter fungus isolate (Stich and Dabydeen, 1995); however, percent germination was considerably lower (3 %) compared with seeds of *P. ciliaris* (T. Stich, pers. com.). Currently, Stich is a doctoral student at Clemson University and is working closely with Tom McInnis to further investigate the symbiosis associated with orchids of the southern Appalachians.

Figure 1: Spiranthes cernua seedlings

For a duration of two years, L. Zettler continued symbiotic micropropagation at Furman University (SC) which involved the assistance of four undergraduate students. Contrary to earlier reports which suggest that light has a stimulatory effect on seed germination (McKinley, 1995; Rasmussen *et al.*, 1990; Rasmussen and Rasmussen, 1991; Zettler and McInnis, 1994), C. Hofer and L. Zettler (1996) concluded that white light had an adverse effect on the seed germination of *S. odorata* during the initial establishment of the symbiosis. This would imply that members of the Orchidaceae react differently to light prior to, or at the time of fungal infection, depending on the species. Clearly, this matter deserves further attention by those who intend to propagate terrestrial orchids from seed. In addition to investigating light's role on symbiotic seed germination, the effect and importance of prolonged seed and fungus storage was also studied at Furman. Deborah Mariner and L. Zettler (1996) reported that *P. integrilabia* seeds remained viable after 6 years of cold storage (-7 and 8 C), but failed to germinate when stored at ambient temperature (23 C) for the same length of time. They also

concluded that a symbiotic fungus (SC1-Pi-70 = UAMH 7632, *Epulorhiza* sp. nov.) maintained its ability to germinate *P. integrilabia* seeds despite 6 years of subculturing. Due to a recent event that led to the destruction of nearly 1,000 *P. integrilabia* inflorescences in Tennessee (Zettler, 1996), it is clear that other studies which address long-term viable storage of seed and fungi must be considered, especially involving species threatened with immediate extinction. In *P. integrilabia*'s case, where only a single large population remains (Shea, 1992; Zettler et al., 1996), viable seed storage may be the only means to guarantee the preservation of genetically-diverse gene pools.

Projected Work

Presently, symbiotic micropagation seems poised for advancement in North America. At SERC (Maryland), H. Rasmussen is attempting to continue orchid seed germination in collaboration with D. Whigham. Should this joint venture re-materialize, additional significant progress may be expected. In the southern Appalachians, research under the direction of T. McInnis is continuing, and physiological/mycological questions related to the symbiosis will likely be answered. In the Midwest, the research initiated by A. Antlfinger is a positive step in an area of the country that harbors understudied taxa. Beginning in 1997, a joint venture between L. Zettler (recently hired at Illinois College) and the Chicago Botanic Garden to propagate terrestrial orchids of the Midwest is anticipated. In Canada, R. Currah's group continues to study the orchid/fungal symbiosis, especially concerning fungal taxonomy, and it seems likely that this will continue.

Conclusion

At the conclusion of the North American Native Terrestrial Orchid Propagation and Production Conference in Chadds Ford (1989), it was clear that our native orchids were in serious need of additional scientific study. Since that time, encouraging progress has been made in both Canada and the United States, and it appears that symbiotic micropropagation may finally be gaining a foothold in North America. Assuming current trends continue, and in all likelihood they will, we can expect more taxa to be successfully propagated from seed, accompanied by more effective techniques to do so. Whether or not this progress will match the extinction rates projected in the next century remains to be seen.

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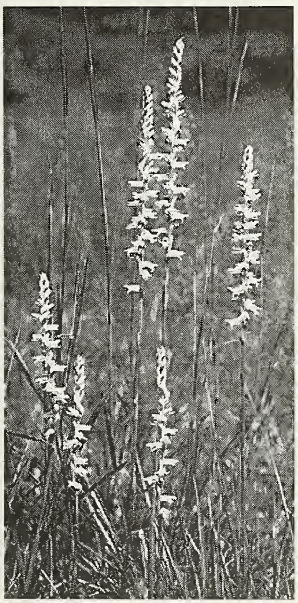

Spiranthes vernalis, photo courtesy Paul Martin Brown

Platanthera leucophaea, photo courtesy Paul Martin Brown

Seeds and Seedlings of *Platanthera leucophaea* (Orchidaceae)[1]

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THE EASTERN PRAIRIE FRINGED ORCHID, *Platanthera leucophaea*, is a threatened (State and Federal listing) orchid in Ohio where the stability of its populations is a cause for concern. The extant sites are being closely monitored by the Division of Natural Areas and Preserves, Ohio Department of Natural Resources. The effects of drought, herbivory, plant succession and pollination success can be observed on mature plants but factors affecting seedling establishment are largely unstudied. This paper focuses on the earliest stages of the life cycle, including seed production and release, germination, and early seedling development. It is hoped that knowledge of these critical early stages may aid in management of the surviving populations.

Ecology and Habitat

Platanthera leucophaea occupies mesic and wet prairie, bog and fen habitats in eastern North America (Braun 1967; Case 1987, Homoya 1993, Bowles 1983, 1985, 1991; Bowles & Flakne 1992; Windus, Cochrane and Stoutamire 1994; Sheviak and Bowles 1986). Some of its historic populations have disappeared because of habitat destruction and the decreasing range has led to its listing as threatened in Ohio. Populations occur in five Ohio counties (Clark, Holmes, Lucas, Sandusky, Wayne) in unshaded sites supporting herbaceous perennials such as *Phalaris arundinacea, Calamagrosits canadensis, Eupatorium fistulosum* and species of *Aster, Solidago,* and *Carex.* Woody plants (*Cornus racemosa, Rosa palustris* and *Salix* species) are invading some sites. Soil samples from 7 populations in 5 counties consist of yellow to dark brown fine silty loam and clay, often with a surface layer of decaying plant material. Soil pH at all sites is between 6.6 and 7.1. Electrolyte levels, determined by adding distilled water to an equal volume of soil, range from 120 to 420 mhos/cm.

Fruit and Seed Production

Plants flower in late June-early July on .5-1 M stems carrying 5-35 flowers. Hawkmoths pollinate flowers in nature (Bowles, 1983, Sheviak & Bowles 1986). In the Sandusky County population, small inflorescences produced fewer fruits/flower in 1992 than did larger inflorescences but flower number and fruit set did not show this pattern in 1993 (Table 1). Year to year differences in fruit set may be influenced by pollinator activity, resource limitation and herbivory. Many inflorescences are destroyed by deer at this site and there may be preferential feeding on larger inflorescences. Although the size distribution differed between 1992 and 1993 the percent of fruit set per flower remained almost constant.

[1]Supported by grants from the Ohio Department of Natural Resources and the U.S. Fish and Wildlife Service.
[2]I wish to thank Barbara Natterer, Teresa diPietro and Christopher Burlak for field assistance, and Randy Mitchell, Jennifer Windus and Marlin Bowles for reviewing this manuscript.

Table 1. Inflorescence size and fruit set in Sandusky County plants.

1992				
Flower #/plant	#plants	# flowers	# fruits	% fruits/flower
6-10	10	84	31	37
11-15	17	225	128	57
16-20	10	177	103	58
21-25	10	219	132	60
26-30	2	55	46	84
31-35	1	34	30	88
Year totals:	50	794	470	59
Average flowers/plant= 16; av. fruits/plant= 9.4				
1993				
6-10	13	122	74	61
11-15	36	468	298	64
16-20	29	618	347	56
21-25	13	301	169	56
26-30	7	160	109	68
31-35	3	64	40	63
year totals:	101	1733	1037	60
Average flowers/plant= 17.2; av. fruits/plant= 10.3				

After pollination ovaries develop to the end of the season. Capsules begin to blacken in late September and release seeds (n=30, average length/width= 421/143 mμ, embryo length/width= 164/109 mμ) in October. Seeds from one capsule were weighed and a fraction counted, arriving at an estimate of 4,458 seeds for this capsule. Embryos were present in about half the seeds from 12 collections in 4 counties (Table 2). Using the estimations of 10 fruits/flowering plant, 4,500 seeds/fruit and 50% embryo content one can give a working estimate of 22,500 fertile seeds/flowering plant/year in the Sandusky County Ohio population.

Table 2. Percent seeds containing embryos

Sandusky Co.	1992	51%	Lucas Co.	1980	62
	1993	54		1990	54, 58
	1994	62			
	1995	44			
Holmes Co.	1992	50	Wayne Co.	1982	31
	1993	42		1990	10
	1994	50			

Seed and Embryo

Platanthera leucophaea seeds were removed from ripe capsules, air-dried for 5 days and refrigerated at 5°C in unsealed cellophane envelopes. Data presented here are from October collections from Sandusky Co., 1992-1995. Seed viability of

many orchid species decreases during storage and the rate of decline varies with species and storage conditions(review in Rasmussen 1995). A procedure, described later in this paper, was developed to monitor changes in embryo viability of this orchid.

Platanthera leucophaea seeds, like those of most terrestrial orchids, are water repellent and either float on the surface or adhere to wet surfaces, unlike seeds of epiphytic species which wet readily and are carried within or sink to the bottom of the water volume. Most seeds of *Platanthera leucophaea* remain afloat even after 2 hours of oxidation in a bleaching solution. This flotation is not simply a result of trapped air because seeds aspirated under water also remain afloat. The wetting agent Tween 80 has been often used by others (Rasmussen 1995)in surface sterilization procedures with orchid seeds and it causes *Platanthera* seeds to lose their hydrophobic properties and to sink. Since it also reduces TTC (triphenyl tetrazolium chloride) staining in *Platanthera* and appears to be toxic (Rasmussen 1995), it has not been used as a wetting agent in this study.

Seed coats and embryos of this orchid have a dark brown water-soluble pigment which must be removed before TTC staining can be observed. Pigment was bleached by shaking seed samples in 5 cc of 0.5% sodium hypochlorite (diluted Clorox) followed by four rinses in distilled water. Bleaching periods were varied from 30 to 120 minutes in order to determine the optimum treatment time for viability staining. TTC stain (1% TTC, pH 7, Van waes and Debergh 1986) was incubated with seeds in dark at 35°C for 24 hours, then percentages of colored (pink to red) embryos were microscopically determined. Fluorescein diacetate staining (Pritchard 1985) was tested as a viability stain but was less reliable with these seeds than TTC and was not used further.

Embryo staining increased with bleaching period but embryo damage also became apparent (Table 3). Seeds of species with permeability controlled dormancy are more permeable in some regions than others (Baskin & Baskin 1989) and this is also true of embryos of this orchid. The poles are more permeable than the central region of the embryo to bleach and the poles are the first to be damaged by chemical treatment. Embryo poles become transparent as bleaching continues and these areas do not react to stain. Stainable tissues remain translucent. Percentages of embryos with partial staining continue to increase beyond 2 hours of treatment but so many are damaged that data are not included here.

Embryo staining and apparent viability are decreasing during the current storage (5°C) period. In previous years seed samples have been found to be apparently inviable after 6 months of dry refrigeration. The procedure used here is useful in determining the presence/absence of viable embryos as well as determining loss during storage but probably underestimates the numbers of viable embryos in samples.

Table 3. Percent embryos staining with TTC after 30, 60, 90 and 120 minutes shaking in 0.5% sodium hypochlorite. Seed collected 10/10/95.

Stain date	bleached 30 min.	60 min.	90 min.	120 min.
10/12/95	1%	15	35	39
11/10/95	-	10	20	26
12/8/95	1	10	24	28
1/3/96	-	5	22	26
3/1/96	2	4	14	21

Water repellent *Platanthera* seeds released in October are likely to remain on surfaces of decaying vegetation and soil rather than being incorporated into the soil mass unless surface tension effects are changed naturally. Effective germination and seedling development in most terrestrial orchid species is likely to occur below ground since light inhibits germination (Stoutamire 1974) and protocorms are unlikely to survive the temperature and moisture fluctuations at soil surfaces. Seeds of other temperate terrestrial species such as *Cypripedium reginae, C. acaule,* and *C. parviflorum* (Curtis 1943), *Epipactis helleborine* (Van der Kinderen 1995), *Platanthera ciliaris* and *P. blephariglottis* (Case 1987), germinate below ground. The first leaf of naturally occurring *Platanthera leucophaea* seedlings arises from underground protocorms and no surface seedlings have been observed. How, then, do water repellent seeds become buried? I suspect that native earthworms may be involved to some extent and am currently investigating this.

Germination Studies

Samples (1000-2000 seeds) were prepared for germination by hypochlorite treatment as previously described and stratified either on nutrient agar in the refrigerator or in soil.

In Vivo: Soil germination trials utilized seed samples in 35 mμ Nitex bolt cloth mounted in plastic slide holders (Rasmussen & Whigham 1993). Since bleaching apparently increases embryo permeability, the germination rates of bleached and unbleached seed samples were compared. Seed samples in slides were inserted horizontally 2 cm deep in living prairie sods in greenhouse flats. Flats were wintered at 5°C and placed outdoors at ambient temperatures during the growing season. Cotton blue (Brundrett, Piché and Peterson 1984) was used as a mycorrhizal stain after slides were retrieved and examined.

Non germinating embryos were opaque and remained within the testas while developing embryos enlarged, protruded from the ruptured testas and developed 1-3 elongate nucleated rhizoids. Although numerous fungal hyphae were visible among the seeds, none of the protocorms formed an association with mycorrhizal fungi during the year of observation and most were dead or dying by late summer. Failure to form mycorrhizae could be due to absence of appropriate symbionts in the soil flats or inappropriate conditions for their establishment.

Table 4: Seeds shaken in distilled water or in 0.5 a bleach 1 hr, rinsed x4 with distilled water, buried 2 cm in prairie sods, March 27, 1994. TTC staining of original sample after 1 hr bleach =7 %; after 2 hr . =31%.

examined	Germ. % unbleached	Germ. % bleached
6/25/94	0	82
8/15/94	0	80
10/15/94	26	93
6/9/95	66	96
7/17/95	60	96

Bleached seeds in Nitex slides buried in late winter germinated in large numbers as the soil warmed from *ca.* 5°C to 15-20°C. Unbleached seeds did not germinate until later in the summer or after the second cold period. The germination of bleached seeds was greater than the initial TTC staining indicated and the stain underestimated the viability of orchid seed samples (Lauzer, St-Arnaud and Barabé 1993; Van der Kinderen 1995; Rasmussen 1995).

In vitro: In September 1992 bleached seeds were rinsed in sterile distilled water and sown on a modified Fast medium consisting of $Ca(NO_3)_2 \cdot 4H_20$ 65 mg; KCl 33 mg; KH_2PO_4 33 mg; $MgSO_4 \cdot 7H_20$ 33 mg; NH_4NO_3 65 mg; peptone 1 g; sucrose 7 g; agar 8 g; 1 l distilled water (Rasmussen 1995), pH 6 before autoclaving, 10 ml medium in 50 ml tissue culture flasks. Flasks were refrigerated at 4°C for 8 weeks, then moved to a growth chamber maintained at 24°C in darkness. Seeds germinated, produced protocorms and developed 1-2 roots 5-15 mm long and a 5-10 mm terminal shoot by the end of the summer 1993. Flasks were again refrigerated through the 1993/94 winter, and 32 seedlings were inserted at marked locations in flats of prairie forbs in March. Sixteen seedlings produced single 15-30 mm leaves by midsummer and again became dormant the second winter. Nine plants produced 40-80 mm single leaves the third season and are currently dormant. Their progress will be closely followed.

