Germination in the Cypripedium/Paphiopedilum Alliance

The colourful temperate ladyslippers including *Cypripedium acaule, calceolus* and *reginae* have attracted the attention of many investigators attempting to solve the problem of germinating the recalcitrant seeds (Arditti, 1967; Arditti et al, 1982; Curtis, 1942; Oliva and Arditti, 1984; Stoutamire, 1974, 1983; Withner, 1953). Germination of *Cyp. reginae* seed has perhaps attracted the most attention given that this species is particularly showy. Harvais (1973, 1974, 1980, and 1982) was the first Canadian investigator to approach the problem of axenic culture. He succeeded not only in germinating the seeds of *Cyp. reginae* but also in producing leafy seedlings. His death in 1982 cut short a promising research program and was a great loss. Frosch (1986) outlined a procedure to asymbiotically germinate and grow *Cyp. reginae* to flower in three years. More recently, Ballard (1987), has presented detailed results of his experiments in the sterile propagation of the same species, using seeds taken at early stages of development and at maturity. Of particular interest was his discovery that dormancy in *Cyp. reginae* seeds can be broken by refrigeration of the seeds at 5˚C for two to three months prior to incubation at room temperature. He has achieved from 19–98% germination after three to four months using Knudson's “C” medium (Knudson, 1946) with seed taken 42 to 60 days after pollination.

Cypripedium calceolus is a particularly attractive species, native to both North America and Europe. Carlson (1940) examined the formation of the seed of *Cyp. parviflorum* to gain a better understanding of the problems involved in germination. She examined ovaries, 2–72 days after pollination, finding that fertilization occurred after 26 to 33 days. The ovaries of Cypripediums and Paphiopedilums possess two phases of growth in diameter and one in length (Duncan and Curtis, 1942). The first phase of growth in diameter takes place when the ovules are maturing, after pollination but prior to fertilization. Stort (1984), working with the Cattleya alliance, has shown that unless the pollen tubes penetrate the ovary, the fruit is not formed and the ovary dries. The second phase of ovary growth in diameter is after fertilization when the embryos are growing rapidly. Duncan and Curtis also noted that the growth of the ovary almost ceases when fertilization is taking place. Muick (1978) reported the propagation of *Cypripedium calceolus* from seeds. His method seems to be symbiotic though few details were given. The report stated that blooming size plants could be obtained

in three years, not 12 years as was commonly assumed. Apparently *Cyp. calceolus* seedlings, raised in Europe, are now being imported into Canada for horticultural purposed, (Huronview Nurseries, Bright's Grove, ON: personal communication). No details as to the methods of raising these plants are available.

I first became interested in the germination of *Cyp. calceolus* seed when a friend who grows a more than 50 year old clone of the orchid posed the question, "Can you germinate the seed?" After reading the available literature, I tried to germinate mature seed on a variety of media including a commercial preparation, at room temperature, in the light and in the dark, with no success. The following year, 1986, I decided to take the seeds at an early stage of development, 42 and 66 days after pollination, as well as when the capsule was fully mature, close to the time of dehiscence, at 90 days. The flowers were self-pollinated and were all of the same clone. A new commercial medium, Mother Flask Medium IV (G & B Orchid Laboratories, Vista, CA), recommended for terrestrial seed germination, had become available in 1986. I prepared it according to manufacturer's instructions (final pH 5.5–5.8). Other media tested included Mother Flask Medium II (G & B Orchid Laboratories). The capsules were harvested at random, surface sterilized in 1:10 chlorine bleach solution for 10 minutes, opened aseptically, the seed being sown on slants of sterile media in borosilicate glass tubes (20×150 mm) fitted with permeable membrane polypropylene caps. Approximately 100 seeds were sown per slant. Six slants of each medium were used for each age of capsule. Slants were incubated in the dark at 20±2˚C and were examined periodically. Visible germination in the form of swollen, white protocorms was evident as early as 24 days after sowing the 42 day seeds on Mother Flask IV medium. Germination after three months averaged 10%. No germination occurred with any other medium tested or with any other age of seed. Some of the protocorms were replated to Replate Medium IV (G & B. Orchid Laboratories), a medium designed for replating terrestrial seedlings. However, the protocorms quickly turned dark brown and died. Those protocorms left on the Mother Flask IV slants in the dark grew, developing first a branched rhizome-like structure then extensive hairy roots (Jan. 1987). The plantlets were removed from the slants, 10 months after sowing (April 1987), and were planted in a pot with an adult Cymbidium. The potting medium was a mixture of redwood bark chips, leafmould, fired clay gravel and marble chips. The seedlings developed a green shoot but no expanded leaves. They received the regular watering and weekly feeding schedules of the Cymbidium and were exposed to climatic variations as the Cymbidium was out-of-doors all summers. Six months after potting, (Sept. 1987), one of the plantlets was removed from the pot and examined. It appeared healthy although still without expanded leaves. The root tips showed signs of a recent growth spurt. One root was removed, sectioned with a razor blade, wet-mounted and observed microscopically for

