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Asymbiotic germination of immature seeds and the seedling development of *Cypripedium macranthos* Sw., an endangered lady's slipper orchid

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ABSTRACT

Cypripedium macranthos is a lady's slipper orchid with high horticultural value. We document a reproducible protocol for asymbiotic seed germination based on a defined time frame. The optimum germination percentage was obtained from immature seeds collected at 6 weeks after pollination (WAP). At this time, the embryo reached the early globular stage. As seeds matured, the germination percentage declined sharply. Histological observation revealed that two distinct layers of seed coats enveloped the embryos tightly at maturity. The histochemical staining by Nile red and TBO indicated the presence of hydrophobic substance and phenolic compounds (e.g. lignin and cuticular material) in the seed coats. These features reflected the strong hydrophobic nature of seed coat that may cause the seed dormancy. Among the surveyed media, the modified Harvais medium was the optimum for germination. Among the surveyed organic supplements, potato homogenate (50 g l^{-1}) or banana homogenate (25 or 50 g l^{-1}) was suitable for the growth of seedlings.

1. Introduction

Cypripedium macranthos is one of the most attractive species among the lady-slipper orchids and widely found from Eastern Russia, Northern China, Japan, Korea and Taiwan (Cribb, 1997). Because of its high value in the ornamental market, many plants have been illegally collected from the wild population, making this species under the risk of becoming endangered. In China, according to the long-term investigation of *C. macranthos* by Zhang et al. (2005), the sizes of many wild populations have decreased rapidly as a consequence of over-collection or environmental disruption. Therefore, a reliable micropropagation protocol would be beneficial for conservation programs and commercial demands.

Asymbiotic germination has been a useful technique for commercial propagation of orchids (Arditti, 1967). However, the seed germination in vitro of several terrestrial orchids, such as *Calanthe* (Lee et al., 2007), *Cypripedium* (Rasmussen, 1995) and *Epipactis* (Rasmussen, 1992) is still intricate as compared to the epiphytic orchids in tropical areas. The mature seeds of terrestrial orchids usually have rigid seed coats of strong hydrophobic nature (Rasmussen, 1995). Furthermore, inhibiting substances, such as abscisic acid is found to accumulate in mature seeds of some terrestrial orchids (Lee et al., 2007; Van der Kinderen, 1987; Van Waes and Debergh, 1986). These characteristics appear to result in the poor germination of terrestrial orchids. According to the report by Miyoshi and Mii (1998), the germination percentage of mature seeds of *C. macranthos* is usually less than 5%. For improving the germination of *Cypripedium* mature seeds, various strategies have been used, such as pretreating seeds with the solution of sodium or calcium hypochlorite (Bae et al., 2010; Miyoshi and Mii, 1998; Vujanovic et al., 2000), ultrasound treatments (Lauzer et al., 1994), pre-chilling treatments (Masanori and Tomita, 1997; Miyoshi and Mii, 1998) and the symbiotic culture methods (Shimura and Koda, 2005).

To avoid the seed coat-imposed dormancy at maturity, culturing the immature seeds is an alternative way to maximize germination percentage of several *Cypripedium* species (De Pauw and Remphrey, 1993; Light and MacConaill, 1990; St-Arnaud et al., 1992). However, the determination of optimum stage for seed harvest requires a defined time frame of seed development, e.g. our previous report in *Cypripedium formosanum* (Lee et al., 2005). Currently, little is known about the time frame of seed development and the optimum stage of seed harvest of *C. macranthos*. In order to provide a reproducible protocol for culturing immature seed in vitro of *C. macranthos*, the aims of this study are to investigate the histological and histochemical changes during seed development based on a precise time frame, to determine the optimum stage for seed collection, and to

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Fig. 1. The plant of *C. macranthos* at a natural habitat in Bai Hua Mountain, Beijing, China. Scale bar = 2 cm.

examine the medium compositions for seed germination and the growth of seedlings.

2. Materials and methods

2.1. Plant material

Developing capsules of *C. macranthos* were collected from the natural population at Bai Hua mountain (1800 m above sea level), Beijing, China. Anthesis usually occurs in June, each year. For good capsule setting (approximately 80–95%), the flowers were labeled and hand-pollinated by transferring the pollinia onto the stigma of the same flower as the flowers fully opened (Fig. 1). After capsule setting, at least five capsules were collected at a two-week interval (six different developing stages) for the histological study and asymbiotic seed germination experiments.