Discussion and Conclusions

Data from the Ohio populations indicate that only half the potential embryos develop in naturally pollinated flowers. This may be a result of the brief contact of the sectile pollinium with the stigma, leaving fewer pollen grains than available ovules or it may reflect nutrient or other constraints. These alternatives may be clarified by hand pollinations utilizing varying pollen loads.

Two or more months of moist stratification are required for germination in this species (Stoutamire, unpublished) and extensive germination is not apparent until mid-summer of the following year. *Platanthera leucophaea* seeds fit the Type II winter transient type (Leck 1989) of short-term seed banks although ungerminated seeds may possibly continue to germinate in nature during the following growing season. This is currently being investigated. Some seeds germinate the second year *in vitro* although counts have not been made. Bowles (pers. comm.) has also observed second season germination *in vivo.* Oxidation of the embryo coat by hypochlorite accelerates germination, suggesting that seed coat permeability may be an intrinsic germination control factor associated with stratification. The air-filled testas do not inhibit TTC absorption and staining of

embryos, thus included air is probably not a factor in controlling the germination rate. The water repellent testas strongly affect the way seeds move on water and adhere to solid objects. Loss of the repellent effect probably allows seeds to be more effectively incorporated into the soil mass.

An unresolved problem is the difficulty in determining the optimum bleach period for estimating viability. All embryos in a sample do not stain uniformly, some being very pale. I do not know whether these faint color reactions represent viable seeds and/or whether they may represent a delayed germination mechanism.

Seeds germinate in vitro and seedlings continue growth when transplanted to prairie sods. Successful mycorrhizal associations are assumed. Any seeds which germinate but fail to acquire mycobionts will not survive. Failure of mycorrhizal formation in Nitex slides is unexplained and experiments are continuing.

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Platanthera leucophaea, photo courtesy Paul Martin Brown

Cypripedium seedlings in flask.

Cypripedium Ulla (*flavum* x *reginae*), first year in soil.

Orchid Propagation: Theory and Practice

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A Provocative Introduction

The search for methods of orchid propagation on sterile nutrient media has many similarities with other scientific research, especially in the study of biological systems. This includes controlled methodology and comparative studies of well-defined entities. This controlled methodology makes it possible to study each component of the system step by step.

However, the feeling of security and continuity that such a well-defined, well-used methodology gives also can lead to restraining effects on the research result. There is always a risk that the following of the methodology is more important than the results. Individual methods may be like many politicians: they have good ideas and some good results in the beginning, but never understand when to retire and leave room for new ideas and new politics.

In other words, the entities of a study might be well-defined, the methodology well-defined and the statistics satisfactory. But are the results *interesting*?

One important motivation for risking the dominance of methodology over "interesting results" is, of course, the time aspect in a complicated research field. Orchids, for example, have a maximal growth speed which is not always in line with time schedules for academic careers. If one's research field were the generation cycle of the African elephant, one would be in big trouble. Orchids reproduce a little faster than elephants and need less room, but it is still difficult to speed up the results.

This is the main reason why, at least in Europe, most of the methods for orchid propagation and most large scale propagation in practice have been and still are made by interested and patient amateurs outside the academic world. This is not antagonistic, but just a question of scientific method. Both ways are necessary compliments to the research.

The "amateurs" might have a sometimes poorly defined methodology with partly unknown or uncontrolled entities. But, with a rather unrestricted trial-and-error method, they look at the *results* and are possibly interested in the *explanation* in a second step. In another biological field, that of surgery, mankind needs both the well-trained operating surgeon *and* scientific researcher studying surgical problems on a micro level with scientific methods. However, life is too short to get well-experienced in both fields; very few surgeons are in the front line of practice and research.

To summarize, one individual's life is probably not enough for finding out all the mysteries of orchid propagation. We have just started. And many results will come in areas and with methods that we never even thought of when we began. Science is the accumulation of knowledge step by step. I hope to be able to give you a few new pieces.

Orchid Propagation - Complicated or Easy?

Even a very interested observer soon gets discouraged when looking at the theory of orchid propagation, including plant physiology and construction of complicated growing media, and at the practice of orchid propagation, including technological skill and expensive laboratory equipment. The main purpose of this paper is to show how very much all of this can be simplified. Some very simple, cheap, and almost universal growing media can easily be made. In addition, a few different seed treatment methods give 50-100% germination of mature seeds of not all but almost all species and hybrids. With very primitive technology, one can do all laboratory work in the kitchen and survival in soil can be 90-100% in the garden.

I will in turn discuss the following topics: seed storage; sowing and seed treatment; growing media; temperature, light, soil composition, and growing in soil.

1) Seed storage

There is not much to say about this. I store dry seeds in the refrigerator, either inside the dry capsule or taken from the capsule in locked glass tubes. They will have almost the same germination rate for at least three years. I have not tested greater lengths of time.

2) Sowing and seed treatment

This topic is very important. For a large number of terrestrial orchids, poor germination is or has been a very restricting factor in propagation. Several species germinate poorly, if at all. The method of seed treatment is the most important factor for germination, as a suitable medium is the necessary component for continued growth. Poor germination sometimes is due to wrong medium, sometimes to wrong seed treatment. It is very important to distinguish between these two causes.

The problems with germination are just partly understood and explained. Germination definitely depends in part on the structure of the seed coat. Some species have a very thin seed coat that easily lets in water and allows germination; other species have a thick seed coat needing chemical agents for seed coat breaking. It is probable that this seed coat is of variable chemical material.

Temperature may also be of importance for germination. Some species need longer or shorter cold treatment either of the mature, dry seeds, or after sowing. Other factors may inhibit or retard germination. Some species germinate after 3-4 days, others after 5-6 months, with a definite (chemical) alarm clock to wake them, independent of temperature or broken seed coat.

In addition, the fact that a few species still only germinate to an acceptable degree if sown from half-mature capsules indicates other factors not yet understood. It is not just a question of a thick seed coat.

One problem in practice is that two species that are very closely related might be totally different with respect to seed germination. Methods appropriate to each can only be found in a trial-and-error process.

However, almost all species can be germinated with at least one of the following methods: half-mature seeds: hypochlorite bath, hypochlorite bath + cool treatment, sulfuric acid + hypochlorite bath, sulfuric acid + hypochlorite bath +

cool treatment.

a) Half-mature seeds

Most species that do not germinate with the "common" hypochlorite method (Ca-hypochlorite or Na-hypochlorite) germinate from half-mature capsules. However, big problems arise when one might not *have* half-mature seeds. In addition, one never knows at first how many weeks are "half-mature." There is a large variation from species to species. European *Nigritella nigra* have the correct degree of "half-maturity" after 3 to no more than 4 weeks, *Gymnadenia conopsea* after 5 weeks, *Orchis militaris* after 6 weeks, *C. reginae* after 7 weeks, *C. calceolus* after 8 weeks, *C. speciosum* after 9-10 weeks, et cetera. The number of weeks also varies from one area to another with genetically different plants! The interval between "too little" and "too much" half-mature usually is just 4-5 days.

The *timing* of sowing is also of big importance for some species. If one sows half- mature seeds of certain species in late summer, one might totally miss the time needed to get plants in 'potting-in-soil' size next autumn. Some species grow faster than others. For example, the magnificent *Orchis purpurea* should be sown in February or March. A few *Cypripediums* need 15-18 months development on medium.

b) Hypochlorite bath and
c) Hypochlorite bath + cool treatment

If the common hypochlorite method for sterilization and seed coat breaking does not work, some species achieve an acceptable germination rate if the flasks/tubes are kept cold (2-5° C) for two to four months.

d) Sulfuric acid + hypochlorite bath and e) Sulfuric acid + hypochlorite bath + cool treatment

A very interesting and in many cases very efficient method for germination is the combination treatment. This is performed sequentially, with seeds first put in a sulfuric acid solution and then immediately in a diluted hypochlorite solution. The explanation of the success of this method in many otherwise problematic species is probably due to the chemical content of the seed coat; being sometimes cellulose, sometimes other polysaccarides, or sometimes a wax layer. Plants other than orchids, such as some grass seeds, germinate in laboratories only after treatment with sulfuric acid..

However, using solely sulfuric acid on orchid seeds does not promote germination and is not enough for sterilization. If the seeds are *first* treated with a 0.5-2% solution (approximately = 0.05-0.2 Molar) of H_2SO_4 and *then* directly taken to a diluted hypochlorite solution, the time needed for seed bleaching in the hypochlorite solution is greatly shortened and many species have an excellent germination rate in comparison with only hypochlorite treatment.

In using this method, optimal concentration/time ratios can be found for each species. As a rule of thumb, most *Cypripediums* with thick seed coats do best with 2% H_2SO_4 for 10 minutes, a few needing fifteen minutes of soaking., followed by NaClO. Time is the crucial point for the degree of seed bleaching. The breaking point comes rather quickly, so do not use too strong a hypochlorite solution after pre-treatment with sulfuric acid!

To illustrate the benefit of this method, *Orchis militaris* and *O. purpurea* germinate with luck to maybe 10% from half-mature seeds. From mature seeds

treated with hypochlorite, with or without cool treatment, germination is very poor. If *O. purpurea* seeds are treated with 2% H_2SO_4 for ten minutes and then NaClO until "half-bleached," germination with be 90-100% in four to eight weeks. The same treatment on *O. militaris* seeds gives a germination rate of 20-30%. But, cooling for 2-3 months after sowing results in 50-100% germination. On the other hand, their relatives, *O. morio* and *O. spitzelii,* which sometimes grow side to side with *O. militaris* in Sweden, germinate to almost 100% within 4-6 weeks if just treated with NaClO.

Gymnadenia conopsea, Swedish *Nigritella nigra,* and *Platanthera bifolia* are other species that behave in a similar way. Half-mature seed germination is 20-50%. NaClO alone results in very poor germination. H_2SO_4 + NaClO gives some germination. But H_2SO_4 + NaClO + cool treatment lead to 90-100% germination.

Cypripediums differ greatly in method for seed treatment. I sow almost all *Cypripediums* from mature seeds, several with H_2SO_4 + NaClO + cool treatment. Some of them still do not germinate well, a few not at all. *C. calceolus* germinates better from half-mature seeds, as does *C. speciosum* and similar species. *C. flavum, C. reginae, C. californicum* and a few more germinate to almost 100% with just the NaClO method. I have not tested all methods with all species. In most cases, I use the combined H_2SO_4 + NaClO method and test if they need cool treatment for germination or not. *C. guttatum, C. passerinum* and *C. parviflorum* germinate to nearly 100% after H_2SO_4 + NaClO + cool treatment. C. *kentuckiense* (sown in 1995 for the first time) germinates to 50%, *C. pubescens* and *C. acaule* just 10%. *Cypripedium* hybrids always germinate as the mother plant and can thus be sown any desirable month of the year if the method works with the mother plant.

In England, researchers at Kew Botanic Gardens had problems germinating *Himantoglossum hircinum* for a conservation project. They sent me a capsule and with 1% H_2SO_4 + NaClO treatment, I had 100% germination within 6-8 weeks. The problem was not a question of medium but of seed treatment.

This method has been used by me for 8 or 9 years and reproduced several times, year after year, with many species and hybrids. The method deserves to be explored and studied more systematically.

3) Growing media

This is an area which science has given a much too complicated reputation. There are dozens and dozens of studied and published growing media. Of course, some components are of crucial importance. On the other hand, many components are of very little importance and a very large variation is possible. One can have a large variation in the mineral content, but *must* be careful with the nitrogen component.

The watershed in orchid propagation, and a large part of the solution to the problem, lies in the nitrogen component. Orchid species may prefer NH_4, NO_3, or organic nitrogen such as polypeptides or amino acid combinations. Most terrestrial orchids seem to prefer organic nitrogen. Many are definitely harmed by especially NO_3, at least at a young age. A few cannot utilize only organic nitrogen on sowing medium. In that case, NH_4 seems to be better than NO_3.

Some species, such as *Orchis morio,* can be grown on almost any medium containing sugar. I have grown *O. morio* on 12 different media. Most other orchids have a more particular taste concerning the nitrogen meal.

Rather important in the medium is the use of a suitable complex organic

material like pineapple juice, coconut milk, boiled potato, or other similar substances. These contain vitamins and plant hormones often in very suitable combinations and concentrations for orchid propagation. The content is partly known, partly unknown, but the effect very good in many cases.

So, forget about details in mineral content, forget about all the extra microelements, and forget about distilled water. I have used a very large number of different and complicated media throughout the years, and only one with small variations in the last eight to ten years. I raise 5,000-10,000 plants every year on the following medium:

$(Ca)_3(PO_4)_2$	50-100 mg
$MgSO_4\ 7H_2O$	50-100 mg
KH_2PO_4	50-100 mg
sugar	10 g in sowing medium, 15-20 in growing medium
agar-agar	approximately 6 g
pineapple juice	10-25 ml; has ph of 5.5-6
Vamin (amino-acids)	300 mg amino acids approximately = 5 ml solution
Soluvit (vitamins)	0.1-0.3 amp
tap water	1000 ml
charcoal	approximately 0.5 g

(kinetine 2-5 mg; for some Cypripediums)

The pH of this medium automatically sets at 5.5 to 6. Any combination of the three mineral salts is possible and gives the same result. The tap water where I live now contains approximately 13 mg Ca/L and approximately 22 mg SO_4, with other components of even smaller amounts. The pineapple juice adds approximately 30-40 mg K per liter medium, very little of other minerals, but obviously enough microelements. Note that I add no extra Fe.

Since the vitamins and the kinetine are added before 15 minutes of sterilization, it is very likely that some unknown amount of it is destroyed. However, the medium works excellently.

The crucial component is the amino acid component. Vamin is an amino acid solution, used in hospitals in Sweden for intravenous nutrition of poorly nourished or surgical patients. Soluvit is the vitamin solution used for the same purpose. The pineapple juice has a very stimulating effect on root formation and growth.

All *Ophrys, Orchis, Gymnadenia, Platanthera, Nigritella,* and a large number of *Cypripedium* species and hybrids can be raised in any desired number on this medium. *Dactylorhizas* grow very big on it, but have a faster initial growth if 50-100 mg NH_4NO_3 is added to the sowing medium. A few *Cypripediums* do not grow as well on this original version. These are: *C. parviflorum, C. guttatum, C. yatabeanum* and a few more. However, if just the amino acid combination - *the nitrogen component is still most crucial* - is somewhat changed, these species and their hybrids grow without problem. I have approximately 800 *C. guttatum* on the following medium this year:

Glutamine	100 mg
Cystein	25 mg
Phenylalanine	10 mg
Vamin	30 mg amino acids approximate = 0.5 ml solution

all other components as listed previously

However, I still have problems with *C. speciosum, C. macranthum, C. himalaicum* and *C. tibeticum.* They germinate as half-mature seeds rather well, but 98% die within a few months. However, the surviving plants grow very big on the medium. Nevertheless, another sowing medium must be found.

The question is if the amino acid combination or possibly the hormone content in the pineapple juice is the problem. Obviously, these red-flowering *Cypripediums* have different demands for sowing and for growing medium. For all other orchids, I use the same components in sowing and growing medium, just raising the sugar content. Another interesting observation is that several *C. tibeticum* and *C. himalaicum* hybrids can be propagated on a large scale without any problem on these media, but just a very few *C. speciosum* and *C. macranthum* hybrid plants!