signs of mycorrhizal infection and mycorrhizal peloton formation. Williamson and Hadley (1970) showed that fungal infection can be controlled by the host; successful mycorrhizal infection is only through epidermal root hairs. They added that peloton formation is evidence of initiation of symbiosis. Hadley (1970) pointed out that root infection and peloton formation may occur with a variety of fungi and not necessarily with the same fungus that can stimulate germination of a particular orchid species. Examination of the root of the potted seedling showed that infection had occurred via the epidermal root hairs and that few pelotons had formed beneath the epidermis (Figs. 1, 2). These seedlings have been kept over winter at approximately 10˚C.

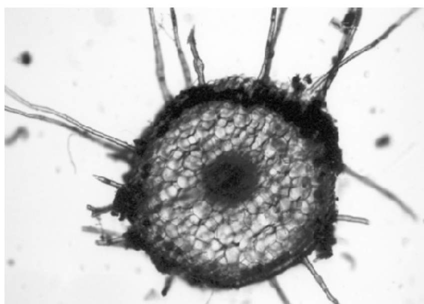

Fig. 1: Cross-section of root of Cyp. calceolus showing root-hair infection

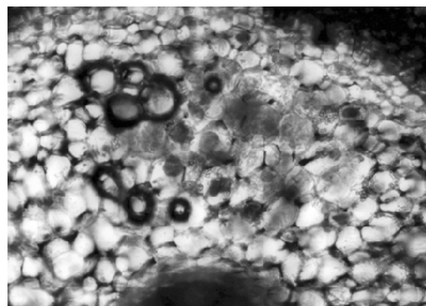

Fig. 2: Detail of peloton formation in cross-section of root of Cyp. calceolus.

The germination experiment was repeated in 1987 with capsules harvested at 30, 35, 43, 49, 58, and 73 days after self-pollination. The capsules were all harvested from the same clone. This more extensive experiment was designed to bracket the stage of optimum germination and to gather as much evidence as possible to identify it. Days after pollination was an absolute figure but I was concerned that climatic variation could play a role in the rate of development of the seeds.

The capsules were surface sterilized as previously described and the seed was sown on slants of Mother Flask Medium IV. Twelve replicate slants were prepared for each age of seed. Approximately 100 seeds were sown on each slant though it was difficult to ascertain numbers of seeds with the 30 and 35 day samples; the seeds were difficult to separate from each other. Some of the same seed was mounted in water, examined under the microscope and photographed to show the average stages of embryonic development in a test capsule. The slants were incubated at 20±2˚C in the dark. Three additional replicates at each stage were prepared and stored for either 1,2, or 3 months at 5˚C in the dark, then were

Table 1

Germination of pre-mature seeds of Cypripedium calceolus v. pubescens, with and without cold pretreatment: Results after nine months.

Days after pollination	Diameter of capsule (mm)	Embryo Status		% Germination*
		Integuements	Embryo	
0	4	Not studied	—	
30	8	Visible	0	
35	11	Elongated	0	
43	11	Final size	3 – celled	1
49	13		8–12 cells	50-75
58	14	Darkening	Final size	5-50
73	14	Collapsed	Mature	0
110	not measured	Capsule dehisced		not studied

*: 12 replicated, approximately 100 seeds/slant, on Mother Flask Medium IV (G & B Orchids, Vista CA) at 20 ± 2˚C.
Essentially no germination was seen in triple replicates stored at 5˚C for the first one, two or three months, and subsequently held at 20˚C.

moved to the 20±2˚C area, still in the dark, where they were observed at regular intervals.

One capsule of the same clone, pollinated at the same time as the others, was marked with waterproof ink at the widest point, and was measured at each time a capsule was harvested. The capsule diameter was plotted against the time after pollination.

