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AXENIC SEED CULTURE AND MICROPROPAGATION OF *CYPRIPEDIUM REGINAE*

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ABSTRACT. An efficient method of axenic seed propagation of large numbers of *Cypripedium reginae* Walt. was developed as a means of supplying seedlings for a restoration attempt in the northeastern United States. Seedlings were grown on ¼ strength Murashige and Skoog basal salts supplemented with 100 ml/l coconut milk and solidified with 7g/l agar. The development of *Cypripedium reginae* was studied with both light and scanning electron microscopy. Dose response experiments of seed germination versus exposure time to sodium hypochlorite indicate germination and developmental progress was greatest at longer exposure times. Experiments on seed germination subsequent to exposure to increased concentrations of sodium hypochlorite (0%, 0.125%, 0.25%, and 0.5%) indicate germination and developmental progress was greatest at higher concentrations. Earliest germination was seen at 14 days after initial plating of seed. Stage 5 seedlings were present 27 days after initial plating and in greatest proportion in seeds exposed to longer times in sodium hypochlorite. Micropropagation experiments showed a success rate of 32% with an indication of difficulty in cutting the seedlings to allow for both shoot and root development. Approximately 10,000 seedlings have been successfully germinated and have developed both healthy roots and shoots.

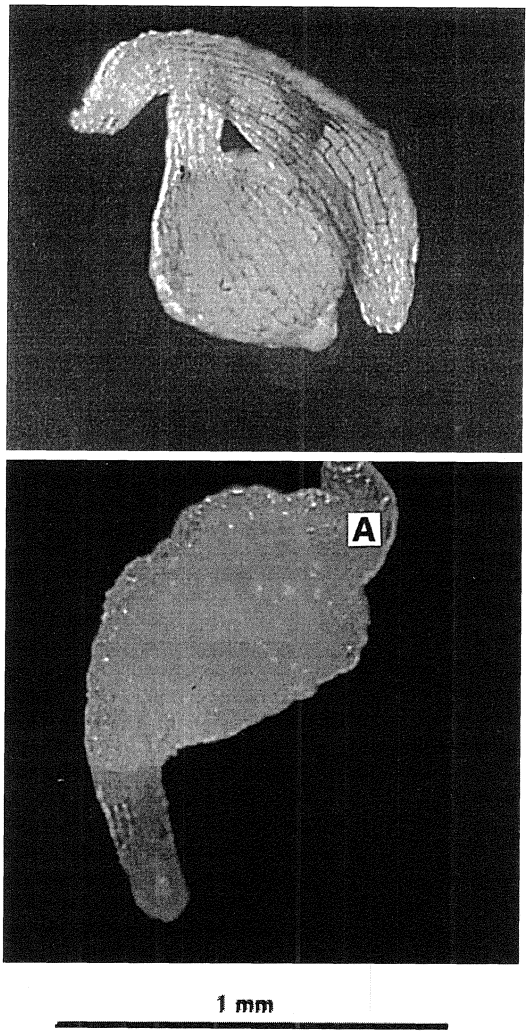
INTRODUCTION

The showy lady's slipper (*Cypripedium reginae* Walt.) is a terrestrial orchid found in the north-central and northeastern regions of North America. In New Hampshire this species commonly grows in marshy areas or fens preferring the more alkaline soils found on the border of Vermont and New Hampshire. Although these plants have probably never been found in great numbers in New England, partly owing to habitat loss, they are now critically endangered in much of northeastern North America.

Cypripedium species require the help of insects for pollination. Orchids, in general, display high pollinator specificity. In some cases, orchids can only be fertilized by the males of a single species of insect. However, this is not the case in lady's slippers (Withner 1974, Vogt 1990). The physical arrangement of these flowers creates a tight space through which the insects such as syrphid flies and bees (Vogt 1990) have to squeeze. A pollinating insect first passes by the stigma, and upon exiting the trap rubs against the anther. The insect that now carries pollen must reenter a lady's slipper to successfully carry out pollination (Dressler 1981). When successfully pollinated, like many orchids, *Cypripedium* seed pods contain thousands of seeds. Because the seeds do not contain endosperm (Dressler 1981), they seem to require a more rigorous set of conditions to germinate and develop than seeds with endosperm.

The showy lady's slipper is often regarded as the most regal of its genus. It usually has two flowers occurring on plants up to three feet tall. Because this species has suffered substantial habitat loss, reproduces slowly, and has been the object of illegal collection, it is a prime subject for a restoration project using *in vitro* culture. Although *in vitro* seed culture of the various *Cypripedium* species has recently made significant progress, optimal conditions for germination and development are uncertain. A comparison of results is difficult since media and growing conditions vary widely. A partial list of basic media includes Murashige and Skoog (1962), Harvais (1973), and Clements and Ellyard (1979). Modifications to media are also quite varied. While Harvais (1973) uses a medium with potato extract, Ballard (1987) and Steele (1995) use unmodified pieces of potato and DeMarie *et al.* (1991) use yeast extract. Coconut milk is also commonly used (Chu & Mudge 1994, Arditti & Ernst 1992). Procedures used to surface disinfest seeds differ greatly with the most usual agent being 0.5% sodium hypochlorite. Exposure times to sodium hypochlorite vary widely from minutes to hours (Steele 1995, Chu & Mudge 1994, Ballard 1987). Chu and Mudge (1994) have found in *Cypripedium calceolus* that the practice of seed prechilling was advantageous. They also found liquid medium supported the highest levels of germination.