Theoretically innumerable combinations of 18 different amino acids are possible and all cannot be tested. However, even small variations in components can give big variations in the result, and problems can probably be solved with just variations of the following amino acids. The following is a variant of the Norstoog medium as used by Finn Haugli in Norway. He has grown just a few *Cypripediums* on it. He has faster initial growth of *Cypripedium* protocorms of some species, but plants eventually grow bigger on my standard amino acid medium.

Glutamine	400 mg
Alanine	50 mg
Cystein	20 mg
Arginine	10 mg
Leucine	10 mg
Ph-alanine	10 mg
Tyrosine	10 mg

Potato instead of pineapple, kinetine, NAA and 2,4-D.

These amino acid based media also have another very good property: they have very low toxicity even after long time use. Very rarely do plants show sign of toxicity. Crowded plants might starve of sugar deficiency, but eight months on the same medium is never any problem, and 12 months is harmful for just a few *Cypripedium* species. On the other hand, plants are almost never kept for 12 months on the same medium. The low toxicity is helpful for species/hybrids that need/prefer periods of cold treatment in a period of their life on the medium. The flasks/tubes can go in or out of the refrigerator whenever needed and the plants are simply transferred to fresh medium when size and crowding make it necessary.

4) Growing temperature

Most species prefer to grow on medium at room temperature, that is 15-20° C. However, there are some species were *temperature sequences* over the growing cycle are very important and sometimes even necessary for growth. For example, Mediterranean *Ophrys* in nature has 5-6 months of (rainy) winter to germinate, grow, and produce a tuber before the dry, hot summer. On medium, they still hurry and quickly develop leaves, roots and tubers. During that period, they prefer temperatures of 15-20° C. in winter, even if Mediterranean winter is cooler, usually 10-15° C.

European *Dactylorhizas, Gymnadenias, Nigritellas, Platantheras,* et cetera, prefer to grow from small protocorm to big plant on medium in 15-20° C. However, when the plants seem to be fully developed in autumn exhibiting short leaves or a fat crown bud and some roots, they are usually potted in soil. If they are instead kept on medium with a much lower temperature, root growth starts again. This is seen much more on amino acid media than on media with inorganic nitrogen. At just a few degrees above 0° C, roots can grow several centimeters more on medium, and thus produce bigger plants when they finally are potted in soil in winter or early spring. Sometimes I have chilled plants of these species with a 5-10 mm protocorm size for 2-3 months, simulating normal winter development. In just a few of the *Dactylorhiza* and similar species, this gives bigger plants the next autumn.

For a few *Orchis* species, cold treatment in one or even two well-defined growing stages is quite necessary for further growth. Most *Orchis* species, however, do not need any cold treatment. *O. morio* and *O. spitzelii* illustrate this fact. They germinate within 2 to 4 weeks and to a rate of 100%. They keep growing all autumn and winter, at room temperature, then develop leaves and tubers on medium in spring.

Orchis purpurea, the biggest and one of the most beautiful of European orchids, growing 70-80 cm tall, germinates after 4-6 weeks to a rate of 90-100% after H_2SO_4 + NaClO, without further cool treatment. These plants grow to 2-3 mm protocorm size before growth stops, when they must have a cold period just above 0° C. After 6 weeks, still in the same low temperature, the protocorms start to grow again with a much faster speed than earlier. If they are taken from the refrigerator and transferred to fresh medium, they quickly develop long roots, leaves, and tubers within a few months.

Its close relative, *O. militaris,* germinates to a rate of 20-30% after H_2SO_4 + NaClO without cool treatment, and to a rate of 50-100% after H_2SO_4 + NaClO + cool treatment. Like *O. purpurea,* these plants stop growing at the 2-3 mm protocorm size, continuing only after a cold period. However, this species differs a little if the plants come from Sweden versus Central Europe. Swedish *O. militaris* plants develop 2-3 cm leaves and 2- 3 roots on medium but no tuber. To develop a tuber, plants need one more cold treatment. After the second cold treatment, when temperatures are just a few degrees above 0 C, new long leaves quickly develop, followed by a good tuber in a few months. *O. militaris* from central Europe (England) in most cases develops a tuber after just one cold period. The Swedish *O. militaris* probably has adapted to a colder climate. Central European *Orchis laxiflora* and Swedish *O. laxiflora var. palustris* differ in just the same way; the Swedish version needs two "winters" to form tubers. There are other examples of the importance of temperature sequences, but I leave that now.

Cypripediums usually prefer to grow in room temperature, although several need cold treatment after sowing of mature seeds in order to germinate. A few species, though not many, are stimulated in further growth if winter temperature are somewhat decreased. On the other hand, several *Cypripedium* species are given cold treatment to germinate from mature seeds.

5) Light

There is not much to say about light, except that I use second-hand fluorescent lights both for plants on medium with leaves and when I "start" plants in soil.

6) Soil composition and growing in soil

This subject has given many growers big problems. Relatively scientific compositions of soil or substitutes for soil have been constructed and described. A lot of different fertilizers for this purpose are also described, from camomile tea to vitamin solutions. More or less artificial conditions for growing are also constructed. The rationale behind these precautions is the idea that the little orchid plant, coming out of the aseptic medium, would be sensitive to infecting agents. This is also the reason why the symbiotic method for propagation a priori is said to be better for large scale propagation.

However, in nature orchids grow in soil, and the easiest way is to grow them in a soil similar to the natural conditions of that species. I use "natural" soil from areas where orchids grow or seemingly could grow. I use four different "soils," sometimes plus peat. With variable mixtures of these, I can grow almost any species/hybrid with survival rates of 90-100%. Usually, I use the "pure" soil from a certain area for most species. For *Cypripediums*, however, I mix 2 or 3 different soils.

Sometimes, I am asked if I need "very much" soil for potting a lot of orchid plants. Actually, I usually grow in 500 ml (10.5 cm) clay pots, and in one pot 8-15 plants are potted, depending on plant size. Thus, a bucket (10 l) of soil can give me soil enough for growing 150-300 plants. The next autumn, the plants are taken up, placed in a garden or in nature, and the soil is recycled. So, potting in soil is a question of experienced gardening much more than the making of an artificial substitute.

What about the mycorhiza? I do not know and do not care very much about it. Perhaps the orchids do not need the mycorhiza at that size or they have several to select from in the "natural" soil. Chinese *C. flavum*, American *C. reginae*, as well as the hybrid *C. flavum x C. reginae* can grow with a 99% survival rate in a soil from a Swedish forest on sandstone. Several thousands of Mediterranean *Ophrys* have been grown with survival rates of 90% in a soil from a Swedish meadow on limestone.

So, if you have problems with plants in soil, let in the gardener!

Summary

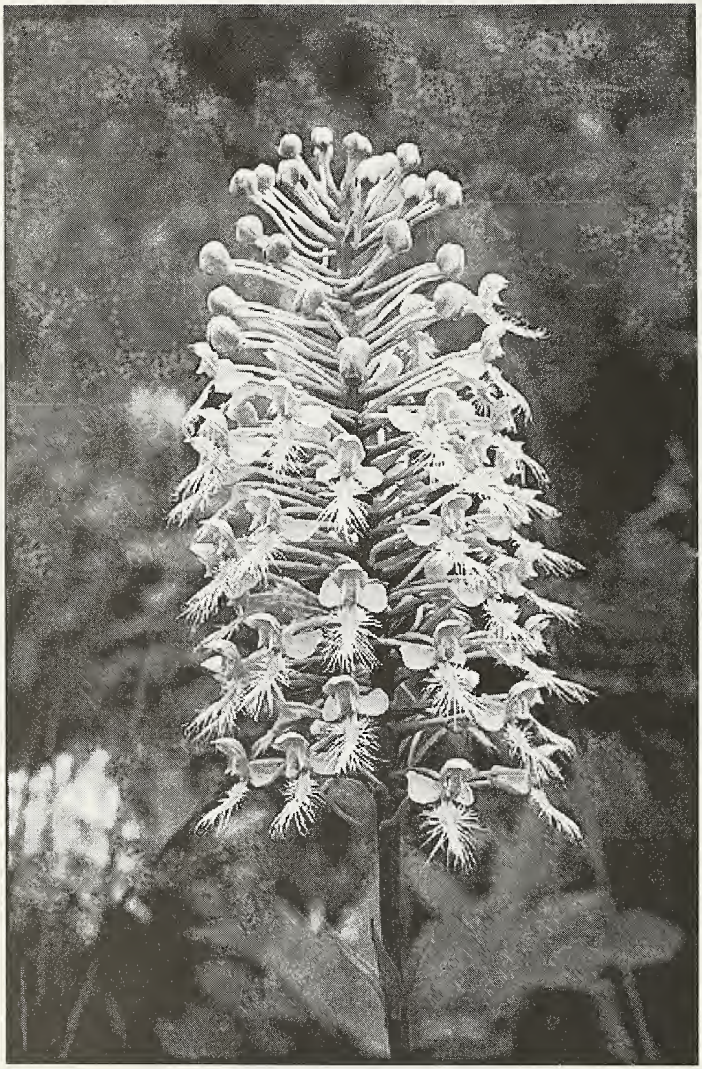
So far, not all but a very large number of terrestrial orchid species and hybrids can be raised on a large scale with an asymbiotic method. Very simple technology can be used. Most species/hybrids can be germinated from mature seeds with several different methods. Sowing and growing media can be simplified, but the

nitrogen source seems to be the crucial agent for success or failure. Organic nitrogen, such as amino acids, in the medium seems to give a much more universal media than inorganic nitrogen, with few exceptions. Potting and growing in soil is mostly a question of experienced gardening.

Thus, the theoretical basis can be simplified and standardized. On the other hand, the much less defined quality of experience is necessary. One must have seen hundreds and thousands of orchid protocorms and plants to know if they are healthy and to know what to do next with them. One cannot become a surgeon, a piano player, or a successful orchid propagator by a correspondence course. One needs two things most of all: patience and a long life.

Cypripedium Ulla (*flavum* x *reginae*), second year in soil.

Cypripedium Ulla (*flavum* x *reginae*), third year in soil.



Platanthera ciliaris, photo courtesy Paul Martin Brown

The Reintroduction of *Platanthera ciliaris* in Canada

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The past occurrence of *Platanthera ciliaris* near Leamington in Ontario, Canada is confirmed by herbarium specimens lodged in major herbaria (CAN, DAO and TRT). Dodge (1914) also reported its existence near Leamington and Windsor. This orchid has not been seen in recent times despite the efforts of many field naturalists and botanists and is presumed extinct in Canada (Whiting and Catling 1986). Although a wide ranging species in the Eastern United States, it is possibly extirpated in Massachusetts and New Hampshire,endangered in New Jersey and Rhode Island, threatened in Indiana, Michigan and Ohio and rare in Connecticut, Delaware, Illinois and Missouri (Argus and White 1982).

Materials and Methods

A site in Michigan was chosen to be the donor of seed for this experiment as the prevailing winds are from the West and most likely to be the source of the original plants in Ontario. In the Fall of 1993, with the help of Fred and Roberta Case, seed and three pieces of root with yellow brown discoloration indicating the presence of the symbiotic fungus were harvested and taken back to the laboratory. The seed were air dried and stored at 4°C. The roots were sterilised in 10% commercial bleach, rinsed twice in sterile water and thin sections through the infected areas were placed under a dissecting microscope in a laminar flow hood. Five fungal coils (peletons) from each of the three root pieces were removed by using a fine insect mounting pin. The peletons were incubated on water agar with 0.1% streptomycin sulfate for 7 days at 22°C then transferred to potato dextrose agar. The isolates were sent to the University of Alberta microfungus collection where they were identified as *Ceratorhiza goodyerae-repentis.*

The seed was vacuum treated and sterilised in 10% commercial bleach (Anderson, 1989) for 1/2 to 2 hours. The optimal bleaching time for the seed was 1 hour. Fewer seed germinated in the 1/2 hour treatment and some of the embryos were killed in the one and a half and two hour treatments. The seed were sown on a variety of symbiotic media with the fungus, and asymbiotic media. All treatments were incubated in the dark at 22°C. The symbiotic germination was consistently poor with only 1% or less germination but the resulting protocorms continued to grow. All the asymbiotic media gave excellent germination in excess of 90% over a period of 12 months. A modified Lucke's medium (TABLE 1) with ammonium nitrate replaced with 100mg myo-inositol and 10mg L-glutamine (Waes and Debergh, 1986) gave slightly better growth.

Table 1

Modified Lucke's Medium

Potassium nitrate KNO_3	0.4 g
Magnesium nitrate $Mg(NO_3)_2$	0.11 g
*Phosphate buffer	18 ml
Ammonium ferric citrate	0.025 g
Myo-inositol	100 mg
L-glutamine	10 mg
Coconut milk	50 ml
Glucose	10 g
Fructose	10 g
Agar	7 g

Made up to 1 litre with glass distilled water.

* Phosphate buffer: Mix 975ml of monopotassium phosphate KH2PO4 solution (13.6g/l) with 25ml of dipotassium phosphate K2HPO4 solution (17.4g/l)

Germination was prolonged over a period of 12 months and protocorms were removed every 4 weeks and placed on fresh media 4 per flask. Protocorms and seedlings were transferred to different symbiotic media at regular intervals. Symbiotic and asymbiotic cultures were kept in the dark at 22°C for 5 months. The cultures were then cooled over a period of 18 day to 4°C then placed in a cold room at 4°C for 8 weeks.

Results

The results of transferring protocorms and seedlings from asymbiotic culture to a symbiotic medium varied depending on the stage of growth. Young and older protocorms mostly survived and grew well on both oats medium (Clements and Ellyard, 1979) and corn meal agar from Difco which contained 50 g of infusium from corn meal and 15 g agar per litre. A slightly higher survival was obtained by transferring the protocorms to water agar with the fungus for seven days then to the oats or corn agar. There was slightly less mortality of protocorms on the corn meal agar. Mortality was high when protocorms were transferred to H1 oats medium (Clements et al., 1986, modified by Rasmussen et al., 1990) (Table 2)

Table 2

$Ca(NO_3)_2.4H_2O$	200 mg
KCl	100 mg
KH_2PO_4	200 mg
$MgSO_4.7H_2O$	100 mg
Yeast extract (Difco)	100 mg
Sucrose	2 g
Finely ground oats	3 g
Agar	12 g
Glass distilled water	1000 ml

After the seedlings had been given a two month cold treatment some

seedlings were transferred to H1 oats medium and placed in the light and at this stage survival was much better. Only 1 flask out of 10 did not survive and this may have been because the seedlings were at a slightly younger physiological age than the others.

On the 22 September 10 symbiotic plants of *Platanthera ciliaris* were planted on crown land adjacent to the Ojibway prairie in Windsor. Some of these plants had a root 8cm long and a well developed bud. The site will be visited in July 1996 to determine the survival rate and more plants will be introduced. Greenhouse grown plants of *Platanthera* have bloomed in 32 months to 3 or 4 years (Linden, 1980; Stoutamire, 1974; Fast, 1985). Hopefully the plants reintroduced will flower within the next 2 years. There are about 450 seedlings at different stages of growth in pots and flasks.