The results of the 1987 experiments are summarized in Table 1. The first signs of germination were observed 18 days after incubation of the 49 day seed at 20˚C. Germination averaged 50%–75%, varying widely among the replicates. Seed taken at 58 days after pollination began to germinate in just 8 days (5–50% germination); however since that time most but not all of the protocorms darkened and ceased growth. They were not necessarily dead, however, since a few of the darkened bodies have resumed growth several months later. The 49 day seedlings are developing as in the previous experiment; their roots are now penetrating the medium (March 1988). Microscopic examination of the 49 days after pollination seeds (Fig. 3) showed immature embryos, 1/3 developed, consisting of approximately 9-12 cells.

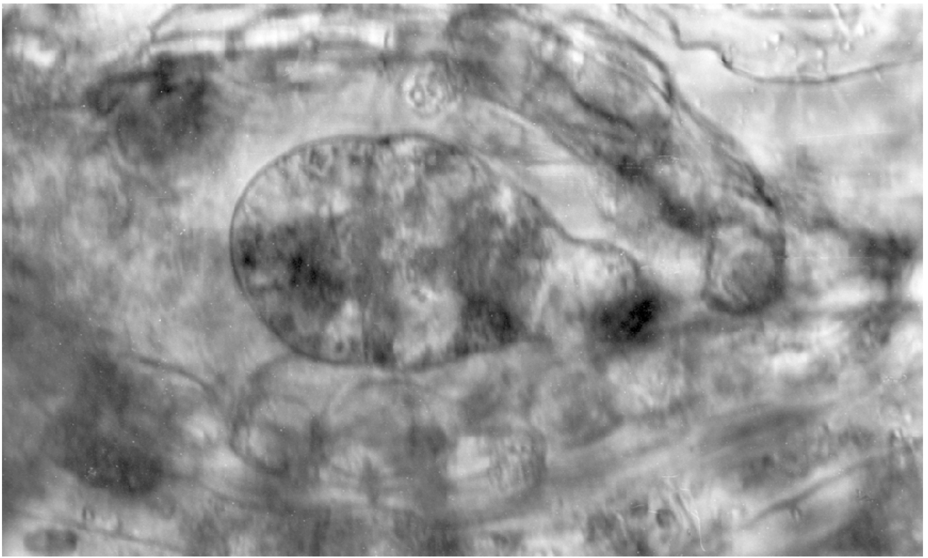

Fig 3: Immature embryo of Cyp. calceolus at 49 days post-pollination.

Measurement at this stage showed that the capsule was midway in the second stage of growth.

Correlation of the stage of capsule enlargement and of the stage of seed development is interesting and may provide the amateur orchidist with a tool to determine the stage for successful, asymbiotic germination of *Cypripedium calceolus* v. *pubescens.* That is, 1) days may be counted after pollination, 2) capsules may be measured and harvested midway during the second growth-in-diameter phase (Fig. 4), and 3) seed may be examined microscopically for the 1/3 embryo development stage (Fig. 3). To allow for natural variation, I would recommend harvesting several capsules at regular intervals, 40–55 days after pollination. After germination, seedlings should be kept in the dark at about 20˚C, on the same slant, until they have developed an extensive root system (approximately 12 months). The seedlings may then be removed from the culture vessel, rinsed free of medium and planted. The potting medium should be organic, finely divided yet free-draining, and contain a few marble chips. The seedlings should never be allowed to dry out. It may take several years before the first expanded leaves appear. A cold annual rest period may be necessary for development.

Germination of Paphiopedilum species and hybrids has also presented problems to investigators. A few detailed studies exist (see Arditti, 1967; Arditti et al, 1982; Cribb, 1987 for reviews); many of the published reports present conflicting results. My investigations began as before with a question from an orchidist. “Can you germinate the seed of this primary Paphiopedilum cross?” For me, fundamental questions were “When can the capsule be harvested?” and

"What are the optimum conditions for germination?"

Duncan and Curtis (1942) studied the fruit development of *Paph. fairrieanum, bellatulum, villosum* and *Paph. Maudiae.* They found, as with *Cyp. calceolus,* that the ovary growth in diameter took place in two stages separated by a slow growth period during fertilization. They reported that fertilization took place approximately six weeks after pollination in all test plants; the slow growth period continued for a further four to ten weeks, varying with the species, and within clones of the hybrid *Paph. Maudiae.* Thus, if one measures the capsule diameter at a marked point over time (Fig. 5), one should be able to pinpoint 1) the commencement of fertilization, marked by a slowing of growth, 2) commencement of rapid seed development, marked by a second rapid increase in capsule diameter, and 3) maturation of the seed, marked by a second and final slowing of capsule enlargement. This is a simple way for anyone with a ruler or with an inexpensive pair of callipers to determine the desired stage to harvest seed.