2.2. Histological and histochemical studies

Capsules of different developing stages were sliced and fixed in 2.5% glutaraldehyde and 1.6% paraformaldehyde buffered with 0.05 M phosphate buffer, pH 6.8, for 24 h at 4 °C. After fixation, the samples were dehydrated in an ethanol series and infiltrated gradually (3:1, 1:1, and 1:3 100% ethanol:Technovit 7100, 24 h each) with Technovit 7100 (Kulzerand Co., Germany), followed by two changes of pure Technovit 7100. The samples were then embedded according to Yeung (1999). Median longitudinal serial sections, 3 μm thick, were cut using a Ralph knife on a Reichert-Jung 2040 Autocut rotary microtome. Sections were stained with Periodic Acid-Schiff's (PAS) reaction for total carbohydrate and counterstained with either 0.05% (w/v) Toluidine Blue O (TBO) in benzoate buffer for general histology or 1% (w/v) Amido Black 10B in 7% acetic acid for protein (Yeung, 1984). For detecting cuticular substance deposition in the seeds, the sections were stained with 1 μg ml^{-1} of Nile red (Sigma Chemical Co., St. Louis, MO) for 10 min, briefly washed in distilled water, and mounted in water containing 0.1% n-propyl gallate (Sigma Chemical Co.), an antifading compound as detailed in Yeung et al. (1996). The fluorescence pattern of Nile red was examined using an epifluorescence microscope (Axioskop 2, Carl Zeiss AG, Germany) equipped with the Zeiss filter set 15 (546/12 nm excitation filter and 590 emission barrier filter). The sections were observed and the images were captured digitally using a CCD camera attached to a light microscope (Axioskop 2, Carl Zeiss AG).

2.3. Measuring water content of developing seeds

Seeds of different developmental stages were dissected from placenta and dried in an oven at 70 °C for 48 h. Water content was estimated as the percentage of water loss; fresh weight minus dry weight, to its fresh weight.

2.4. Effect of collection timing on seed germination in vitro

Developing capsules were collected at intervals of two weeks, 4–14 weeks after pollination (WAP), for seed germination experiments. Five capsules were collected per time interval. The capsules were taken to the laboratory, and were surface sterilized with a 2.5% sodium hypochlorite solution for 10 min and rinsed three times with sterile distilled water. After surface sterilization, the capsules were cut open, and then the seeds were scooped out with forceps onto the culture medium. To ensure the seed quality and developmental stages of each capsule, the remaining seeds of each capsule were fixed and examined under a microscope. Approximately 90% of seeds contained fully developed embryos (as confirmed by microscopy). Seeds from each capsule were mixed and evenly distributed into twelve flasks (replicates). The culture medium used in this experiment was the Harvais medium (Harvais, 1982), which was modified by supplementing with 2 mg l^{-1} glycine, 0.5 mg l^{-1} niacin, 0.5 mg l^{-1} pyridoxine HCl, 0.1 mg l^{-1} thiamine, 1 g l^{-1} tryptone, 20 g l^{-1} sucrose, 100 ml l^{-1} coconut water, 0.5 g l^{-1} activated charcoal, and 7 g l^{-1} agar. The pH was adjusted to 5.6 before autoclaving at 121 °C for 15 min. Twenty milliliters of medium were placed into each Erlenmeyer flask (125 ml). The experiments were performed in a randomized design. Each treatment comprised twelve flasks and was conducted three times. After sowing, the cultures were incubated in a growth room in the dark at 20 ± 1 °C.

2.5. Effect of basal medium on seed germination in vitro

In the preliminary studies, seeds collected at 6 WAP had constant good germination percentages. Therefore, to investigate the influence of basal medium on seed germination in vitro, seeds collected at 6 WAP were inoculated on four different basal media: Murashige and Skoog (MS) medium (Murashige and Skoog, 1962); Knudson C (KC) medium (Knudson, 1946); Harvais medium (Harvais, 1982); Vacin and Went (VW) medium (Vacin and Went, 1949). These four basal media were modified by supplementing with 2 mg l^{-1} glycine, 0.5 mg l^{-1} niacin, 0.5 mg l^{-1} pyridoxine HCl, 0.1 mg l^{-1} thiamine, 0.5 g l^{-1} tryptone, 20 g l^{-1} sucrose, 100 ml l^{-1} coconut water, 0.5 g l^{-1} activated charcoal, and 7 g l^{-1} agar. The pH was adjusted to 5.6 before autoclaving at 121 °C for 15 min. Twenty milliliters of medium were placed into each Erlenmeyer flask (125 ml). The experiments were performed in a randomized design. Each treatment comprised twelve flasks (replicates) and was conducted three times. Six capsules were collected, and the seeds from each capsule were evenly distributed into the replicates of four basal media. After sowing, the cultures were incubated in a growth room in the dark at 20 ± 1 °C.

2.6. Effect of organic supplements on seedling growth in vitro

In our preliminary investigations, Harvais medium was suitable for seed germination and the survival of protocorms. Therefore, the Harvais medium supplemented with 2 mg l^{-1} glycine, 0.5 mg l^{-1} niacin, 0.5 mg l^{-1} pyridoxine HCl, 0.1 mg l^{-1} thiamine, 0.5 g l^{-1} tryptone, 20 g l^{-1} sucrose, 1 g l^{-1} activated charcoal, and 7 g l^{-1} agar was selected as the basal medium to investigate the influence of organic supplements on seedling growth in vitro. Different organic supplements: 50, 100 ml l^{-1} coconut water, or 25, 50 g l^{-1} banana homogenate, or 25, 50 g l^{-1} potato homogenate were tested. The