Discussion

During symbiotic germination a suitable fungus enters the basal cells of the embryo through the suspensor end (Hadley, 1989). Poor germination rates in symbiotic culture may be due in part to the timing of introducing the fungus. The first stage in seed germination is the imbibing of water and swelling of the embryo. Colonisation of the basal cells by the fungus may not be able to take place until the cells are fully imbibed and the ones that are not may be parasitised by the fungus. After germination has taken place the protocorm forms hairs and the fungus can often be seen growing down into the cells of the protocorm through these hairs.

The use of organic nitrogen in the asymboitic medium seems a logical step in improving germination and growth, as in nature the fungus is the sole source of nutrients for the developing protocorm and the nitrogen would be in the organic form.

Acknowledgements

The author thanks the Southern Ontario Orchid Society and The Ontario Heritage Foundation for financial support. Fred and Roberta Case for their valuable help in the field and Peter Croezen and Jenifer Line for their technical help.

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Reproductive constraints in *Cypripedium*: Horticultural and Conservation Viewpoints

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When we propagate orchids we usually have horticultural and/or conservation goals in mind. Horticultural objectives can include vigor, ease of cultivation, floriferousness, flower color and form. When selecting parents to achieve these objectives, it is practical to choose fertile parents giving rise to vigorous offspring. Once vigor has been established in a breeding line, further selection for flower color and form can commence. Vigor and reliability should remain primary objectives throughout a breeding program for without these traits, even the most beautiful orchids may not endure in cultivation.

Conservation is by definition the wise use of a resource. It follows that wise use requires an in-depth knowledge of the resource. Conservation projects such as the hand-pollination of wild orchids to increase fruit set and seed yield, the re-introduction of nursery-raised plants to a habitat that is known to have once supported the species, or the transplantation of plants whose habitat is threatened to a new locality, require considerable understanding of orchid biology and careful attention to experimental design. For example, when attempting to conserve a population of orchids, the size of population to be preserved must be defined. Must all individuals be preserved or need only some individuals be retained to maintain the population? When attempting to answer such questions, the role that individuals play within a population becomes an important consideration. How much do we really know about the selection pressures that have determined which individuals still exist in a population and why some individuals have not survived? Our evidence suggests that we are only at a very preliminary stage of understanding this important aspect of orchid biology.

It has been suggested that species in which seed production is pollinator-limited often have flowers that are relatively long-lived, with the result that the opportunity for pollination is maximally extended (Catling & Catling, 1991). Cypripediums have been reported to be particularly susceptible to pollinator limitation. Fruit set can be highly variable, from year to year and from site to site. Gill (1989) related fruiting failure in *Cypripedium acaule* to pollinator inefficiency. After many years of disappointingly low fruit set in the populations he studies, he has recently reported a serendipitous event involving massive defoliation of forest trees by Gypsy Moth (*Lymantria dispar*) followed by greater orchid flower and capsule production than previously observed. He suggested that the increase of light on the forest floor led to the growth and flowering of ericaceous companion plants, which attracted bumblebee pollinators in sufficient number to also pollinate nearby orchid flowers (Gill, 1996 - this publication). Joyce and Allan Reddoch have found consistently higher fruit set with *C. acaule* in the region of Ottawa, Canada, recording 10 - 40 percent or higher capsule set on 474 plants in

five sites over a number of years (personal communication). Tremblay (1994) observed that fruit set in *C. parviflorum* var. *pubescens* (*C. calceolus* v. *pubescens*) in Gatineau Park varied from 9 to 32 percent over two years but that herbivory significantly reduced the number of fruits reaching maturity without damage.We have noted in experiments with the same species in the same habitat that there are inter- and intra-clonal differences in compatibility (Light & MacConaill - in press). Ballard (1990) reported that hand pollination results with *Cypripedium calceolus* var. *parviflorum* were as poor as those achieved by insect pollination. We have observed with *C. arietinum* that while there is generally high fruit set, many of the capsules become infested with weevils so that little or no seed is released at the time of dehiscence. Still, all of these orchids have reproduced occasionally, in one habitat or another, if only in small numbers. The plants seem to be relatively long-lived. It is only when the populations and possible habitats have been reduced either through natural disaster or by development pressures that limited seed production might become problematic for a species. It is then that observations of unpredictable and inconsistent fruit set even with hand pollination raise concerns. We have investigated some of the factors which may play a role in the variability observed in *C. parviflorum* v. *pubescens*.

Experimental Design

To minimize bias, choice of flowers for any particular treatment was determined well before anthesis. Each flowering stem was tagged at the base using a polyethylene square marked indelibly with an alpha numeric code. If flower buds were lost or damaged after the experiment began, they were considered as 'missing data'. Where two flowers were produced on a flowering stem, only the lowermost was used.

To prevent insect pollination, flowers were blocked just before anthesis with a 6 to 8 cm square of light-weight, water-repellent, spunwoven polypropylene fabric (Reemay®) placed in the pouch. The fabric could be compressed then placed in the pouch opening without damaging a flower and would rebound to effectively block pollinator entry.

An experiment to test the effect of different pollen-stigma combinations and post-anthesis pollination times was designed by assigning pollen and seed parent combinations by block, and post-anthesis pollination times 2-days by 2-days to 14 days across blocks by Latin Square design (Table 1)

Pollen quality

Pollen quality is seldom tested as a possible explanation of seed yield and seed germinability. To sample pollen for assay, entire anthers (one per flower) were removed. A new wooden toothpick was used for each flower. A portion of the pollen of the order of 20 000 monads was taken for pollen germination studies. We assessed pollen germinability using the protocol outlined in Appendix 1. Remaining pollen, estimated as 80,000 monads using the method outlined in Appendix 2, was used to pollinate flowers of known age.

We observed that pollen of *C. parviflorum* v. *pubescens* retains germinability if the flowers are removed at anthesis and placed in a sealed container at 4°C (Table 2). Refrigerated anther contents become somewhat granular. Older flowers stored

under similar conditions remained fresh but the pollen lost some of its germinability. Isolated anthers stored for one month in an open container at 25°C dried quickly: pollen still would germinate although only at a low level. Pollen from the same flowers stored dry at 4°C retained germinability.

Even when pollen germinability was tested and proven, it was not necessarily a reliable measure of fertility. Pollen collected from flowers representing five different plants generally germinated in 0.5 M sucrose within 12 hours. Pollen samples collected from older flowers was sometimes contaminated with fungus especially following a period of heavy rain: contaminated pollen did not germinate nor were capsules formed when it was used in hand-pollination experiments. Pollen germination was of the order of 30 - 70% in all plants excepting one where only one sample reached this range. We found some variability in pollen germinability within and between plants of *C. parviflorum* v. *pubescens* (Table 3). While fungus infection of older anthers could explain poor pollen germinability in some of the samples, we have no explanation for low pollen germinability from young, uninfected anthers of the same plants. Seed production was highest when pollen of one particular plant was used to pollinate flowers of certain other plants suggesting that pollen germinability is not the only factor to be considered: seed parent influences must also be weighed when doing assays.

Effect of flower age on fruit set

The maximum age at which pollinated flowers set fruit varied between plants from two to ten days (Table 4). The interval after which pollinations were unsuccessful was not related to visible aging of the flowers except at 12 days when unpollinated flowers of all plants withered. We noted two instances (marked in Table 4) where the ovary became darker green after pollination but aborted further development three to four weeks later.

Plants varied considerably in capsule production from flowers aged 10 days or less, ranging from 17 to 67%. Seed parent fecundity was not correlated to total leaf area, number of leaves and/or shoots. There was no obvious predictor of the observed behavior.

The effect of water limitation on seed development

We have observed that the fruits of *C. parviflorum* var. *pubescens* are susceptible to the lack of water if the severe drought occurs during critical stages of seed development. In 1993, when less than 2 mm of rain fell during the six weeks of early seed development (mid-May to early July), we observed that while the plants were never visibly wilted and fruits formed and developed normally, the capsules were spongy to the touch: when harvested 50 days post-pollination, all seeds showed arrested embryo development and none germinated during *in vitro* germination experiments. Rainfall was average during our 1995 experiments: embryo development was within the expected range.

Discussion

Long term observations of orchid populations add to our understanding of their demography, population flux, and response to changing climate and to

disturbance. Experiments on individuals within a population can lead to a greater understanding of how orchid populations function. It has been suggested that species in which seed production is pollinator-limited often have flowers that are relatively long-lived, with the result that the opportunity for pollination is maximally extended (Catling & Catling, 1991). In *C. parviflorum* var. *pubescens*, flowers can remain fresh for two weeks or longer if the weather is unseasonably cool.. Using dyed pollen, Tremblay (1994) was able to monitor pollen movement in an isolated deme. He observed that flowers were visited by pollinators once only during the experiment. When pollinator movement occurred relative to flower age at pollination was not reported. Our data show that in 1995 certain plants in this same population became ineffective as seed producers as little as two days after the flowers have opened. We have also observed that pollinated flowers do not senesce after pollination but remain fresh as long as ten days after pollination or until the time that nearby, similar-aged, unpollinated flowers fade. Why do flowers remain fresh after they have either been pollinated or have ceased being potential seed producers? Our observation that plants vary considerably in the flower age at which pollination will result in fruit set suggests a maternal influence on fecundity that is separate to a maternal influence on the plant potential as a pollen parent. The opportunity to be a pollen donor is therefore extended through the entire life span of a flower while the ability to be a seed parent is more strictly controlled. What factors play a role in the limitation on fruit set remains to be investigated.

There are several important points raised through our work which should be considered by those pollinating flowers of *Cypripedium* in an attempt to get seed.

1) Pollen should not automatically be assumed to be germinable. The simple test described in Appendix 1 can be used to establish pollen germinability within six to twelve hours.
2) Flower age at pollination has been shown to affect seed production outcome in *C. parviflorum* v. *pubescens*. It may also be an important consideration in other *Cypripedium sp.* Flower age at pollination should become part of the data collected when attempting to get seed. It is recommended to pollinate flowers at 2 - 4 days post-anthesis.
3) We have observed sufficient variation in inter- and intra-clonal compatibility, seed germinability and seedling vigor in *C. parviflorum* v. *pubescens* to recommend that the breeding characteristics of a colony be established using a balanced, randomized design before beginning any propagation program.
4) Water appears to be critical to seed development in *C. parviflorum* v. *pubescens*. Careful attention should be taken that this species always has adequate water throughout the flowering period and until the fruits are harvested.

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APPENDIX 1

Testing Germinability of Orchid Pollen

You will need:

- Table sugar (sucrose)
- De-ionized water
- Disposable petri dishes
- Marker to write on plastic
- Freshly collected pollen
- Balance to weigh the sugar
- Volume measure
- Dropper
- Two needles
- Microscope

Sucrose solution

Prepare a solution of sucrose and water by weighing 34.2 grams sucrose and adding enough water to the sucrose to make a total of 100 millilitres. Mix well until the sugar is all dissolved. This is a 1.0 Molar sucrose solution. Prepare a series of known dilutions using a quantity of both 1.0 M sucrose and de-ionized water as follows. For example, 1 part of 1.0 M sucrose solution mixed with 9 parts of de-ionized water makes a 0.1 Molar sucrose solution and so on. Use only freshly prepared solution.

Final molarity	0.1 M	0.2 M	0.3 M	0.4 M	0.5 M	0.6 M	0.7 M	0.8 M	0.9 M
1.0 M sucrose	1	2	3	4	5	6	7	8	9
De-ionized water	9	8	7	6	5	4	3	2	1

Method

Pollen of most terrestrial orchids can germinate quickly in a solution of 0.3 to 0.5 M sucrose provided that the pollen is germinable and not defective. One can test for pollen germinability using the hanging drop technique, observing what happens when a small amount of pollen is placed in a drop of sucrose solution. To stop the drop from evaporating, some of the same solution is placed in the base of the petri dish and the lid with drop on its undersurface is placed on top. A range of sucrose concentrations is suggested as a preliminary step to confirm the dilution needed for pollen germination with the orchid you are investigating. Pollen should germinate at room temperature and within 36 hours, often sooner, so you do not have long to wait for results. If no germination occurs after 36 hours, you can conclude that the pollen either would not germinate in the particular solution used, or could not germinate for some other reason. The latter explanation is particularly true if no germination occurs over a wide range of sucrose dilutions.

Details

Mark a number of half-inch circles on the outside of the petri dish cover using a marker. Note the sample code beside each circle and the date, time, etc. on the dish. Within each circle place a small drop of the chosen sucrose solution. The drop must be small enough such that when you invert the lid, the drop does not fall off! Using the needles, tease a small amount of pollen from the pollinium and

place it in the drop. Do not worry if the pollen floats. Enough will be suspended in the drop. Some kinds of pollen are difficult to sample: pollen of the Yellow Lady's-slipper is sticky. I have found that two needles can be used to scape off a small sample into the drop. Remember that a small sample represents many thousands of pollen grains. Once all samples are in place on the lid, put about 5 ml (1 teaspoon) of the same sucrose solution in the dish. Invert the lid over the dish. Later, examine the drops through the dish lid using the low power of a microscope. The first stage of germination is the appearance of a finger-like projection, the pollen tube, from the pollen grain. Once germination starts, the pollen tubes will elongate and if there are many, become tangled. If you are counting the number of grains germinating, it is easier to do it sooner than later.

APPENDIX 2

Separating pollen grains of *Cypripedium parviflorum* var. *pubescens* from sticky matrix using various solvents.

It is extremely difficult to count pollen grains dispersed in a sticky matrix. We investigated the possibility of using solvents to dissolve or soften the matrix so that the grains could be separated and more accurately counted. With the exception of acetone, polar solvents were poor solvents. Moderately polar Brodie's Mixture (amyl alcohol/heptane) was adequate for separation and counting purposes. Pollen treated with solvents was not used in germination experiments.

Relative dissolution of pollen matrix by various solvents.

Water	Polar	No effect
1M HCl	Polar	Matrix swells but no grains released
1M NaOH	Polar	Matrix swells but no grains released
Methanol	Moderately polar	No effect
Trichlorethylene	Moderately polar	Grains separate from matrix
Toluene	Moderately polar	Grains separate from dissolving matrix
Hexane	Non-polar	Matrix dissolves releasing grains
Acetone	Polar	Grains separate from dissolving matrix

Table 1

Clonal compatability in two demes of *Cypripedium parviflorum* var. *pubescens*.

	Site 1						Site 2				
	Pollen from plant						Pollen from plant				
On	A	B	C	D	E	On	1	2	3	4	5
A	Y	Y	Y	Y	Y	1	Y	Y	Y	Y	Y
B	Y	*	*	n	n	2	n	n	Y	Y	n
C	*	Y	Y	*	Y	3	n	n	n	Y	Y
D	n	Y	*	Y	Y	4	n	n	n	n	n
E	*	*	Y	*	*	5	Y	Y	Y	Y	n

Table 2

Effect of storage conditions on *in vitro* pollen germination of differently aged flowers of *C. parviflorum* var. *pubescens*.

		Stored 25 days at:	
Age	Immediate	4°C	25°C
-2	31	90	0
-1	50,57	90,90	0,44
1	55,60	90, f*	10,48
2	46	f*	0
4	86	f*	10

* Pollen heavily contaminated with fungus. No germination.