Cypripedium reginae take about 8 years to mature to a flowering state in the wild (Withner 1974). Any approach, such as *in vitro* culture, which speeds this process may prove to be an important adjunct to restoration attempts. Consequently, any approach which increases either axenic or *in vitro* culture efficiency should be of value. When considering efficiency, *in vitro* methods that affect percent germination and rate, along with subsequent development and survival, are central factors. Chu and Mudge (1994) obtained acceptable germination rates when seeds were surface sterilized in bleach for twelve minutes. Further



FIGURES 1–2. 1) Light micrograph taken at 40× of a stage 2 (ungerminated) and stage 3 (germinated) seedlings. A seed was considered germinated when its embryo had enlarged. 2) Light micrograph taken at 40× of a stage 5 seedlings. The seedling has broken through.

studies (Dovholuk & Faletra 1996) showed an increase in percent germination when exposed for up to thirty minutes in bleach. Various other culture factors that could affect germination, such as seed density and temperature, have also been investigated (Faletra *et al.* 1997, Sokolski & Faletra 1997).

The intention of this investigation was to seek an efficient means of axenic seed propagation and/or micropropagation of *Cypripedium* species to eventually support a restoration effort for endangered species of this genus in the northeastern region of the United States. Of the local species that are critically endangered, (U. S. Department of Resources and Economic Development 1994) *Cypripedium reginae* was chosen since it was the only dependable seed source. Analysis of seedling development with light microscopy (LM) and scanning electron microscopy (SEM) was done prior to all other investigations to allow for a benchmark understanding of seedling morphology and histology. This information was valuable as a guide to micropropagation studies. A nomenclature for seedling stages similar to that of Chu and Mudge (1994) was used.

MATERIALS AND METHODS

General Procedures

Fully mature seeds were harvested from partially dehisced seed capsules of *Cypripedium reginae* which were gathered in early October from eastern Vermont and western New Hampshire. The seeds were removed and stored in the dark at room temperature. The medium used in seed germination was ¼ strength Murashige and Skoog salts (Sigma Chemical Co., MS-6899), 1% sucrose, and 100 ml of liquid endosperm/liter of medium (coconut milk). The endosperm was drained from a ripe coconut and filtered through #1 Whatman filter paper. The media was mixed, the pH was adjusted to 6.0, and when desired, the medium was solidified by the addition of 7 grams of agar/liter of medium. About 20 ml of medium were distributed into culture containers which were capped and autoclaved at

TABLE 1. Seed germination in groups exposed to 0.5% sodium hypochlorite for 12, 20, 28 and 36 minutes. There were 50 cultures per group. Values are means ± 1 standard deviation.

Days in culture	Time in bleach			
	12 minutes	20 minutes	28 minutes	36 minutes
20	0.15 ± 0.47	0.92 ± 0.82	1.32 ± 0.98	2.49 ± 0.76
27	3.35 ± 1.13	5.32 ± 1.22	7.13 ± 1.94	9.47 ± 2.79
37	27.02 ± 8.84	87.56 ± 3.58	90.40 ± 3.75	88.40 ± 3.24
47	81.14 ± 4.87	89.40 ± 2.78	85.84 ± 4.20	87.25 ± 4.58

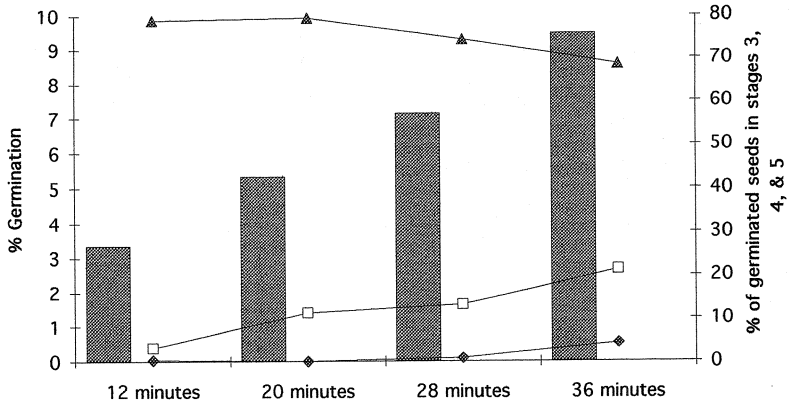


FIGURE 3. Germination and development of seeds in culture for 27 days plotted against time exposed to 0.5% sodium hypochlorite. Bars represent percent germination for different times in sodium hypochlorite. Lines represent percent of seedlings in stages 3, 4, or 5. Black triangles are stage three seedlings; open boxes are stage 4 seedlings; black diamonds are stage 5 seedlings.

120 C for 20 minutes. All solid medium cultures were in 25×150 mm glass tubes. Seeds were removed from storage and placed in 100 ml of a 0.5% sodium hypochlorite with two drops of detergent as a wetting agent. The seeds were swirled every two or three minutes throughout the exposure time. The seeds were filtered (Whatman #1) in a conical glass funnel, rinsed five times with sterile distilled water and transferred into sterile media with an inoculation loop. Solid cultures were placed inside a dark incubator at 22 C. Liquid cultures were kept in the dark on an orbital shaker at 60 rpm. Each experiment was observed weekly for signs of germination, growth, and development. Seed development was monitored through a stereo dissection microscope at magnifications between 20× and 40×.

Germination and Development Responses to Increasing Times in Bleach

In addition to following the general procedures described above, seeds were soaked in 10% bleach (0.5% sodium hypochlorite) for 12, 20, 28 and 36 minutes. Fifty cultures/group were used.

Effects of Exposure to Increasing Concentrations of Bleach

General procedures described above were followed with the exception that seeds were soaked in 0%, 2.5%, 5%, or 10% bleach (0%, 0.125%, 0.25%, and 0.5% sodium hypochlorite) solution for twelve minutes. Ten cultures/group were used.

