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In-Vitro Seed Germination of *Calanthe sieboldii*, an Endangered Orchid Species

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Immature seeds from unripe capsules of *Calanthe sieboldii*, were sown on one of three sterilized media: MS; modified MSH [i.e., the inorganic salts of MS plus the organic elements of Schenk and Hildebrandt]; Hyponex. Germination and protocorm development occurred on the MS and MSH media within eight weeks, but percent germination was low. The addition of putrescine (1 mg L^{-1}) or adenine sulfate (25 mg L^{-1}) to the MSH medium enhanced germination. Resultant protocorms were subcultured on the Hyponex medium, where they developed into plantlets after 12 weeks of additional culture. Plantlets were then successfully transferred to community pots.

Keywords: adenine sulfate, *Calanthe sieboldii*, protocorm, putrescine

Many Asian orchids are threatened by extinction because of over-collection and habitat destruction. Procedures must be developed to conserve these valuable species. *Calanthe sieboldii*, an endangered, native terrestrial orchid of Korea, produces attractive flowers and has commercial value. Although in-situ conservation and preservation of natural habitats is the most suitable method for sustaining these endangered species, the development of techniques for their mass propagation and reintroduction to natural habitat is also becoming popular. In-vitro seed germination can be a powerful tool for preserving rare native orchid species. With this method, a large number of orchids can be produced while maintaining a more variable gene pool than can be obtained through clonal propagation.

To our knowledge, asymbiotic seed culture has not been reported for *C. sieboldii*. Our main objective was to develop a methodology for in-vitro germination culture of this species. Here we tested the effects of different media, additions of putrescine (polyamine) and adenine sulfate, as well as light versus dark incubation on germination.

MATERIAL AND METHODS

Plant Material

Plants of *C. sieboldii* were collected from nature and maintained under greenhouse conditions. The flowers were hand-pollinated, and ovary development was noted a week after fertilization.

Surface Sterilization of Capsules and Sowing of Seeds

Immature, 80-day-old capsules of *C. sieboldii* were used. (Normally, 120 days post-pollination are required for capsule maturation.) These capsules were surface-sterilized in 2% sodium hypochlorite for 20 min, then washed several times with sterile, distilled water. Afterward, the capsules were cut open and seeds were removed with a sterile scalpel. The seeds were placed in a centrifuge tube with 15 mL of sterile, distilled deionized water, then agitated for 2 min to disperse the clumps. The seed suspension was diluted and re-sampled until the suspension contained approximately 100 seed per 50 μL. Approximately 500 μL of suspension was cultured on each Petri dish.

Culture Media

Three basal media were evaluated for efficacy of germination: 1) a Murashige and Skoog (1962) medium; 2) a modified MS medium- (MSH)-(-inorganic salts of MS plus organic elements from Schenk

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Abbreviations: MS-medium, Murashige and Skoog (1962); MSH medium-modified MS medium containing MS inorganic salts and Schenk and Hildebrandt (1972) organic elements.

and Hildebrandt (1972); and 3) a Hyponex medium [Kano, (1965); 6.5-6-6, Hyponex Co. Ltd., Tokyo, Japan]. In another set of experiments, different concentrations of putrescine (0.5, 1, 5, and 10 mg L^{-1}) and adenine sulfate (10, 25, and 50 mg L^{-1}) were added to the modified MSH medium to determine their effect on seed germination. All media were supplemented with 2% (w/v) sucrose; pH was adjusted to 5.5. Where indicated, 0.8% agar (Sigma, St. Louis, MO, USA) was used to solidify the medium. For the seed germination studies, the 100- x 20- mm Petri dishes contained 30 mL medium. The dishes were sealed with Parafilm M (American National Can, Chicago, IL, USA). For protocorm culture, 500-mL culture jars containing 200 mL of medium were used. The media were sterilized by autoclaving for 20 min at 104 kPa and 121°C.

Alginate Encapsulation

A sodium alginic acid type IV (Sigma), solution (3% w/v) was prepared with the MSH, MS, and Hyponex liquid media. Seeds were suspended in this alginate solution and dropped into a sterilized calcium chloride solution (50 µM) via an MP-2 microtube pump (Eyela, Tokyo Rikakikai Co. Ltd. Japan). After 15 min the alginate capsules were washed in sterile, deionized water and used for the culture studies. One hundred encapsulated seeds (diameter 5 to 8 mm) were sown per Petri dish.