Table 3

Variation of *in vitro* Pollen Germination (%) with Flower Age (Days Post-Anthesis) in *C. parviflorum* var. *pubescens*

Age	B	C	D	E	Y
2		59,41		1	28
3		70			
4		81			10
5	40		1	29,37	48
6	60,41	72,42			
	43	53,64			
7	55	42,18	40		
8		63		54	44
9	10			0	
10		38	9	0	
11			0		
12	5	19	0	1	
13		20		36	
14		40		27	5
15		30			27

Table 4

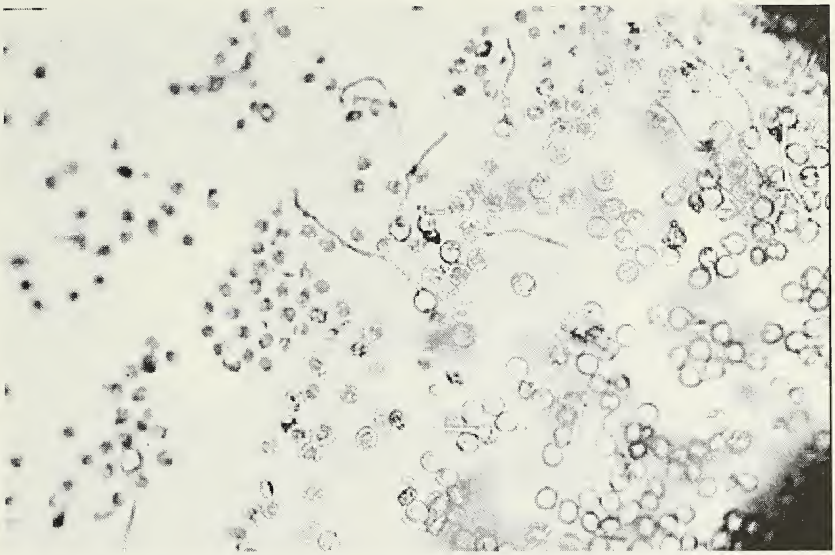
Effect of pollen recipient flower age upon fruit set in *Cypripedium parviflorum* var. *pubescens*.

Recipient Age (Days)	Pollen Donor B	C	D	E	Y	Total
2	0/2	4/3	0/1	0/1	0/1	4/8
4	2/2	1/3	2/1	1/1*	2/1	8/8
6	1/2†	2/3	1/1*	0/1	1/1	5/8
8	0/1	2/3†	0/1	0/2	0/1	2/8
10	0/2	1/3	0/1	0/1	0/1	1/8
Sub-totals (1st 10 days)	3/9	10/15	3/5	1/6	3/5	20/40
12	0/2	0/3	0/1	0/1	0/1	0/8
14	0/1	0/3	0/1	0/2	0/1	0/8

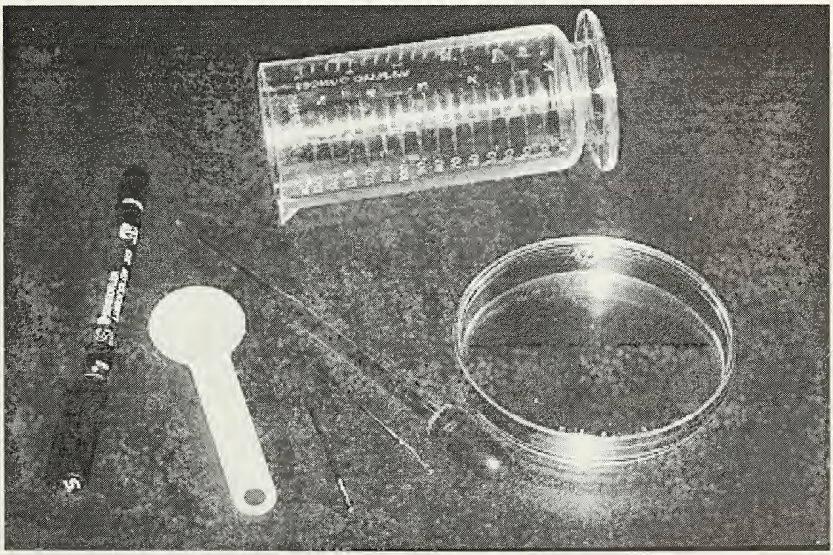
Recipient Age	Pollen Recipient					
(Days)	B	C	D	E	Y	Total
2	2/2	0/3	0/1	1/1	1/1	4/8
4	2/2	5/3*	0/1	0/1	1/1	7/8
6	1/2	1/3*	1/1†	0/1	1/1	4/8
8	1/1†	0/3	1/1	0/2	0/1	2/8
10	1/2	0/3	0/1	0/1	0/1	1/8
Sub-totals (1st 10 days)	8/9	6/15	2/5	1/6	3/5	20/40
12	0/2	0/3	0/1	0/1	0/1	0/8
14	0/1	0/3	0/1	0/2	0/1	0/8

* One capsule in each group greened up initially, and then aborted development within the first four weeks.

† One capsule in each group weevil infested, and thus not useable for seed germination study.

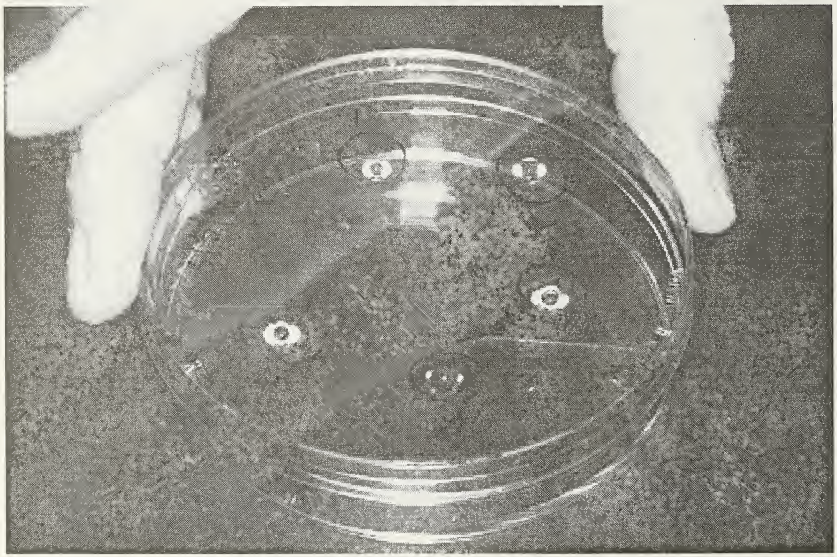


Pollen of *Cypripedium parviflorum* var. *pubescens* germinating after 12 hours incubation. Only a few pollen tubes have developed suggesting that this pollen should not be used.

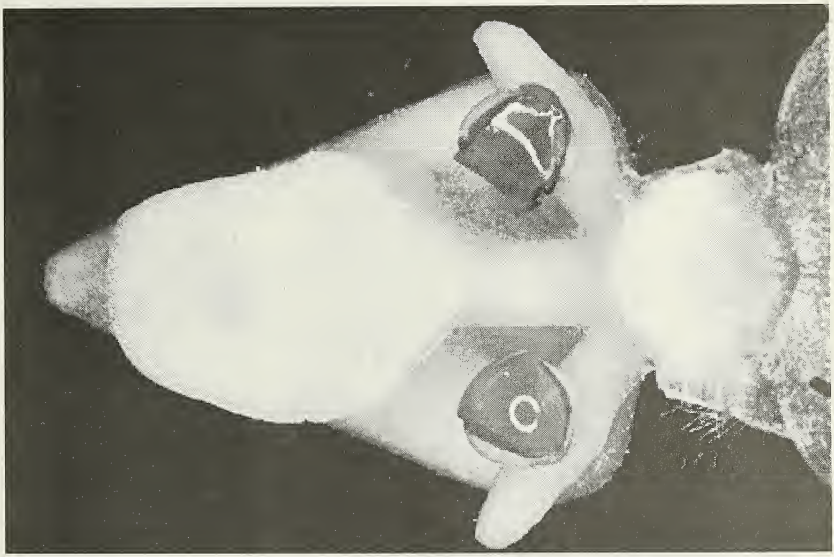


Equipment needed to assay pollen germinability.

First step in pollen germinability assay. Place drops of sucrose solution on petri dish lid.



Pollen samples in hanging drops of sucrose solution.



During a pollination visit, pollen may be deposited on any one of three parts of the stigma. Pollen must be sticky if it is to be effectively removed by an insect or human pollinator.

Sagittal section of a flower of *Cypripedium parviflorum* var. *pubescens* showing the position of the stigma, anther and ovary contents.

Cypripedium arietinum, Ottawa, Canada. Of the many fruits formed annually, only a few will contain mature seed. Weevils and other predators are primarily to blame.

The Natural Population Ecology of Temperate Terrestrials: Pink Lady's-Slippers, *Cypripedium acaule*†

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Abstract

Long-term field studies of nearly 6000 individuals of the pink lady's-slipper, *Cypripedium acaule* Ait, in Maryland and Virginia produced many surprising results, including: 1) persistent reproductive failure - only 5% of flowers naturally set fruit in the first 20 years, most individuals never flowered during the study; 2) deceptive pollination biology - no rewards were produced by the orchids or received by pollinators; indeed the trap-flower appears to be hostile to potential pollinators; 3) annual survival of 98% - mature plants are functionally immortal, and I infer that many individuals are older than the trees in the forest in which they reside; 4) subterranean life - a minority of plants disappeared underground and remained there for years, even decades, before reappearing above ground; and 5) lack of inbreeding depression - hand-pollination experiments that ranged from autogamous selfings to distant outcrossings produced equivalent germinations and protocorm growth in growth chambers. The features of repeated failure to set seed, and deceptive pollination biology are known in other species of orchids, especially the classic studies by Charles Darwin, and defy easy explanation in terms of adaptation and evolution by natural selection.

The extensive destruction of mature oaks and other canopy trees by gypsy moths, *Lymantria dispar*, in 1990-91 had a surprisingly positive impact on the pink lady's-slipper. There was a 64% increase in sprouting, a 6-fold increase in flowering, and the visitation by bumblebees jumped from 23% in 1989 to 75% 1992. Successful pollination increased from a previous per-year average of 2.6% to 20.7% in 1990. Other understory plants, especially nectar-rich ericaceous shrubs, became luxuriant and attracted bumblebees that then visited and pollinated the orchids incidentally.

My results indicate that pink lady's-slippers require sunny, open habitat conditions to stimulate flowering and attract the requisite pollinators. Conversely, the explanation for the repeated poor reproductive performance is that my study populations (and most stands in the species range) were trapped in heavily shaded, regenerating mature forests where pollinators do not regularly forage. The survivorship data indicate that my study populations are remnants from the last century when these plots of land were totally deforested. Fire must have been the natural disturbance upon which *C. acaule* depended for reproduction.

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† Key Words: *Cypripedium acaule*, deceptive pollination, fire, gypsy moth, human-altered landscapes, inbreeding depression, orchids, pink lady's-slipper, reproductive failure, survivorship

Introduction

The pink lady's-slipper, *Cypripedium acaule* Ait., is a common terrestrial orchid of the mixed coniferous-deciduous forests of northeastern North America. It ranges from Newfoundland and Quebec to Alberta south to New Jersey and northern Indiana, and along the coastal plain and Appalachian Mountains to South Carolina and Alabama (Gleason and Cronquist, 1963). Also known as the Moccasin-flower, the species is partial to acid, usually well-drained soils, such as sandy pinelands and dry woods on sandstone, but also occurs in swamps and bogs. While most (90%) individuals of a stand are non-flowering throughout the growing season, flowering occurs from April to June and individual flowers may last over two weeks. Individual mature plants flower erratically over their lifetime. Pollination is accomplished by large, globose, hairy insects, such as bumblebees (*Bombus* spp.) and their nest parasites *Psithyrus* spp. (Davis, 1986; Plowright *et al.*, 1980). Yet successful fertilization and seed set (fruit production) is very low in most stands in most years (personal observation; Cochran, 1986; Primack and Hall, 1990). For example, only 29 flowers out of 1078 were naturally pollinated in the first 13 years of a thorough study of a large stand in Virginia (see below).

My observations of repeated and widespread reproductive failure by the majority of plants in well-defined stands is puzzling in both ecological and evolutionary contexts (Gill, 1987). Perplexing ecological questions include: How does a population persist if no reproduction takes place? What is the cause of the reproductive failure? Are the plants infertile, or is there a failure of effective pollinator service? How can such a large, showy, exquisite and intricate flower not attract appropriate (bumblebee) pollinators? Are the appropriate co-evolved pollinators absent in contemporary habitats? Is *C. acaule* in the process of extinction? Can this orchid serve as a model for other orchid species that have low rates of fruit set?

The evolutionary questions raised by the observation of persistent reproductive failure in nature are even more perplexing. By definition, natural selection favors those individuals in a population that are most reproductively successful in their lifetimes. We expect most individuals of a species to be equipped with adaptations from past years of selection. Why, then, are the overwhelming majority of individuals failing to reproduce? Why has this species resorted to deception and entrapment of it pollinators rather than rewarding its visitors with nectar (Cochran, 1986)? Why is a self-pollinating individual not instantly favored by selection and why has selfing not replaced the failing, vector-dependent pollination system? After all, an individual with a genetically altered floral morphology that permits autogamy will have 100% reproductive success every time it flowers instead of the dismal 2.7% probability of success that contemporary individuals experience.

These questions were stimulated by observations from my two decades of field research on the pink lady's-slipper. My long-term field studies and experiments have provided answers to some of the questions. Here I report some salient results and my interpretations of them.

Site Descriptions

Field studies were conducted annually and thoroughly in three stands of *C. acaule* in Maryland and Virginia for one to two decades. In addition, several stands throughout eastern USA were examined intermittently for fruit set. The detailed, long term studies were sited at 1) Orchid Hill along Long Run Road in the George Washington National Forest, Rockingham County, Virginia,
2) Greenbelt National Park, Greenbelt, Maryland, and 3) Beltsville Agricultural Research Center (BARC), Beltsville, Maryland. The sites where casual records of fruit set (i.e. single mid or late summer counts of flower stalks and % fruits) were recorded were of two kinds: those visited by myself and those examined by enthusiastic colleagues. Such sites located were in Maine, Massachusetts, New Hampshire, glaciated and coastal localities in New Jersey, Piedmont and coastal Maryland, highland Pennsylvania, Ohio Michigan, Tennessee, and northern Alabama. All the stands were in mature, mixed deciduous-conifer forests with closed or broken (light gap) canopies.

The canopy of Orchid Hill was dominated by chestnut oak, *Quercus prinus*, and table mountain pine, *Pinus pungens*, with some tupelo, *Nyssa sylvatica*, Virginia pine, *P. virginiana*, and black locust, *Robinia pseudo-acacia*, and a few hickories, *Carya* spp., and scarlet oak, *Q. coccinea*. The woody understory was dominated by ericaceous shrubs, including blueberries, *Vaccinium* spp., huckleberries, *Gaylussacia* spp., mountain laurel, *Kalmia latifolia*, and fetterbush, *Leucothoe racemoa*, and bear oak, *Q. ilicifolia*. Tree ring counts indicate that the mature trees on Orchid Hill are in a uniform age bracket of 60-80 years; the oldest found was a 107 year-old *P. pungens*. Photographic records and/or verbal commentaries from long-time local residents confirm that, in all cases, the woodlots with stands of *C. acaule* are similarly aged and were either clearcut or active pastures in the first 2-3 decades of the 20th century. Pink lady's-slippers are found in mature forests which nearly always contain some conifers: in New England red spruce, *Picea rubens*, and white pine, *Pinus strobus*, are common; in the mid-Atlantic coastal regions Virginia pine, *P. virginiana*, pitch pine, *P. rigida*, and short-leaf pine, *P. echinata*, are associates; in Appalachia table mountain pine, *Pinus pungens*, and red cedar, *Juniperus virginiana*, are typical. Invariably, the ages of the conifers are 50-100 years.