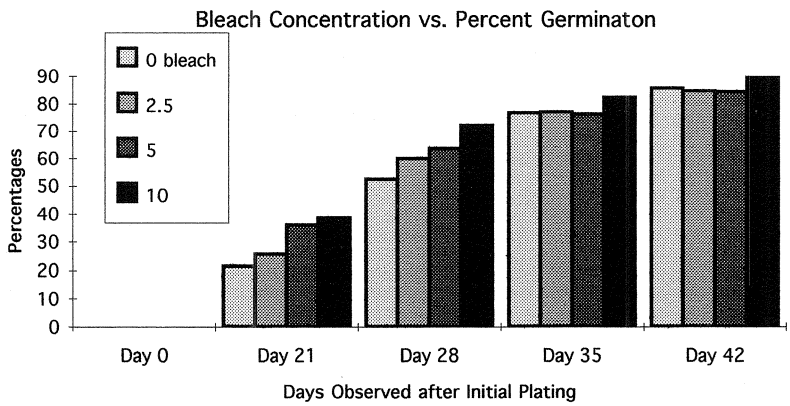


FIGURE 4. Each bar represents % germination after 0, 21, 28, 35, and 42 days in culture for seeds exposed to 0%, 2.5%, 5% and 10% bleach (0%, 0.125%, 0.25%, and 0.5% sodium hypochlorite). The 5% and 10%-bleach groups were significantly ($p<0.05$) higher than the 2.5% and 0% groups on days 21 and 28 and were not significantly different thereafter.

TABLE 2. Seven experiments on micropropagation are shown. The numbers of groups in each experiment ranged from 6 cultures in the smallest to 40 cultures in the largest. Successful cultures were those that grew both roots and shoots on both halves after sectioning.

Experiment	% Success
1	10
2	0
3	70
4	33
5	38
6	32
7	0
Mean % success	33

Micropropagation of *Cypripedium reginae*

Four to ten-month-old seedlings were used. Experimental seedlings were cut approximately in half (one or two roots on each section and half of a shoot) with a sterile blade, while control seedlings remained uncut. Seedlings were replated in new sterile tubes containing the same solid medium as before. Cultures were incubated at 22 C in the dark for approximately 2 months.

Embedding and Sectioning of Specimens for Light Microscopy

The seedlings and seedling pieces, which had been fixed in 4% glutaraldehyde for 3 weeks and washed in 50% ethanol, were infiltrated with three ninety-minute changes in JB-4 monomer A (Polysciences Inc.). One ml of JB-4 monomer B was added to 25 mls of fresh catalyzed monomer A. Specimen wells were filled with plastic, a specimen was placed in each well, and the well was covered with a GMA block holder. When the plastic hardened, specimens were sectioned on a Sorval Ultramicrotome, mounted on slides, stained with toluidine blue, and observed under light microscopy. Pictures were taken with a Zeiss compound light microscope.

Fixation of Seedlings for Scanning Electron Microscopy

Seedlings were fixed in 4% glutaraldehyde solution in 0.1M Pipes buffer with a pH of 7.0 for 1 week. Seedlings were then washed in distilled water (dH_2O) five times for 20 minutes each. The seedlings were dehydrated with three changes of increasing percent solutions of ethanol starting at 50% and ending at 100%. Final

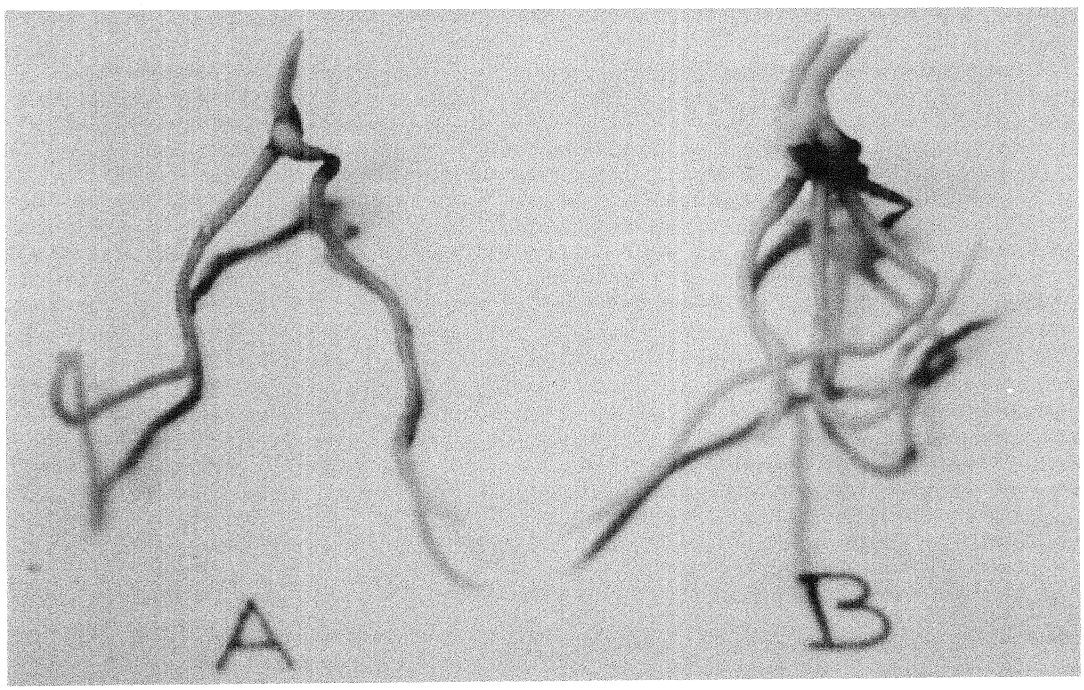


FIGURE 5. Seedlings A and B were sectioned three months prior from a 4 month old seedling with a 7 mm long shoot and roots at least 2 cm long.