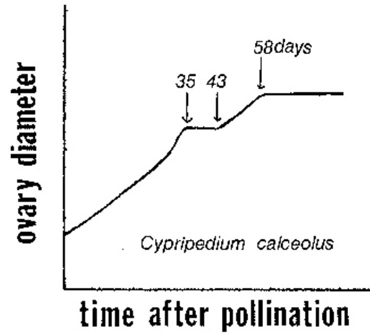

Fig 4: Time-course of post-pollination ovary enlargement of Cyp. calceolus.

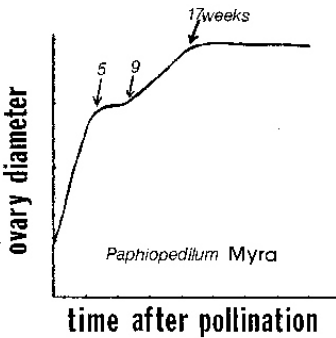

Fig 5: Time-course of post-pollination ovary enlargement of Paph. Myra × self.

The choice of germination medium can be a problem, given that species and hybrids may have varying nutritional requirements for seed germination and growth. Hegarty (1955) concluded that Paph. seed germinated best at 70˚F (21˚C) in subdued light. Ernst (1974;1975) studied the use of activated charcoal in asymbiotic seedling culture of several genera including Paphiopedilums. Fridborg and Eriksson (1975) showed that activated charcoal probably absorbed some media compounds and thus was effective in modifying growth of tissue cultures. Some investigators have believed activated charcoal to be beneficial in that it darkens the medium, creating a more favourable medium for roots. Dallo Rosa and Laneri (1977) prepared several variants of Knudson's "C" medium including additions of banana pulp (8%), activated charcoal (0.2%), and coconut

milk (10%), as well as varying the mineral content.

After reviewing these studies, I developed media recipes including 8% banana pulp and 0.2% activated charcoal, and decided to germinate the seeds at about 20˚C in subdued light. I found that the addition of activated charcoal (Activated Coconut Charcoal, 50–200 mesh, 5-690A, Fisher Scientific Co. Ltd.) to two different media definitely influenced germination of Paphiopedilum seeds, incubated in subdued light at 20±2˚C, and most definitely did not interfere with the passage of light (the charcoal particles sank to the bottom of the culture tube!). Various Paph. hybrid seeds, taken before capsule dehiscence, germinated well (> 75%) on media containing 0.2% activated charcoal and poorly on media with out charcoal. Media used included Mother Flask Medium II (G & B Orchid Laboratories) and an homemade recipe (LRX) containing 8% banana pulp. Since this study, trials with Mother Flask Medium IV have shown it to be an excellent medium for germination of Paphs. No trials have been carried out with additions of activated charcoal to this medium.

Seedlings of *Paph. exul*, germinated asymbiotically in subdued light at 20˚C on Mother Flask Medium II + 0.2% Activated Charcoal, were removed from flask after one year and were planted into pots containing a mixture of fine redwood bark and horticultural charcoal. After six months, a plant was removed and an apparently healthy root was sectioned and examined under the microscope. Mycorrhizal infection and peleton formation were observed. These observation, similar to those reported with *Cypripedium calceolus* seedlings show that asymbiotically raised seedlings may be successfully colonized with mycorrhizal symbionts. The seedlings continue to grow and prosper.

To summarize:

1. A method to germinate pre-mature seeds of *Cypripedium calceolus* var. *pubescens* has been developed. Seeds harvested approximately 49 days after pollination, when the embryos are 1 /3 developed will germinate in 14 to 21 days on a commercial medium, Mother Flask Medium IV (G & B Orchid Laboratories, Vista, CA) when incubated at 20±2˚C in the dark.

2. A simple method for determining the optimum time for harvesting capsules of the *Cypripedium/Paphiopedilum* alliance is presented.

3. Asymbiotically raised seedlings of *Cyp. calceolus* and *Paph. exul* were shown to be successfully colonized with mycorrhizal symbionts after planting out into bark medium.

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