Culture Conditions

Following inoculation, the culture vessels were maintained at 22°C and 60% relative humidity. Two sets of cultures were prepared; one was kept in continuous dark, the other under 16 h-per-day cool, white fluorescent light of 40 µmol m^{-2} s^{-1}. A randomized experimental design was used, with 10 replicates per treatment. Percent seed germination was calculated at the protocorm stage, during Week 8 of culturing. Protocorms were counted under a stereomicroscope. Average seed germination was calculated by comparing the number of seed sown and the number of protocorms that formed.

Culture of PLBs and Transplantation of Plantlets

The well-developed protocorms were subcultured on a 1-g L^{-1} Hyponex medium (6.5-6-6). Young plantlets (4 to 6 cm tall) were transplanted to plastic pots containing peat moss and perlite (1:1). They were then raised in a growth chamber under day/night temperatures of 25°C/15°C, and a 16-h photoperiod of 200 µmol m^{-2} s^{-1}. After two weeks, the plantlets were transferred to a greenhouse, under day/night temperatures of 25°C/15°C and a 16-h photoperiod of 500 µmol m^{-2} s^{-1}. The plants were fortified with a 6.5-4.5-19.0 Hyponex solution once in 15 days.

RESULTS AND DISCUSSION

Media Effect on Seed Germination

The effects of different media and culture conditions on seed germination of *C. sieboldii* are depicted in Figure 1. After one week in culture, seeds became swollen. Germination began in the second week and continued through Week 6. The Hyponex medium did not promote seed germination. In contrast, seeds germinated on both the MS and the MSH media, but percent germination was low (Fig. 1). No differences were seen between either the liquid and solid media cultures, or the dark and light incubation treatments. However, on the MSH medium, 17.65% of the encapsulated seeds germinated and formed protocorms in eight weeks.

In-vitro germination rates for terrestrial orchids are generally low (Stoutamine, 1974; Arditti, 1979, Arditti et al., 1982). In fact, the genus *Calanthe* is the most difficult in terms of embryo germination (Kano, 1979). Miyoshi and Mii (1988, 1995) examined *Calanthe discolor* and found that ultrasonic treatments and phytohormone pre-treatments promoted better

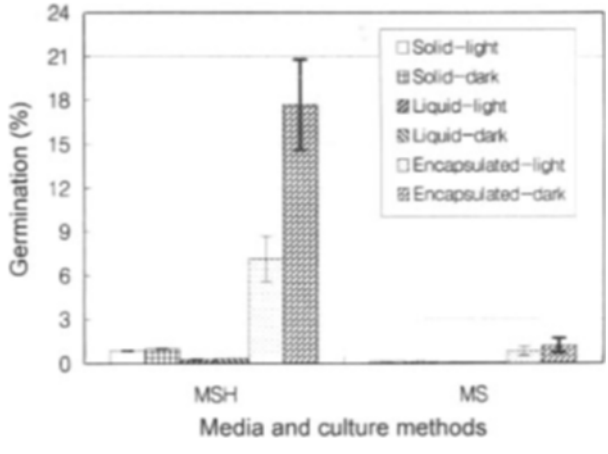

Figure 1. Effect of MSH and MS media, light and dark incubation, and liquid- and solid- media culture treatments on seed germination after eight weeks of culture. Values represent mean ± S.E. response of 10 replicates.

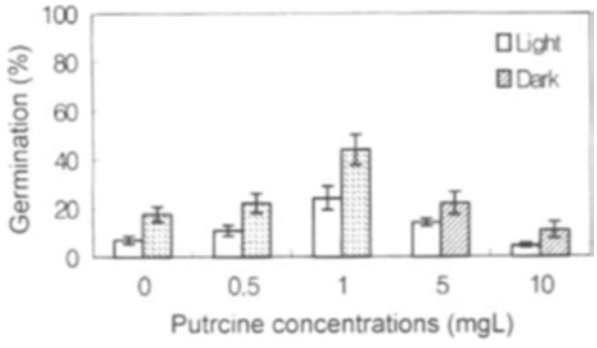

Figure 2. Effect of different concentrations of putrescine and light- or dark incubation on seed germination after eight weeks of culture. Each value represents mean ± S.E. response of 10 replicates.

germination compared with the control. In contrast to our results with *C. sieboldii*, the Hyponex medium was suitable germination of *Calanthe satsuma* (Fukai et al., 1997). This suggests that the physiological requirements for germination vary among species of the same genus. Encapsulation of the minute seeds also made handling more favorable, thereby overcoming the effect of overcrowding during germination and at the protocorm stage.