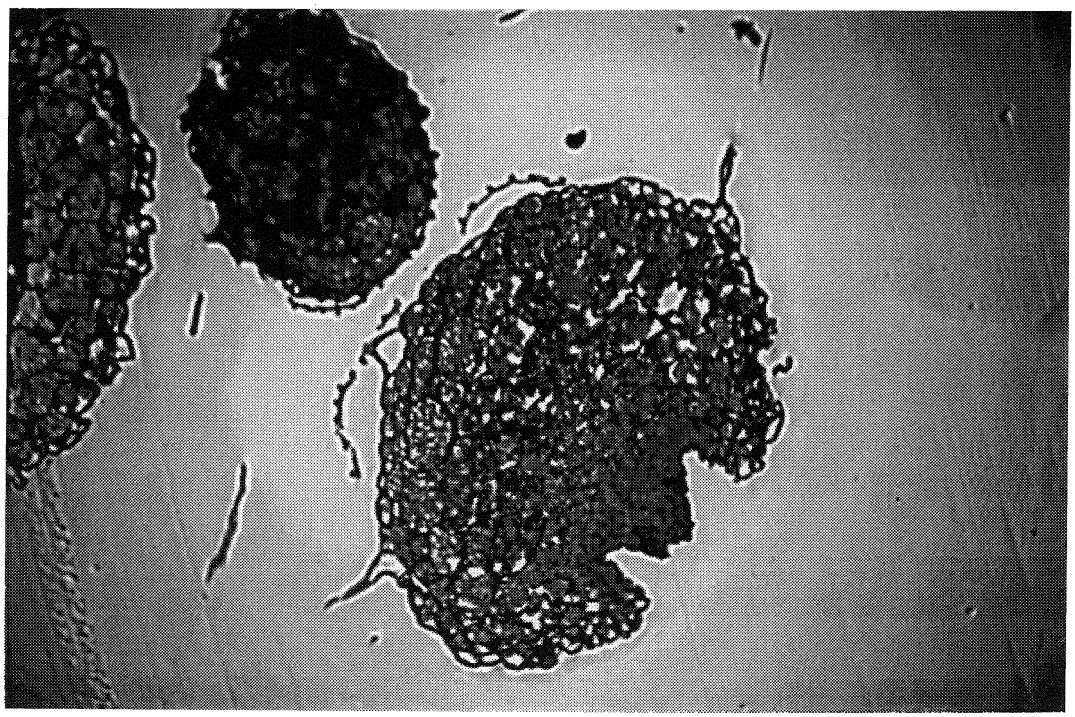


FIGURE 6. Light micrograph of stage 4 and 5 seedlings taken at 40×. The larger of the two seedlings is the stage 5 seedling (longitudinal section) with a developing apex. About 1/3 of this seedling is made up of meristematic tissue. The smaller seedling shows primarily parenchymal cells and no evidence of meristematic tissue.

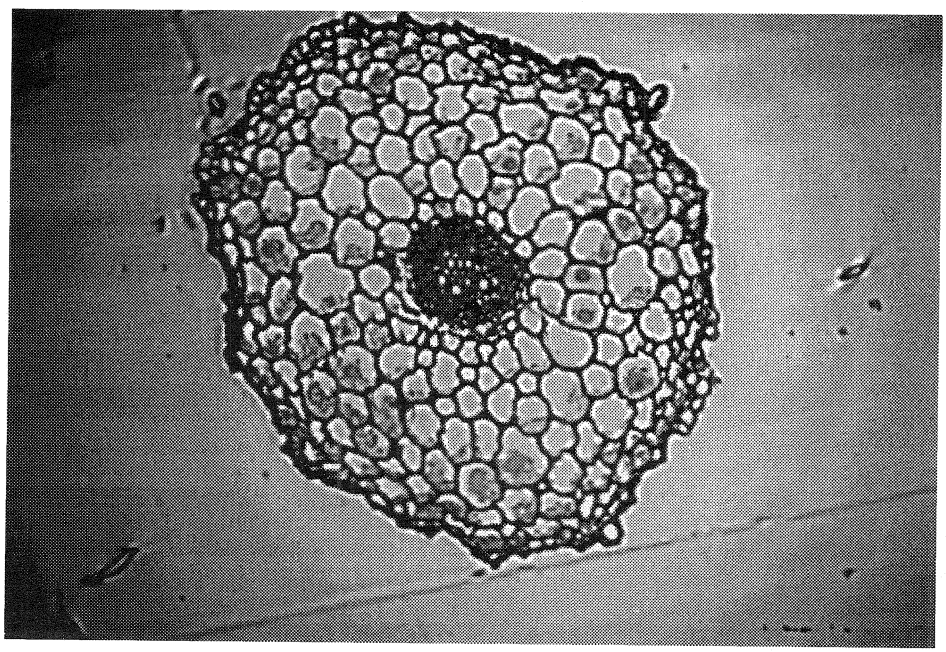


FIGURE 7. Typical cross section of root tissue showing central vascular tissue, cortical parenchymal cells containing numerous amyloplasts, and no evidence of meristematic tissue.