Methods

Since 1978 over 6000 individuals of *C. acaule* have been monitored at the main study site at Orchid Hill, which lies at 736 m elevation on the crest of a spur ridge with north- and south-facing slopes that differ in ambient climate. A sample of plants were marked in 1977, but in 1978 the 0.5 ha area was gridded into 4m x 4m quadrats, and all orchid individuals (5721 as of 1996) were mapped. All plants were marked with 1 x 3 cm aluminum numbered tags, which were cut from beer/soft drink cans, embossed with an ordinary ballpoint pen, and anchored in the ground with a 20p galvanized common nail. Since 1978 annual censuses were conducted in May and June (throughout and after the peak flowering). The grid was traversed thoroughly, every previously marked plant (or tag) was relocated and evaluated, and every new plant was tagged, mapped, and recorded. Evaluations included presence or absence of above-ground leaves/flowers, vegetative or flowering states, state of development and color and condition of flower, presence

or absence of pollinia, presence or absence of pollen on the stigma, and measurements of length and width of the inner leaf and flower stalk, if present. The data were transcribed from field record sheets to individual record cards and then loaded onto a LOTUS 1-2-3 spreadsheet and analyzed by an IBM PC computer.

Only those plants that have ever flowered were tagged in the Greenbelt Park (353 as of 1996) and in two Beltsville stands (316 and 351 as of 1996). At these sites I used 11 cm, white plastic (Pylon Plastics, Inc.) plant markers, numbers written with indelible black magic markers. Progress of flower and fruit development was monitored daily or weekly.

This paper focuses on the results of the two decades study on Orchid Hill, VA. The contrast between the period 1977-1989 and the period 1990-1996 will be emphasized because a dramatic and abrupt change in the forest structure on the plots occurred in 1990-1991.

Results

Gypsy Moth Invasion - Throughout the 1970's and 1980's the study sites were unchanging, broken canopied forest with well shaded understories. In 1989 the first larvae and adults of gypsy moth, *Lymantria dispar,* were found on Orchid Hill and the two Maryland study plots, but foliage damage was inconspicuous. In May and June of both 1990 and 1991, the infestation of gypsy moths was tremendously destructive - the mature hardwoods and pines were completely defoliated, the canopy was totally opened, and much of the understory vegetation was stripped. Larval frass rained down from the canopy in May, a curtain of gypsy moth instars dangled (bungee-jumping) from their silk threads in June. By early summer of both years the forest floor was covered with larval frass and scores of larvae of other lepidopterous species that had been evicted from the canopy by the gypsy moth army. For the two successive summers of 1990 and 1991, the forest floor was exposed to full sunlight, and was hotter and drier than the previous decades. Only minor damage was done to *C. acaule* by the gypsy moth larvae during the invasion. Nevertheless, the results from the pre- and the post-gypsy moth periods were strikingly different.

Population Size - The size of all stands of pink lady's-slippers was predictable for the first twelve years of the study, but changed dramatically after 1990 (Figure 1). The number of visible/countable plants on Orchid Hill averaged 765.6 per year on the study plot between 1978 and 1989; only in one year (1980) did the countable number exceed 1000. A massive recruitment of new plants in 1990 and 1991 pushed the census counts to well over 1200 visible plants throughout the post-gypsy moth period; the peak of 1590 visible plants in 1992 was 64% higher than the 1989 census. The average population size of the Orchid Hill stand during the second (post-gypsy moth) period is obviously very significantly greater than the size of the same stand before the invasion.

Three census points (Figure 1) do not reflect the true sizes of the visible population: 1) the 1977 initiation - only 100 flowering plants and 100 large non-flowering plants were marked arbitrarily and temporarily with colored clothespins. I estimate the true number of plants in the Orchid Hill study plot in 1977 was at least 757, based on the 20-year average of 13.4% flowering; 2) the 1978 census - the first complete census was conducted in 1978, and it is possible the census value of

435 is less than the true value because I had not yet learned how to discover every minute seedling in the leaf litter and under vegetation; 3) the 1995 census - in 1995 an unwanted wildfire swept through the center of the study plot on the afternoon of 18 June, before the annual census was completed. The twenty-eight quadrats that were not censused in 1995 held 40.0% of the visible plants in 1994. Hence, I estimate the incomplete census number of 842 visible plants in 1995 should be increased by 40%, or to 1179 plants.

The actual population size of pink lady's-slippers was much greater than the visible, above-ground, countable plants: there are many plants living in an invisible, subterranean state. Besides the myriad protocorms (which are germinating seedlings that have not yet projected their first above-ground green leaf), many subterranean older plants were discovered as they appeared/disappeared/reappeared suddenly in successive censuses. They clearly subsisted underground without above-ground green foliage for years; most returned above ground after one year absence, but others have remained subterranean for nearly two decades. They are clearly a minority of the total population, but their actual numbers are difficult to determine because some are still reappearing for the first time in two decades and those that have disappeared and not yet reappeared may be dead or alive.

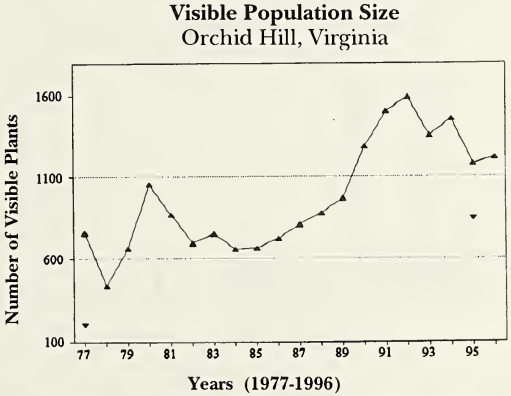

Figure 1. Population size of the main stand of pink lady's-slipper Orchids, *C. acaule*, at Orchid Hill, Rockingham County, Virginia. Numbers depict the plants visible above-ground; portions of the actual population are alive but underground and invisible. Gypsy moths invaded the study site and destroyed the canopy of the forest in 1990 and 1991. Population size increased significantly in the years after 1990.

Population Structure and Survivorship - Throughout the two decades of the study the majority of pink lady's-slippers in all study sites were non-flowering. For example, on Orchid Hill 86.6% of 18,620 plant-years were non-flowering. Two-leaf plants outnumbered one-leaf plants only slightly, 1.09 to one. New plants (evidenced by the first appearance of a single small green leaf at an unmarked spot in the quadrats) usually measured 40-60 mm in length. The largest plants had leaves over 260 mm in length, over 150 mm, and flower stalks over 400 mm tall. Individual genets were distinguishable but a minority of plants produced subsidiary buds and multiple pairs of leaves and flower stalks; vegetative plants with

three or more leaves were rare.

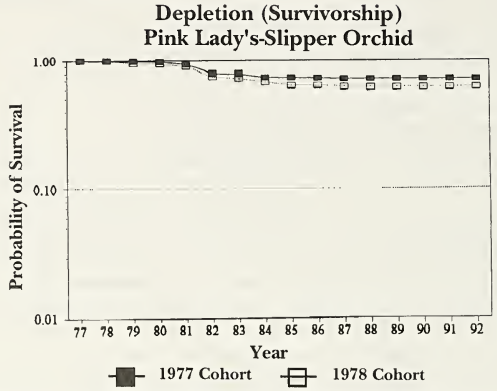

Figure 2. Per cent survival of 170 pink lady's-slippers, *C. acaule,* marked in 1977, and 266 plants first marked in 1978 at Orchid Hill. Because the cohorts are mixed ages and lineages, the curves are depletions rather than age-specific survivorship.

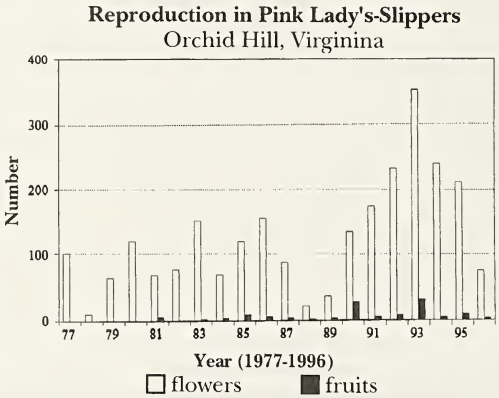

Figure 3. Flowering and fruiting in the main stand of Pink Lady's-Slippers, *C. acaule,* at Orchid Hill from 1977-1996. Most flowers failed to be pollinated and failed to set fruit. Gypsy moths invaded the study site and destroyed the canopy of the forest in 1990 and 1991. Significant increases in the number of flowers and fruits occurred in the years after 1990.

Pink lady's-slippers are long-lived. Every year familiar plants with their labeled tags were predictably in their mapped places to be counted. The plants with the longest records from which annual rates of survival could be calculated were the cohorts of plants marked in 1977 and 1978. Because the plants in these cohorts are of mixed ages and lineages, their mortality generates *depletion* rather than strict *survivorship* curves (Figure 2). The near perfect annual survival rates (over 97.8% for 1977 cohort; 96.5% for 1978 cohort) are clearly depicted in the flat trajectories of the depletion curves. Most of the plants (72% and 61%, respectively) that I marked twenty years ago are still alive and prospering.

Reproduction - At Orchid Hill the average number of flowers per year was 83.4±45.6 (x ± Std. Dev.) during the 13 years before the gypsy moths and 202.9±87.5 for the 7 years after the gypsy moths arrived, an increase of 243% (Figure 3). The average across the two decades was 125.2±82.4 per year, with a low of 9 flowers in 1978 and a high of 353 flowers in 1993. It appears there was a dramatic increase in flowering following the gypsy moth invasion, it reached a peak in 1993, but the number of flowers returned to pre-invasion values by 1996. Many of the newly flowering plants were dwarf in stature.

Most astonishing was the repeated poor fruiting rates in all of my study stands (Figure 4). On Orchid Hill not a single flower set fruit by natural pollination in the first four years (1977 - 1980) of the study. By 1989 only 29 fruits had been produced by natural pollinations from 1078 flowers in 13 years (x = 2.23±2.77 fruits/ year). During and after the gypsy moth invasion, fruiting increased significantly, yielding 97 fruits from 1420 flowers in 7 years (x = 13.86±11.68 fruits per year; t = 3.416, df = 18, P< 0.01). The extraordinary fruit productions in 1990 and 1993 accounted for the significant differences - the fruitings in the intervening and subsequent years were similar to the fruiting rates in the 1980's. These same patterns were observed at all the other study sites.

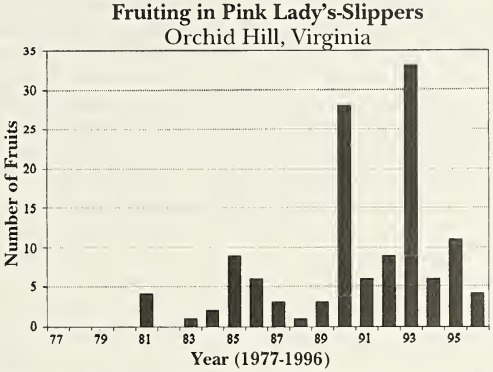

Figure 4. Number of fruit pods produced by the main stand of Pink Lady's-Slippers, *C. acaule*, at Orchid Hill over the two decades 1977-1996. Significant increase in fruiting occurred after the gypsy moth invasion in 1990.

There was a very highly significant positive relationship between the number of flowers and the number of fruits set in a given year over the 20 year study period ($b = 0.6815$, $r^2 = 0.4644$, $p<0.0009$; Figure 5). Clearly, larger floral displays lead to more fruit set, and defoliations of forest canopies produce larger floral displays in *C. acaule* (Figure 3).

In an effort to determine the cause of the failure of flowering plants to set fruits, I tested for fertility by performing hand-pollination experiments in 1981 and 1986. In 1981 all 10 out-crossed flowers and all 10 autogamously self-pollinated flowers set full-sized fruits (100%) while only 3 of 20 unmanipulated controls produced fruit. In 1986 I repeated the experiment with 12 natural controls, 5 sham hand-pollination controls, 12 allogamously and 12 autogamously hand-pollinated flowers. None of the controls set fruit, but 21 of the 24 hand-pollinated flowers set fruit. Clearly, if stigmas receive conspecific pollen of any

kind, full fruits are produced. Under normal ambient conditions pollen was not delivered to stigmas.

In later hand-pollination experiments the relative quality of seeds produced by self-pollinations were compared to outcrossed pollinations. Equivalent rates of germinations and protocorm growth were found in both sets of seeds. There was no evidence of inbreeding depression, or negative genetic consequences of self pollinations in *C. acaule*; the full details will be reported elsewhere (Gill and Stoutamire, in preparation).

Visitations to *C. acaule* flowers by bees are rare. If a robust-bodied bee entered a flower (labellum) and brushed by the stigma and anthers, it left the telltale clues of missing pollinia and reflexed anther caps; actual pollination was evidenced by pollen visible on the stigma. The overwhelming majority of flowers (70-80%) had no signs of visitation - they continued to possess both pollinia and intact anther caps until their brown, wilted flowers dropped. Of those that had reflexed anther caps, only 5% or so had both pollinia missing, giving evidence of at least two visits. The best ever flowering occurred two years after the gypsy moth invasion: in 1993 half of the flowers were visited, 80 (23.4%) had both pollinia missing, and the most fruits (33) were produced.

Examination of the pink lady's-slipper flowers confirmed that there are no floral rewards for flower visitors. *C. acaule* no nectaries and no production of floral nectar (Davis, 1986; Cochran, 1986). The anthers are sequestered in the inner recesses of the flower and protected from the outside by a hard cap. Fragrance is usually absent or weak, and descriptions vary greatly according to time of day and locality but mostly among noses. I have seen queen bumblebees approach flowers and turn away, but I have yet to witness an entry into the flower.

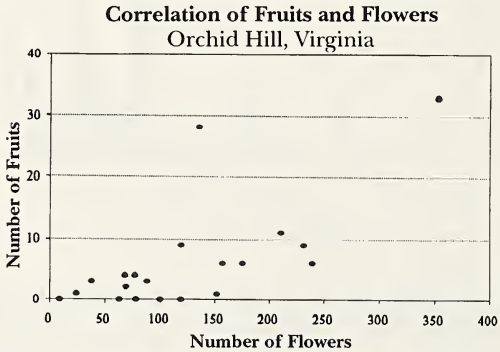

Figure 5. Correlation of number of fruits and number of flowers in the main stand of pink lady's-slippers, *C. acaule*, at Orchid Hill from 1977-1996. The correlation is significantly positive and accounts for 46.4% of the variation.

Discussion

Reproductive Performance - The long-term observations and experimental studies reported here provide new answers to the questions regarding the cause of the widespread and repeated pattern of reproductive failure in stands of *C. acaule*. The serendipitous invasion and destruction of the mid-Atlantic forests by gypsy

moths in 1990-91 severely altered forest conditions that, in turn, precipitated beneficial changes in the breeding biology and population ecology of pink lady's-slippers. It was the long-term feature of these studies that revealed the answers; had the studies been of much shorter duration, the context and magnitude of the changes wrought by the gypsy moths would have been missed.