Effect of Putrescine and Adenine Sulfate Supplements in Culture Media on Germination

The effects of putrescine and adenine sulfate in the media are summarized in Figures 2 and 3. When 1 mg L^{-1} putrescine was added, germination increased two-fold over the rate for seeds in the basal MSH medium (Fig. 4B). However, higher levels (10 mg L^{-1}) inhibited germination. Maximum rates were 24 and 44% for seeds cultured in light and dark conditions, respectively. Protocorms formed by the eighth week

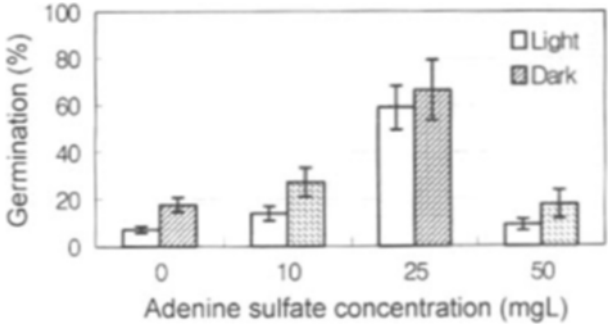

Figure 3. Effect of different concentrations of adenine sulfate and light- or dark incubation on seed germination after eight weeks of culture. Each value represents mean ± S.E. response of 10 replicates.

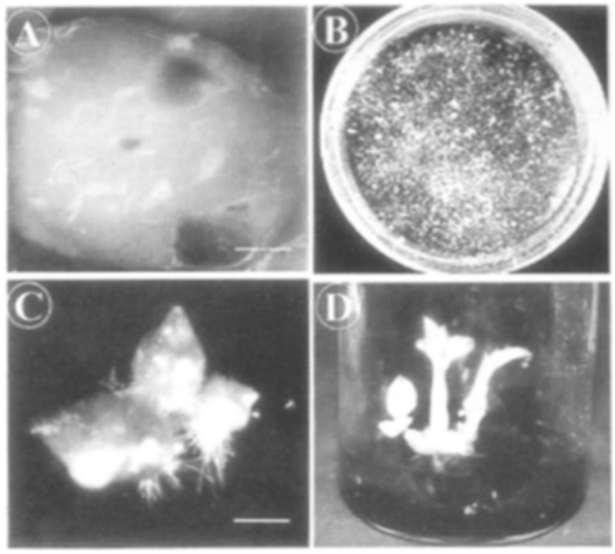

Figure 4. In-vitro seed germination of *C. sieboldii*. **A.** Encapsulated seeds. Scale bar = 2 mm. **B.** Seeds at different germination stages on MSH medium supplemented with 1 mgL^{-1} putrescine after six weeks of culture. **C.** Prorocorms of *C. sieboldii*. Scale bar = 2 mm. **D.** Plantlets developing on a Hyponex medium with 0.5% activated charcoal after two weeks of subculture.

(Fig. 4C). Polyamines play an important role in controlling the developmental process in plants. For example, putrescine has a stimulatory effect on shoot regeneration in Chinese cabbage (Pua et al., 1996) and bulblet formation in *Lillium longifolium* (Tanimoto and Matsubara, 1995).

Adenine sulfate is normally used as a growth additive in plant tissue culture (Deora and Shekhawat, 1995, Das et al., 1996). In the current study, seed germination was optimum in the medium supplemented with 25 mg L^{-1} adenine sulfate; rates were 58.8 and 66.2% in light and dark, respectively (Fig. 3). These were three-fold increases over that found with the MSH basal medium.

Germination of terrestrial orchid species is better in the dark than under illumination (Hadley, 1982; van Waes and Debergh, 1986; Rasmussen et al., 1990; Fukai et al., 1997). However, Takahashi et al. (2000) found that transfer embryonic *Habenaria radiata* from dark to light conditions enhanced the germination. In the current study of *C. sieboldii*, germination of dark-incubation culture was enhanced compared with those treated in the light.

Development of Plantlets from Protocorms and Transplantation of Plantlets

Developing protocorms were transferred to the

Hyponex medium, where they developed shoots and roots within the next eight weeks. Young plantlets were subcultured to fresh Hyponex media, with activated charcoal (0.5%) for further development (Fig. 4D). When the plantlets, were 4 to 6 cm tall, they were transferred to plastic pots containing peat moss and perlite (1:1), and kept in a growth chamber under a 16-h photoperiod of 200 μmol m^{-2} s^{-1} and day/night temperatures of 25°/15°C. The plants grew well, with 70% percent surviving at four weeks after transplantation.

In the present study the efficient seed germination was achieved in *C. sieboldii*, with the use of putrescine and adenine sulfate in vitro. The methodology developed here is useful for restoration and reintroduction of endangered species *C. sieboldii*.

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