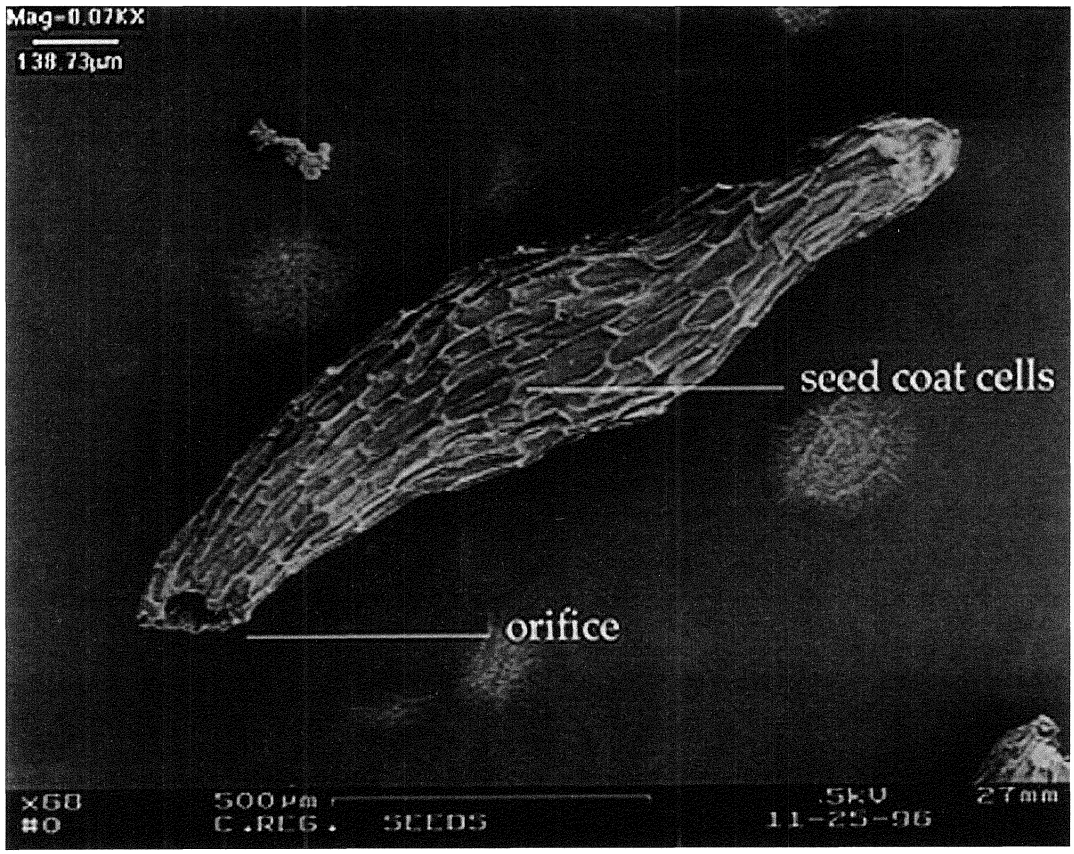


FIGURE 8. *Cypripedium reginae* seed taken at 70× with SEM. A hole at the micropylar end is evident while the chalazal pole is closed.

dehydration of seedlings was done in hexamethyldisilazane. Seedlings and seeds were mounted and coated with gold-palladium in a Hummer sputter coater for about 10 minutes. The mounts were then loaded into a Zeiss DSM 962 scanning electron microscope and pictures were taken.

Statistical Analysis

Analysis of variance (ANOVA) or t-test was used to compute confidence limits among mean levels.

RESULTS

Seed development was observed under 40× magnification. The stages of seedling development (FIGURES 1 and 2) were named as follows. Stage 1 is the seed before it was exposed to bleach. The seed coat has well defined cells enclosing a centrally located embryo of about 300–500 cells. Stage 2 seeds appear identical to stage 1 seeds with the difference being that stage 2 seeds have been exposed to bleach and placed in culture. Exposure to bleach will make large groups of seeds (> 50) appear lighter in color to the unaided eye. Stage 3 (germinated) is characterized by an enlarged embryo which has not yet broken through its seed coat. Stage 4 (seedling) is an embryo that has broken through the seed coat. Pieces of seed coat often remain attached to the seedling. Stage 5 is characterized by the presence of an apex. Seedlings which present an identifiable root and shoot are referred to as late stage seedlings.

Germination and Development Responses to Increasing Times in Bleach

The first sign of seed germination was observed in all groups 20 days after initial plating (TABLE 1). There was a significantly greater ($p<0.05$) mean percentage of germination for successively longer exposure times. There was a leveling off of values by days 37 and 44. On

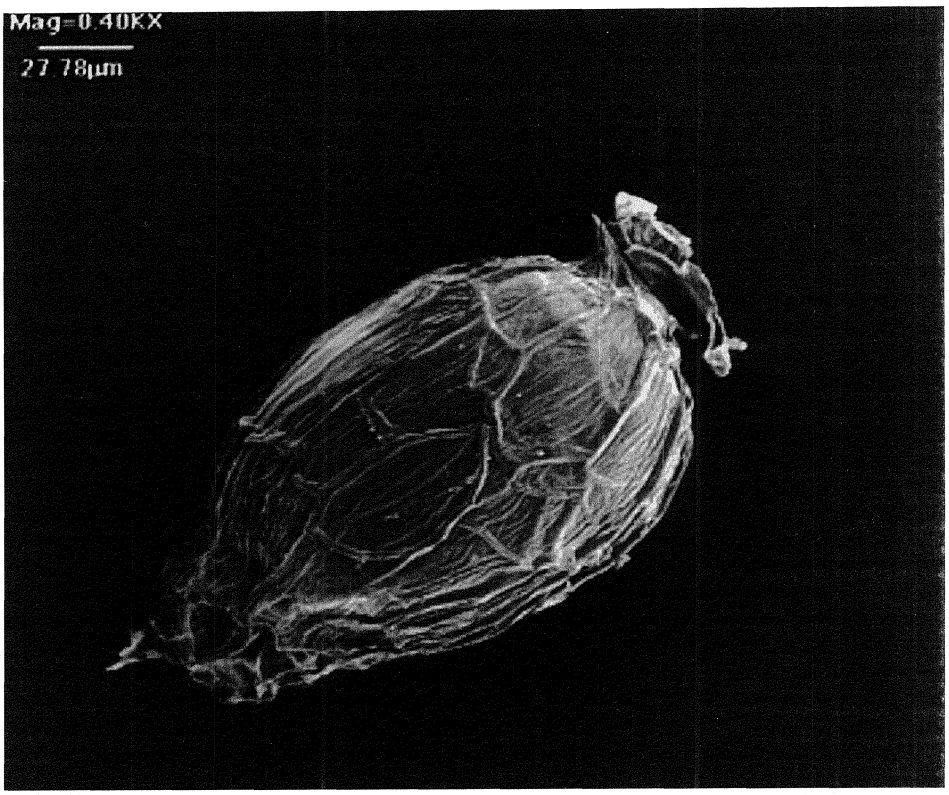


FIGURE 9. After mechanically stripping the outer seed coat the embryo shown here at 400× with SEM reveals an internal seed coat.

days 37 and 44 the values for mean percentage of germination at longer exposure times remained significantly greater than the 12-minute exposure time ($p<0.05$). FIGURE 3 shows percentage of germination and development plotted against exposure to bleach after 27 days in culture. The numbers of stage 3 seedlings declined with longer exposure times while the numbers of stage 4 and 5 seedlings increased. The values for 28 and 36 minutes of exposure were significantly greater than the 12 minute exposure time ($p<0.05$).