The hand-pollination experiments showed unambiguously that the plants in this study were fertile and self-compatible, and the failure to set seed was due to ineffective pollinator service. When I provided stigmas with pollen from either distant plants or self, the flowers produced fruits full of seeds. Obviously, natural pollinators were not delivering pollen to flowers as well as hand-pollinations. Although bumblebees were seen occasionally in the mature forests, they were evidently foraging elsewhere and only rarely visited pink lady's-slipper flowers.

My observations of persistently low percentages of fruit set in stands of pink lady's-slippers during the first 13 years of this study are consistent with the experience of other workers studying *C. acaule* in other localities, including Tennessee (Cochran, 1986), Massachusetts (Davis, 1986; Primack and Hall, 1990; Primack *et al.*, 1994) and New Brunswick (Plowright *et al.*, 1980), as well as other species of orchids (e.g Darwin 1877, Ackerman and Montalvo, 1990; Calvo, 1990, 1993; Bartareau, 1995; Whigham and O'Neill, 1991). The cause of the poor fruit set in some other orchids has often been found to be poor pollination rather than resource limitation (e.g. Zimmerman and Aide, 1989; Montalvo and Ackerman, 1987).

The fact that *C. acaule* has a deceptive pollination system doubtless played a role in the low visitation rates. Like many other species of orchids (Bartareau, 1995, Dafni, 1983, 1984), pink lady's-slipper flowers offer no rewards - there are no nectar glands and no nectar production, the pollen is inaccessible to pollen-gathering insects, and visitors do not gather fragrant terpenoid scents. Davis (1986) describes in detail the deception.

The effect on bumblebees of the non-reward and entrapment must be negative and discouraging to revisitations and hence, no delivery of pollen. Bumblebees are quick to learn to reject such non-rewarding stations in subsequent foraging bouts - the speed of rejection learning in bumblebees is measured in hours and 1-2 non-rewarding visits (Heinrich, 1979; Ott *et al.* 1985; D. Inouye, personal communication). As a consequence, it is easy to imagine how the entire small population of queen bumblebees in an acre of forest learns to reject pink lady's-slipper flowers in a few days. Most of the visits occurred at the beginning of the flowering season in 1993 (Gill, in prep.). Hence, the negative experience of visiting *C. acaule* flowers actually discourages the pollinator and generates poor visitation rates and ineffective pollination.

By changing the forest conditions, the gypsy moth event revealed three additional, perhaps more important factors that explain why reproduction in pink lady's-slippers is so low in mature woodland settings. The first is the shade of canopied forests. Flower visitations tripled and fruiting quadrupled when the study site became open and sunny after the gypsy moths destroyed the forest canopy. Bumblebees and other species of Apidae avoid shaded habitats and are strongly attracted to sunny, open patches of land (Heinrich, 1979). Thus, the plants themselves were in unattractive, namely shaded, habitat for the pollinators.

A second explanation for the traditional poor reproductive performance is relatively small floral display. The number of flowers in the stand increased 600 %

after the gypsy moth invasion. Bees are attracted to large floral displays, especially those in sunlight. My results show that fruiting is directly correlated with flowering. It is likely that the enlarged floral display after 1990 was itself directly caused by the increased sunshine. Adequate light is commonly a stimulus for flowering. Hence, the sparse flower displays of *C. acaule* before the gypsy moth invasion not only were responsible for fruiting but were themselves a response to the shade.

The third explanation for the poor visitation rates during the pre-gypsy moth years is that other understory plants, especially nectar-producing ericaceous shrubs, were flowering poorly in the shade. After the gypsy moth defoliation, the nectariferous shrubs became luxuriant and attracted bumblebees to the study plots. Although no measurements were taken to document the magnitude of the prolific flowering in the abundant blueberries (*Vaccinium* spp.) and huckleberries (*Gaylussacia* spp.) after the gypsy moth invasion, it was noteworthy in my field notes. Bumblebees were conspicuously more common than they had been during the previous decade, and were observed visiting the ericad flowers. I speculate that in their frenzied state of receiving rewards from the profusion of nectar-producing flowers on the sunny hillside after 1990, bumblebees that visited pink lady's-slipper flowers were less discouraged than they were normally in the shaded, flower-poor forests. Pollination in deceptive orchids succeeds when the deceptive species are embedded in fields of other rewarding flowers (Dafni, 1983, 1984).

These results clearly indicate that reproductive performance by *C. acaule* greatly improves in sunny habitats. Ecological disturbances that transform shaded forests into open sunny fields apparently are necessary for reproduction in the pink lady's-slipper. In addition to the enhancement of flowering and fruiting, opening of the forest canopy also increased population recruitment and growth. Conversely, flowering, fruiting, and pollinator activity are actually suppressed in the shaded understory of mature forests. Whereas survival and growth seem to be excellent in the shaded forest habitats, pollinator service is poor. The reason for the widespread pattern of reproductive failure in this common terrestrial orchid is not due to the absence of coevolved pollinators. The pink lady's-slipper is a species requiring light gaps for its reproduction, and its abundance and distribution therefore depends on the dynamics of disturbance patches in forests.

Long-term Survival - One major reason populations of *C. acaule* persist when little or no reproduction takes place is the incredible survival rates. At the extraordinarily high rates of annual survival of 98%, it takes about 34 years to lose half of these orchids to mortality. It is quite possible that they were alive at the turn of the century. Given the current survival rates, the 436 plants I marked in 1977-78 would be a 15.6% remnant of a population of 2800-7100 plants alive in 1900 on Orchid Hill. Conversely, if current rates of survival continue, I can expect 218 of those plants to be alive still in the year 2026.

My estimates of the rates of survival are bound to increase as more plants re-emerge from their subterranean states of dormancy. I have hundreds of records of labeled plants reappearing as green, above-ground plants after years of absence; often they are large in stature and sometimes even flower immediately. As more of these "old" plants reappear in future years, the actual rates of annual survival will increase above the amazing 98% depletions that mixed-aged cohorts already exhibit. Compared to the surrounding forest age, estimated from tree ring analyses, *C. acaule* appears to be longer-lived! Individual plants are functionally

immortal, many individuals are older than the trees in the forest in which they reside. Hence the orchids were on the study plot before there was a forest there, i.e. at the time the entire region was clear-cut and deforested.

Taking the two conclusions together, that reproduction in *C. acaule* is suppressed in mature shaded forests and enhanced in open, sunny habitats, and that the plants are extraordinarily long-lived in forests and apparently older than the trees in most current woodlots, I come to the conclusion that *C. acaule* is a species superbly adapted to dwell in a state of suspended animation in regenerating and mature forests waiting for the next destructive disturbance, waiting for the next light gap. It is relatively indifferent to the regular parade of warm growing seasons and cold winter dormancies that march by for decades; what is important is the advent of a major disturbance that creates a light gap, even if its takes a century.

Temperate Orchids in Human-altered landscapes - If major forest disturbance and open sunny habitats are the natural requirements of *C. acaule* for its successful pollination and reproduction, what was the natural ecology of this species 500 or thousands of years ago, prior to the arrival of white Europeans? And what was the principle agent of forest opening in pre-Columbian times? These studies suggest that *C. acaule* was fundamentally dependent on fire-generated light gaps in the forest. How abundant and widespread the species was during pre-Columbian times would have depended on the frequency and extent of forest fires. Much interest in the importance and impact of anthropogenic fires, those set by native Americans as part of the cultural practices, on American natural ecology and environmental management has been renewed (Blackburn and Anderson, 1993). Sieg and King (1995) and Pleasants and Moe (1993) have drawn similar conclusions to the status of threatened species of orchids, especially *Platanthera praeclara*, in the American prairies.

In a recent, brief article, Primack (1996) suggested that the pink lady's-slipper was a rare plant at the beginning of the 20th century, and has been making a slow but dramatic recovery over the past several decades. He attributed the rarity one hundred years ago to destructive effects of clear-cutting and imposition of agricultural practices on the landscape. Similarly, Keenan (1994) expressed concern about the negative effects of modern development of stands of *C. acaule.* He captioned one photograph of an open field full of flowering pink lady's-slippers "when a woodland in Milton, New Hampshire was cleared for a garden, dozens of *Cypripedium acaule* persisted."

The results of these studies lead to a different conceptual picture of the past, present, and future ecology of *C. acaule.* We agree that the historical, anthropogenic alterations of the ecological landscape are chiefly responsible for the present-day abundance, distribution, and ecological behavior of pink lady's-slippers. I hypothesize, however, that the extensive, "catastrophic" deforestation of North America done by the white European settlers in the 18th and 19th centuries actually had a positive effect on the reproduction of these orchids by virtue of placing them in open fields, pastures, abandoned farmlands, etc. There they reproduced well, hurled vast quantities of seed onto the ground, and produced large populations. These stands were then engulfed in secondary, pine/conifer-

Cypripedium acaule growing in bright light. Note the dwarf stature and light green leaves.

filled regenerating forests as the tracts of lands were abandoned. The individual plants in current stands of *C. acaule* are themselves remnants of those large populations from the last century. The stands have remained trapped in these forests throughout the 20th century as policy/ attitudes promoted mature woodlots, National Forests and Parks were created, and forest fires were suppressed.

It may be that the recovery of pink lady's-slipper populations in New England of which Primack (1996) speaks are the consequence of repeated destructions of patches of maturing forests by recurrent outbreaks of gypsy moths themselves. Gypsy moths were accidentally introduced into the Boston area of Massachusetts exactly 100 years ago, and have undergone several cyclical outbreaks during this century (Barbosa, 1978). Thus New England forests and woodlots, even in suburban areas, were subject to frequent light-opening events similar to the one I observed in Virginia and Maryland in 1990-91. Large quantities of seed may have been produced, and large scale population recruitments could have occurred every decade during this century.

That many of the newly flowering plants after 1990 were dwarf in stature, leaves half-sized and flower stalks very short, was surprising. At first I thought they were abnormal, but then considered the possibility that it is the tall, robust stature of old stands commonly seen in contemporary shaded forests that is the "unnatural" stature. Excessive leaf size and gigantism is often seen in other plants (e.g. *Quercus* leaves) when young plants grow in severely shaded habitats, such as the understory of mature closed canopied forests. In the orchids large size might be the consequence of prolonged (many decades) entrapment in shaded forests. This interesting possibility needs to be investigated experimentally.

Thus, the data that I have gathered over the past 20 years, such as repeated reproductive failure in mature "pristine" forest, is more a reflection of the recent practice of fire suppression of fire in America than the expression of adaptive evolution of the species in its natural habitat. To test this idea, the National Forest Service of the Dry River District in Virginia and I cooperatively performed a prescribed burn in the George Washington National Forest on 22 April 1996. We will eagerly monitor the results over the next decade.

Is *Cypripedium acaule* in the process of extinction? Is *C. acaule* a model for other orchid species that have low rates of fruit sets? Should prescribed burning be a regular/required ingredient of forest management policy for the preservation of rare and endangered species, especially temperate terrestrial orchids?
C. acaule has a deceptive pollination system. Answers to these questions, my speculations and other evolutionary questions about the adaptive nature of the reproductive biology and ecology of *C. acaule* are discussed in greater detail elsewhere (Gill, in prep.).

Conclusions

After a gloomy 13-year history of sporadic seedling recruitment, sparse flowering, poor pollinator service, and poor fruiting in shaded forests, pink lady's-slipper orchids responded positively to the sunny conditions created by gypsy moths in their invasion of 1990-91. The open forest conditions apparently stimulated sprouting of seedlings, provoked exceptional flowering, tripled visitation by bumblebees, and increased fruit production. I interpret my startling

observations as evidence that the orchids were released from shade suppression by this major ecological disturbance. Although the agent of the 1990-91 defoliation was an exotic pest, I infer that the event mimicked the effects of natural disturbances, such as forest fires in pre-Columbian times. Because my marked plants exhibited astonishingly superb survival rates under all forest conditions, I surmise that my study populations of pink lady's-slippers are remnants from populations that flourished in the last century when these plots of land were deforested and converted to pastures by humans. I speculate that the natural ecology of this species, and quite possibly many other terrestrial temperate orchids, depends on forests gaps for their reproductive success. Success of management programs for the preservation of terrestrial orchids in their natural habitats probably requires regular prescribed burns.

Acknowledgments

The National Forest Service, Dry River District, has graciously issued permits and access to the study area. District Rangers George Blomstrom and Terry Slater were especially helpful. Funds from National Science Foundation grants DEB 77-48817, DEB 78-10832, DEB 80-05080, and BSR 86-05197 provided support. Numerous students and colleagues, including K. A. Berven, B. A. Mock, R. N. Harris, T. G. Halverson, C. D. von Dohlen, P. A'Hearn, Kevin Quinlan, N. A. Bourg, and C. Bennett, have generously provided field assistance. N. Bourg and C. Bennett kindly read and improved the manuscript.

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Propagation and Conservation of Native Lady's Slipper Orchids (*Cypripedium acaule, C. calceolus, and C. reginae*)

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Introduction

With their attractive flowers and hardy growth habit, native Lady's Slipper Orchids of the genus *Cypripedium* are receiving increasing attention from orchid hobbyists, horticulturists , and commercial growers as well. There is an increasing demand for species in this genus, especially *C. acaule, C. calceolus, and C. reginae*, as specimens in home landscape. Also, with their dormant growth habit and relatively simple flower forcing, Cypripedium can be easily adapted to pot culture and for use as a cut flower. Propagation of Cypripedium was not seriously studied until a decade ago. Most of the published papers and reports focused on seed germination. Yet, even now seed germination and seedling growth are still problematic. In this paper, we intend to summarize the results from research on Cypripediums we have conducted at Cornell in the past 5 years with the emphasis *on C. reginae, C. acaule, C. calceolus var. pubescens, and C. calceolus var. parviflorum.*

Seed Germination

Flowers of Cypripedium species were hand-pollinated and capsules were collected just prior to dehiscence. Seeds collected from these capsules about 120 days later were sterilized in 10% Clorox solution with Tween-20 for 15 minutes, followed by rinses 3 times with sterilized distilled water, and then placed on liquid or agar solidified germination media. Cultures were incubated at 23°C in dark. Seeds began to germinated in 4 weeks in liquid suspension culture (SLC) or in 6 weeks in agar culture. Of the media tested, we found 1/3 strength Murashige and Skoog (MS) medium with 100 ml/l coconut water at pH 6.0 gave the best germination and seedling survival compared to Knudson C and Vencin & Went media. All four local species, i.e. *C. reginae, C. calceolus var. pubescens, C. calceolus var. parviflorum* and *C. acaule*, germinated well in this medium with germination percentage of 85% to 98%. *C. californicum* (germination percentage 18 %), *C. kentuckiense* and *C. molle* were also tested. The germination of these species were much lower possibly due to seed damage in the mail.

An important finding of our work was that liquid suspension culture of seeds resulted a high and relatively synchronized germination in C. calceolus and *C. acaule* without 8-week pre-chilling at 5°C. SLC can be used to shorten the time needed for seeds to germinate compared to traditional agar culture. Seeds began to germinate in 4 weeks after sowing compared to 6 weeks or 12 weeks for seeds sowed on agar medium with or without pre-chilling respectively. Protocorms developed further into large seedlings for up to 7 months in the same incubation

vessel before browning occurred. Another advantages of using liquid suspension to germinate seeds are (1) simplification of the seed sowing process, (2) less labor and less skill involved, and (3) easier to separate germinated protocorm or young seedling after rooting. However, the disadvantages are that an orbital shaker is necessary, and more incubation space is need to germinate the seeds.