Effects of Increasing Concentrations of Bleach on Germination

Increased levels of germination were found with increased concentrations of bleach. The 10% bleach group showed higher percent germination on 21 and 28 days after initial plating than the 0%, 0.25%, and 0.5% bleach groups. The differences were significant ($p<0.05$) only between the two higher concentration groups and the lowest (0% bleach) group (FIGURE 4). Values for all groups leveled off by 35 days and 44 days.

Micropropagation of *Cypripedium reginae*

Seven experiments on sectioning seedlings for micropropagation showed a mean success rate of 32% (TABLE 2). Problems with sectioning were experienced when attempting to cut very small (< 4 mm) seedlings. Four months later micropropagated plants had developed healthy roots and shoots and were ready for vernalization (FIGURE 5).

Embedding and Sectioning of Specimens for Light Microscopy

FIGURE 6 is a light micrograph of seedlings at stages 4 and 5. Meristematic tissue is indicated by densely packed nucleated cells. There are numerous starch granules throughout the cells. FIGURE 7 shows a cross section of root tissue with xylem cells in the central area immediately joined by phloem. A central area with developing vascular tissue is surrounded by nucleated parenchymal cells containing numerous amyloplasts which have sunk to the bottoms of the cells, apparently owing to gravity. Cross sections of shoot and rhizome tissue showed similar

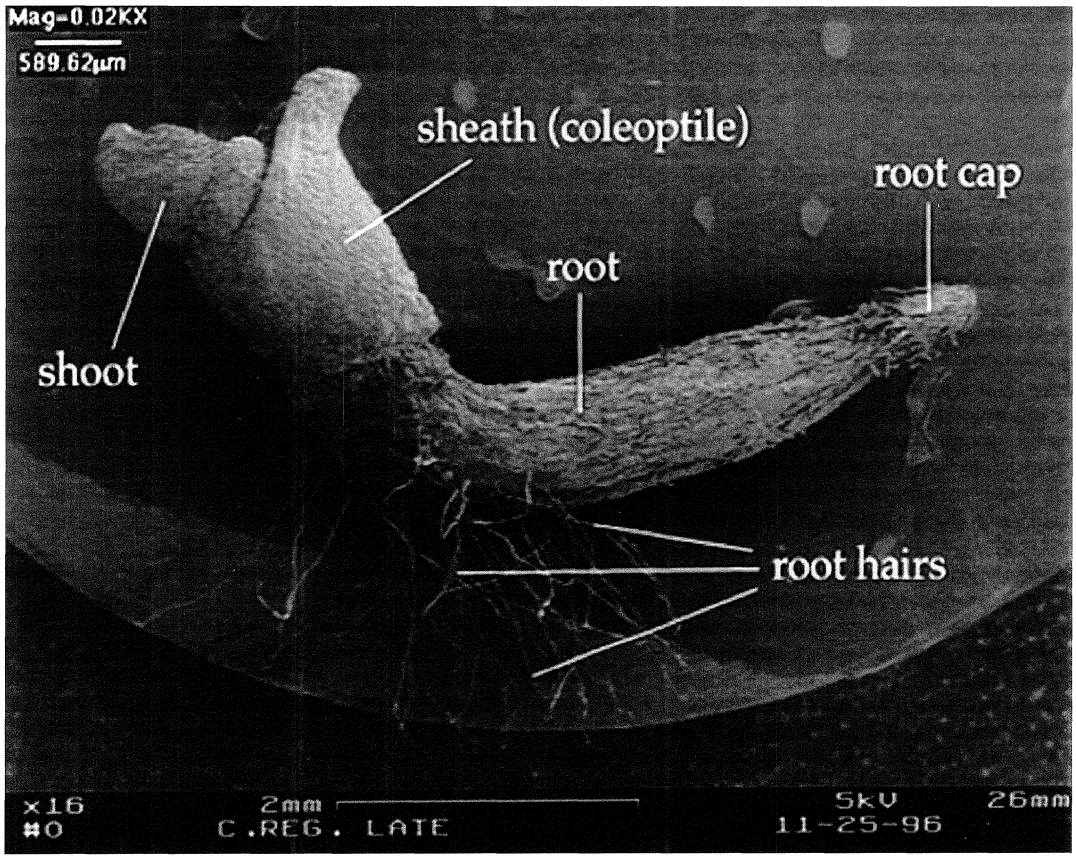


FIGURE 10. A late stage seedling is shown at a developmental state that typically takes about 6 weeks. The seedling has developed a primary root and a shoot ensheathed in a coleoptile-like structure. (20×)

results. Meristematic tissue was only found in large proportion in stage 5 seedlings.

FIXATION OF SEEDLINGS FOR SCANNING ELECTRON MICROSCOPY. FIGURE 8 shows a *Cypripedium reginae* seed. A hole at one end of the seed is evident while the other end is closed. FIGURE 9 shows an embryo stripped of its outer seed coat revealing an inner coat with anticlinal walls over its surface. FIGURE 10 shows a seedling well into development at about two months of age. A shoot emerging from a sheath (coleoptile-like structure) is evident. A root with root cap and root hairs is also seen. FIGURE 11 shows a stoma with accompanying guard cells.

DISCUSSION

Because of severe habitat loss, difficulties in pollination, and a developmental period of 8 to 12 years before flowering, it is no surprise that lady's slippers are among the endangered orchids. Attempts to halt decline or destruction of habitat are of primary importance, since without a place to grow the species cannot survive. Likewise, the importance of successful pollination leading to viable seeds is critical for the survival of the species. Until recently, culturing of showy lady's slippers was difficult. Recent improvements by Chu and Mudge (1994), Steele (1995), and Dovholuk and Faletra (1996), make it reasonably efficient to axenically culture the *Cypripedium* species from seed. Nevertheless, there is considerable room for improvement, especially with respect to micropropagation which has yet to be successful in any member of this genus.

Conditions that affect germination and development affect the efficiency of the system. With respect to germination, since each pod produces thousands of seeds, seed supply should not be of primary concern. With an average plating density of about 45 seeds per culture, a greater than 10% germination rate gave quite adequate numbers of seedlings per culture vessel. With germination beginning as early as day 20, the most pronounced increase in efficiency was seen

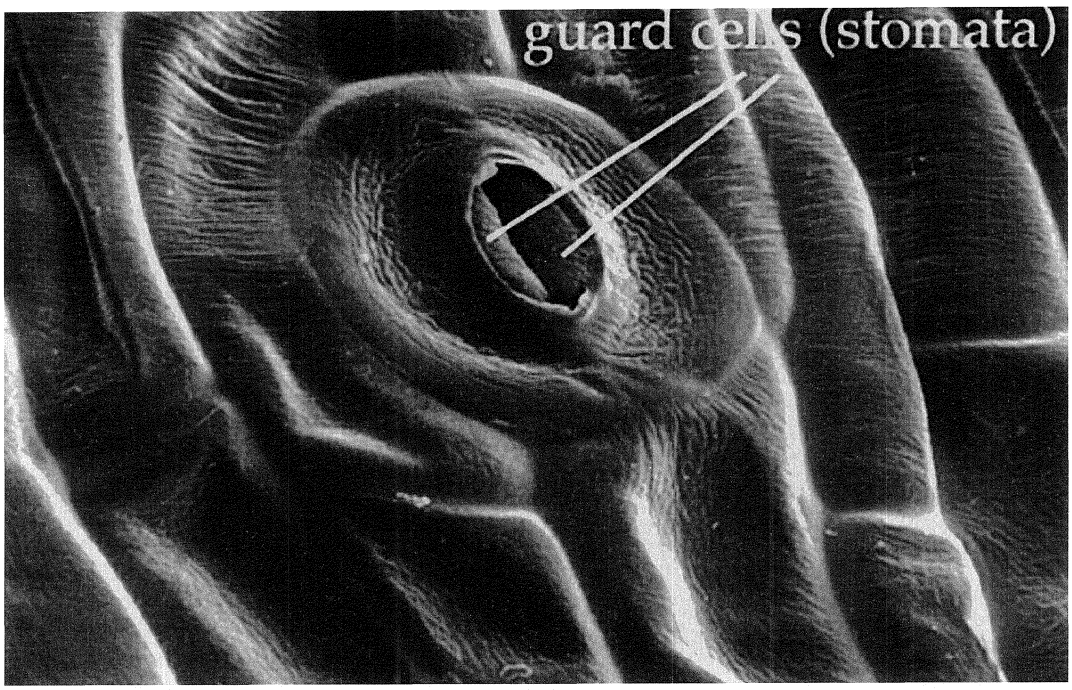


FIGURE 11. A stoma from the shoot area depicted in FIGURE 10 taken at 500× with SEM.

in speeding development of the seedlings by longer exposure to bleach.

The data on day 27 (FIGURE 3) show that the numbers of stage 3 seedlings declined in those seeds exposed to longer sterilization times while the numbers of stages 4 and 5 seedlings increased. This is most likely a consequence of the stage 3 seedlings developing into stages 4 and 5 seedlings. Such results strongly suggest that the most successful method for optimal germination and development in this experiment is to expose seeds to 36 minutes in bleach. It is possible that longer exposure times may extend these results but room for improvement is not substantial. These results also suggest that the overall methods employed to germinate seedlings from the culture medium through exposure to bleach have substantially improved the efficiency of growing *Cypripedium reginae in vitro.* Steele (1995), who has a moderately efficient procedure for propagating *Cypripedium reginae in vitro,* obtains what we refer to as stage 5 seedlings within approximately 72 days. Our approach gives similar results between 27 and 44 days in culture.

Unfortunately, these experiments on seed exposure to bleach did not expose seeds to water as a control. This was not an oversight, since the purpose of bleach is to eliminate any contaminating organisms and without any disinfectant it was thought that cultures would have little chance of survival. Nevertheless, this was problematic since water being the diluent for bleach was the appropriate control. The experiment with 0% bleach as the control was designed to remedy the lack of proper control. It was hoped that with thorough washing with sterile dH_2O minimal contamination would occur. Although 40% of the controls were lost to contamination, there were enough for a sound statistical analysis. In our most recent experiments (not yet completed) we have increased the number of controls to thirty cultures. The significantly higher germination of the two higher bleach concentrations on 21 and 28 days after initial plating shows that although water alone causes substantial germination, the bleach is having a positive effect apart from its disinfectant properties. The oxidative action of the bleach might possibly be softening the interior seed coat which was revealed in scanning electron microscopy (SEM) of the embryo (FIGURE 9). Since the seeds have an orifice (FIGURE 8), the outer seed coat is probably not preventing the embryo from exposure to moisture.