Suspension culture is a very effective way to germinate *Cypripedium calceolus* and *acaule.*

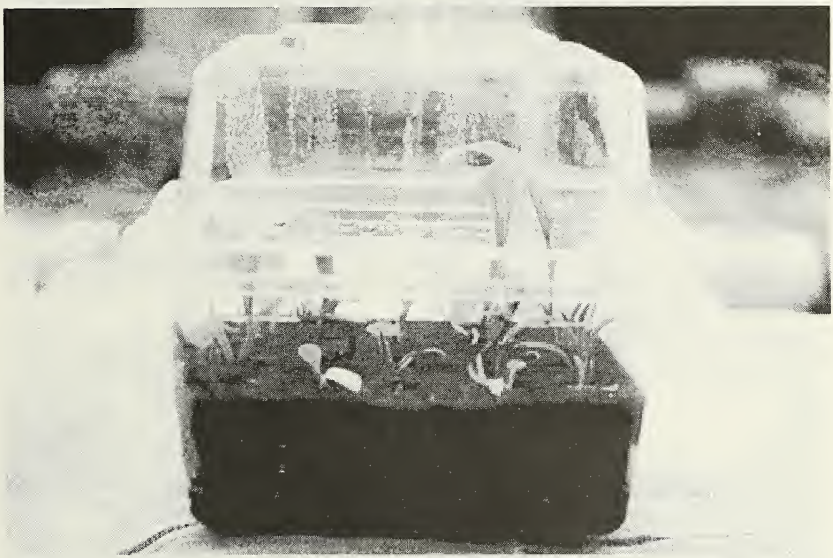
Darkness is crucial for seed germination and early development of seedlings. Adding 100 ml/l of coconut water to medium significantly increased germination and reduced browning. An 8-week 5°C pre-chilling to agar culture greatly stimulated germination, shorten germination period, and synchronized germination.

For germination of *Cypripedium* seeds and early seedling development, we recommend 1/3 strength of MS containing 100 ml/l coconut water, 15 g/l of sucrose, and 7g/l of agar with final pH of 6.0 prior autoclaving. Seeded plates should receive 8 weeks of 5°C pre-chilling treatment after sowing, followed by incubation at room temperature in dark. Germinated protocorms should be transferred before first root elongates beyond 2 cm to prevent roots of seedlings from intertwining with each other and browning. After transfer, young seedlings can be incubated at the same temperature with very dim light condition of about 3000 lux (fluorescent light).

In Vitro *Rockwool Block System for Seedling Growth*

High out-planting mortality has been a major problem associated with Cypripedium seedling production. To increase survival and reduce the impact of out-planting, and eliminate the laborious process of removing roots of seedlings from agar, an *in vitro* rockwool support system was developed in our lab. A 5 cm x 5 cm rockwool block was cut and inserted into Magenta G-7 box followed by a 30-minute autoclave for sterilization. Fifty ml of cooled autoclaved liquid medium

containing 1/3 strength of MS, 100 ml/l of coconut water, and 20 ml/l of sucrose, pH 6.0, was pour into each Magenta box. Five protocorms with a root initial (stage 4 seedling) were transferred to each rockwool block, and incubated at 23°C under 3000 lux cool white fluorescent light. Seedlings developed very vigorously in this system and had much higher survival (both *in vitro* and *ex vitro*) compared to those transferred onto agar medium. seedlings often developed larger dormant buds with a very strong root system . The advantage of this system are (1) higher survival *in vitro* and after out-planting, (2) stronger seedlings, (3) elimination of laborious agar separation from roots, (4) reduction of damage and impact to the root system at time of out-planting, (5) overall simplification of out-planting process: simply leach out sucrose from rockwool block with tap water and "plug" into potting materials.



First year seedlings of *Cypripedium reginae* growing on rockwool block in a Phytatray tissue culture box from Sigma Co.

This *in vitro* rockwool block system is far superior to traditional agar system and may is a key solution to overcome high mortality of Cypripedium seedlings in out-planting.

Out-Planting of Seedlings in Greenhouse and in Natural Habitats

Greenhouse

We have tested various out-planting techniques on several media including sphagnum moss, peat, sand, perlite and their mixture. Agar-based or rockwool-based seedlings of different developmental stages were used in all tests. Seedlings were placed on mist benches under 70% shade with a brief misting once every 30-minute. Dormant seedlings often broke dormancy in 4 weeks after out-planting.

Rockwool -based seedlings repeatedly had very high survival (often 100%), while the survival of agar-cultured seedlings varied from batch to batch, ranging from 0 to 80%. The better performance of rockwool-based seedlings maybe due to stronger buds developed in this system and much more vigorous and intact root system. Also, rockwool-based seedlings were easier to handle and took less time when planting.

Rockwool block system is an ideal system for out-planting *Cypripediums*. Picture shows dormant *Cypripedium calceolus* var. *parviflorum* seedlings on rockwool blocks just being taken out of tissue culture containers.

2-year-old seedlings of *Cypripedium reginae* on rockwool blocks at about 1 year after out-planting.

Sphagnum moss was the best of the media we have tested. Seedlings planted in sphagnum moss had better survival, especially for agar-cultured seedlings. This may due to its ability to maintain a more constant humidity in the root zone which is crucial for the survival of seedlings.

Out-planted seedlings of *Cypripedium calceolus* var. *pubescens* in their natural habitat.

Natural Habitats

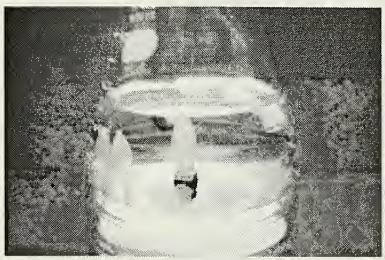
In order to test the possibility of recovery the population in an existing colony or to restore a colony in natural conditions, we have out-planted lab -germinated seedlings in their natural habitats. This experiment was initiated three years ago. The results suggested the possibility of reintroducing seedlings to their natural habitats either to stabilize a declining population or to restore a lost colony. Rockwool-based seedlings has a better survival in the wild. The first year survival was 90%, followed by 70% in the second year and 60% in the third year. Agar-cultured seedlings often disappeared in two years and were difficult to plant and trace in the field.

Asexual Propagation

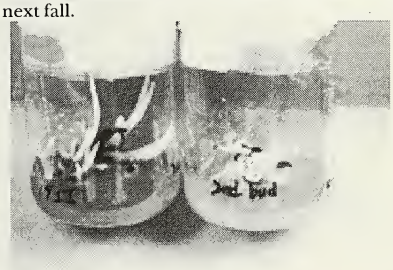
In order not to waste plant materials we collected for various purposes, We launched an attempt on asexual propagation from dormant lateral buds of Cypripediums and have had satisfactory results. Dormant buds of Cypripediums were excised in fall and cleaned with detergent and tooth brush. The outer two scales of dormant bud were peeled off in laminar flow hood and the bud was than sterilized in 10% Clorox solution with a few drops of Tween-20 for 15 minutes. After rinsed three times with sterilized distilled water, shoot tip, lateral buds and stem sections of about 3 mm were carefully excised and placed on media in test tubes containing 1/3 strength of MS, 100 ml/l of coconut water, 20 g/l of sucrose, 7 g/l of agar, and 0, 2, or 5 ppm of 6-Benzylaminopurine (BAP). pH of the media was adjusted to 5.5 prior to autoclaving. Five replicates were made for each medium. Cultures were incubated in dark at 23°C. Contamination was rare due to the nature of well protected dormant bud.

A *Cypripedium acaule* plant with a well formed dormant bud.

A dormant bud contains a flower bud for next Spring and 2 to 3 lateral buds, on of the lateral buds will become a well defined dormant bud next fall.



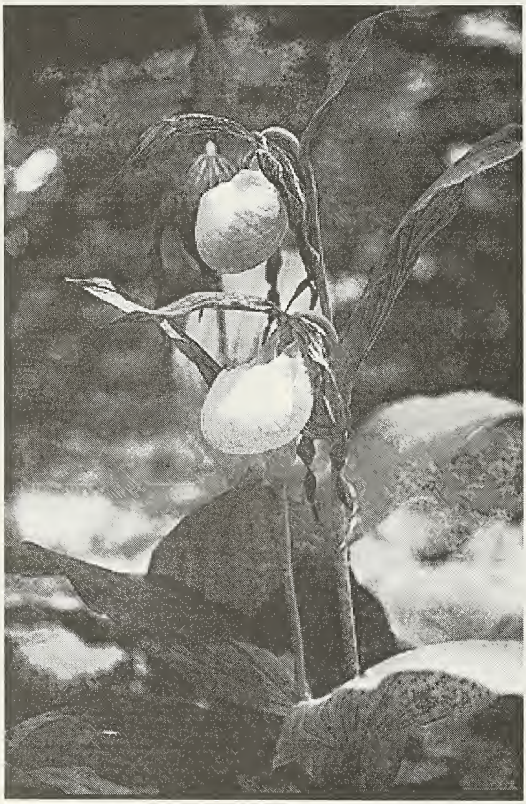
Dormant buds of *Cypripediums* are easy to excise and sterilize.



Larger lateral bud (left) tends to have more shoots than smaller lateral bud (right) from the same dormant bud under the same cultural conditions.

Explants swelled and elongated in 2 weeks. With few exceptions, shoot tips and upper stem sections failed to produce shoots. Lower stem sections produced roots and 0-3 shoots in 3 months. Lateral buds generated 1- 8 shoots and had the best and most predictable results. The most satisfactory result was found on medium containing 5 ppm of BAP. All the shoots generated in culture developed

into dormant buds and can be used for further multiplication without breaking dormancy. When out-planted, dormancy can be broken with a 8 weeks of 5°C treatment. Plants often bloomed in the second or the third years. This asexual propagation protocol could be valuable in mass propagation of field selected individuals or artificially bred varieties of *Cypripedium.*



Cypripedium kentuckiense, photo courtesy Paul Martin Brown

A Challenge to Propagation and Production

Paul Martin Brown
North American Native Orchid Alliance

In 1986 only very few people were engaged in trying to unlock the secrets of propagating our native orchids. As each year passed more names of propagators surfaced and more secrets were unlock to enable them to grow a wider range of species. In 1996 I would estimate that there are more than one hundred persons engaged in growing our North American natives from both seed and with micropropgation techniques. These people vary from professional propagators with elaborate labs to individuals who are growing at home.

Because many of the need to make this business profitable many of the growers are very successfully propagation large quantities of *Cypripediums* and *Calopogons* - two very show and desirable genera.

My challenge to all of you is to go on and beyond lady's-slippers and grass pinks and apply the knowledge you have assembled to some of the other deserving genera. Perhaps not as showy or, at this time popular, but deserving nonetheless.

Why? Because the ultimate solution to preserving our native orchids in the wild is to be able to satisfy the perceived need to grow these plants in our home gardens. They certainly deserve to be grown - but not raped from the wild. In addition, as new species are discovered the time should be taken to immediately try to propagate them to assure that they will not be overcollected and when or if, federal protection is afford these newer species, than they will already be in production and the controversies and difficulties of obtaining propagating material and disseminating plants will be greatly simplified.

I present to you a list of North American native terrestrial orchids that I feel are excellent candidates for propagation for any of several reasons. Take these suggestion seriously and give fair consideration to some of these species. Many of them are quite beautiful and all are captivating in their own special way.

My first candidate is the common **downy rattlesnake orchis**, *Goodyera pubescens.* Heavily overcollected for berry bowls and terreria, this is a species that grows easily in the woodland garden, propagates from both seed and division, and is very attractive. If tissue culture were a possibility there are certain clones in the wild that are exceedingly attractive.

Any number of the *Spiranthes* are desirable candidates (although I will admit I am prejudice!). Most grow quickly from seed, are no fussy to growing conditions and flower prolifically. This is certainly a candidate to satisfy the publics desire for 'wild orchids' in their garden and should be both easy and economically practical.

The showy and curiously colored, **lily-leaved twayblade**, *Liparis liliifolia,* is

certainly a species that grows with the greatest of ease in a good rich garden soil with a little shade. Plants have been sold or traded over the years and have done very well in cultivation. But all of these had to have come from the wild or as divisions. If grown from seed great numbers of plants could be made available to the public and easy the pressure from the plants in their natural habitat.

For the moister areas of the garden the **rose pogonia,** *Pogonia ophioglossoides* offers an excellent subject. It too, grows in large numbers, is showy, has several different color forms and is an easy horticultural subject. Because if it relative abundance seed should be easy to obtain from selected clones in the wild.

Both the **putty-root**, *Aplectrum hyemale* and **crane-fly orchis**, *Tipularia discolor* have been in the trade as collected plants for many years, and both do reasonably well as garden subjects. Although they can occur in the southern states in large numbers, if propagated in quantity they would also make excellent garden subjects, have the curiosity factor of the winter leaf and posses interesting flowers.

More difficult subjects unquestionably exists, such as the several very rare *Platantheras... praeclara, pallida, integrilabia* et al. When we can satisfactorily present these in a viable form to help repopulate the wild then propagation and production will have made it greatest strides. Meanwhile, growing to both satisfy the increasing hunger of native orchid enthusiasts for plants that can be legitimately cultivated and unlocking the secrets of the more difficult species for practical propagation is the primary challenge to leave with you.

Dactylorhiza aristata, photo courtesy Paul Martin Brown

Colophon

The editor and producer are indebted to Paul Martin Brown for the illustrations of many species appearing in this work. Brown is the editor of the North American Native Orchid Journal. Membership currently (1996) is US$26.00 and subscriptions can be sent to:

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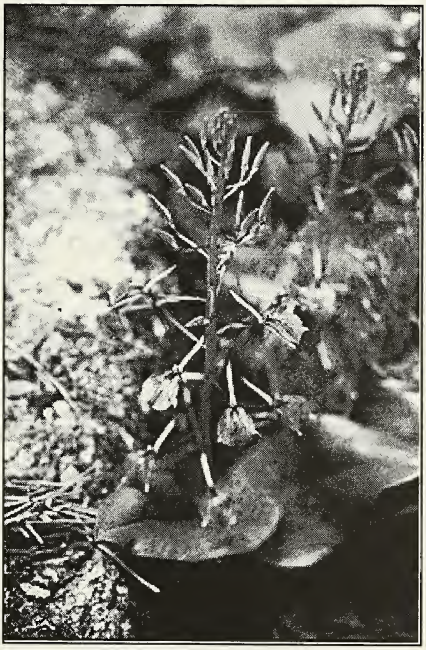
84 Etna Street
Brighton, MA 02135-2830 USA

The *Proceedings* was set in New Baskerville font using Microsoft Word 5.1a and produced in proof on an Apple LaserWriter 360. The *Proceedings* were printed by McNaughton & Gunn, Inc., P.O. Box 10, Saline, MI 48176, USA. Production Services by Clark Riley, clark.riley@qmail.bs.jhu.edu.



Liparis lilifolia, photo by Paul Martin Brown

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ISBN 0-9655075-0-5
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The cover illustration, for which the Conference is most grateful, is the work of Aislinn Adams.