The *Cypripedium* genus has not yielded to attempts at micropropagation. Hoshi *et al.*(1994) recently published results of a fairly exhaustive micropropagation attempt which resulted in limited callus production and no evident protocorms. Our micropropagation attempts using

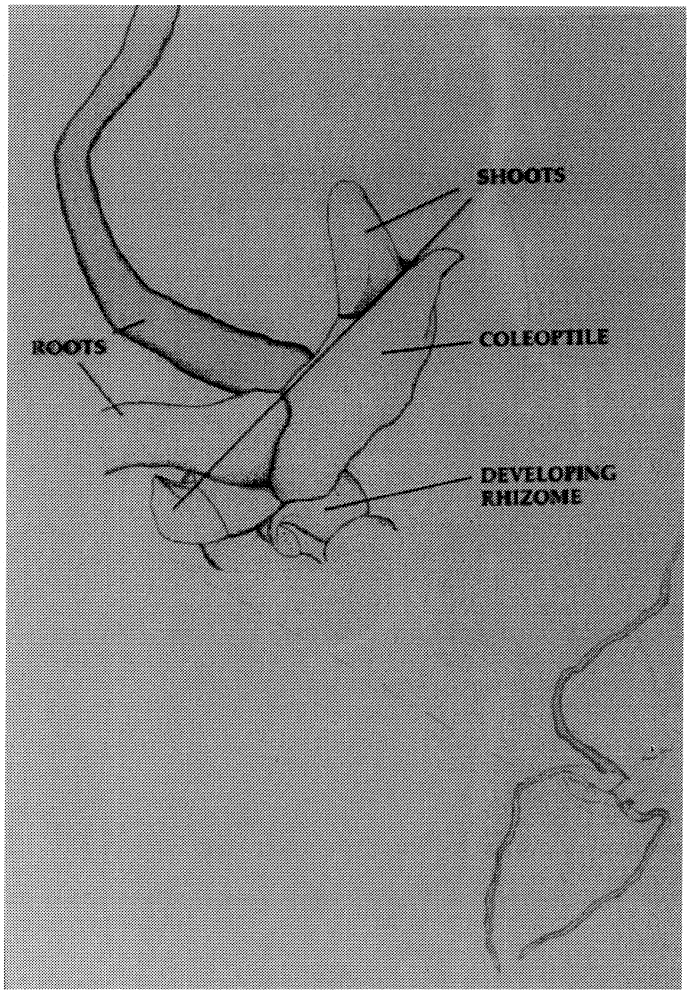


ILLUSTRATION 1. The development of a late stage seedling was observed over a 3 month period. The final product of these observations is depicted here. The lower right portion depicts the overall structure with an enlarged view of the center portion in the upper left. The shoot was observed to develop from the apical area of a stage 5 seedling while the lower two roots developed from 1–2 mm spots of green/yellow tissue. The third or uppermost root grew from the base of the shoot as it continued to develop.

similar approaches to that of Hoshi *et al.* (1994) (i.e., matrix experiments) have not been successful (data not shown).

Attempts to micropropagate by sectioning were reasonably successful. The results from the SEM and LM guided these experiments since positive identification of tissue type was a prerequisite. When seedlings were cut approximately in half, with a piece of a shoot and one or more roots on each half, both halves developed into healthy seedlings and eventually matured to the level in which they were ready to be vernalized (FIGURE 5). In some cases seedlings could be cut in eight pieces. Complications in micropropagation were attributed to the use of very small seedlings (<4 mm) which were difficult to cut. Most recently, we have found the best results when we use seedlings at least 2 cm in overall length with a well defined shoot of at least 7 mm in length. The larger seedlings, being more conducive to sectioning and possessing more shoots, often yielded more than four sections.

Because axenic culture of *Cypripedium reginae* has only recently been successful, studies of morphology and development have been somewhat superficial (Dressler 1981). This is unfortunate since knowledge of development is a key to understanding any plant.

SEM consistently revealed an orifice at only one end of the seed (FIGURE 8). This orifice is probably a remnant of the micropyle. The closed

end was where the seed attached to the placenta. The embryo of approximately 500 cells was enclosed in an inner coat which probably derived from the interior integuments (FIGURE 9). As mentioned above, it is this inner coat that needs to be penetrated by the bleach/water solution.

Light microscopy of embryos shows that the embryos are composed of undifferentiated parenchymal cells with no cotyledons, which is typical of orchids but atypical of the class Monocotyledonae (FIGURE 6). About one third of the tissue in stage 5 seedlings is composed of densely packed nucleated cells. This meristematic tissue is the likely candidate for micropropagation. This apical area has been identified as shoot by the presence of stomata, which appear later in development. We have also observed a single seedling develop over a period of 3 months and found this apical area to develop into shoot while at the base of the apex is a 1–2 mm spot of green/yellow tissue which develops into the root. The final product of these observations is shown in ILLUSTRATION 1.

ILLUSTRATION 1, observations of growth, and SEM all act to reinforce our belief that these orchids grow much like grasses, having successive roots emerging at nodes, and rhizome tissue making up the internodes. This growth pattern will occur until the plant is stimulated to produce an aerial shoot which can be triggered by placing the plantlet in the light.

Light microscopy was intended to locate meristematic tissue which is the choice tissue type for micropropagation. Since multiple sections through root, shoot and rhizome revealed only sparse amounts of meristematic tissue, these tissues were not considered as prime sources for micropropagation. The only meristematic tissue which was found in large proportion to surrounding tissue was found in stage 5 seedlings as shown in FIGURE 6. This has most recently driven us to use stage 5 seedlings in micropropagation experiments, where meristematic cells are mechanically disassociated and cultured in liquid media containing various levels of 2,4-D and Kinetin. Preliminary results are encouraging with some cultures indicating growth after 2 weeks with one culture having a definite protocorm.

ACKNOWLEDGMENTS

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