Seed Development of *Cypripedium debile* Rchb. f. in Relation to Asymbiotic Germination

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Abstract. The histological and histochemical changes in developing seeds of *Cypripedium debile* Rchb. f., a native slipper orchid species with horticultural potential, were investigated. The effects of timing for seed collection, culture media, and cultural conditions were also examined. The optimum germination percentage occurred when mature seeds were collected and sowed on 1/4 Murashige and Skoog basal medium. Besides, the liquid culture promoted germination of mature seeds. This finding is contrary to most other *Cypripedium* species, which are relatively easy to germinate with immature seeds. Moreover, two notable cytological changes of *C. debile* were observed. First, the suspensor protruded beyond the micropyle opening of the inner seedcoat, making the inner seedcoat not substantial. Second, Nile red staining indicated that the deposition of cuticular material on the seedcoat was fragmentary. It is proposed that the less hydrophobic nature of the seedcoat makes mature seeds of *C. debile* easier to obtain water and nutrients for germination.

The genus *Cypripedium* L. is one of the most fascinating groups of orchids, distributing in the temperate region or the high mountains of the Northern Hemisphere (Cribb, 1997). Species of this genus are of high value in ornamental markets because of their exotic flowers. *Cypripedium debile* Rchb. f. is a miniature species (≈10 to 12 cm tall), making it ideal as a compact potted plant. Only a few populations could be found in central China, Japan, and Taiwan (Cribb, 1997; Lin, 1987). Like many *Cypripedium* species, *Cypripedium debile* is threatened as a result of habitat fragmentation and overcollection. Therefore, it is desirable to establish protocols of seed germination for ex situ conservation and reintroduction programs.

Asymbiotic germination is a useful technique to propagate and conserve several endangered orchid species (Arndt and Ernst, 1993; Fay, 1992; Pritchard, 1989), whereas some terrestrial orchids are relatively difficult to germinate in vitro (Rasmussen, 1995). Seeds of terrestrial orchids usually have rigid seedcoats, enveloping the embryos tightly at seed maturity (Lee et al., 2005; Yamazaki and Miyoshi, 2006). As seeds approach maturity, some chemical compounds, e.g., polyphenols, cuticular materials, or lignin, may deposit on the seedcoat and contribute to the hydrophobic nature of mature seeds (Carlson, 1940; Lee et al., 2005; Yeung et al., 1996). These factors may bring about the impermeability of mature seeds and result in low seed germination percentage. Moreover, the accumulations of high levels of endogenous abscisic acid in the mature seeds also inhibit seed germination (Lee et al., 2007; Van der Kinderen, 1987; Van Waes and Debergh, 1986). Consequently, culturing immature seeds was frequently used to maximize germination the percentage of several terrestrial orchids (De Pauw and Remphrey, 1993; Light and MacConaill, 1990). Our preliminary experiments demonstrated that the near mature seeds of *C. debile* had better germination percentage than immature seeds. This is contrasting to the germination profiles of most *Cypripedium* species (De Pauw and Remphrey, 1993; St. Arnaud et al., 1992). Currently, little is known about the seed development and the characteristic of seeds in *C. debile*. Our previous works have demonstrated that the basic knowledge of seed development in several orchids enables a more efficient means about the asymbiotic germination (Lee et al., 2005, 2008). The aim of this study was to investigate the histological and histochemical changes during seed development of *C. debile* and their relations to the seed germination in vitro.

Material and Methods

Plant material. Developing capsules of *C. debile* were collected from a natural population in the bamboo forest located at Hohuan Mountain, Nantou County, Taiwan. Anthesis usually occurred in early June each year. After capsule setting, developing capsules of different stages were collected at regular intervals for histological and asymbiotic seed germination studies.

Light microscopy. 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 ºC. After fixation, the sections were dehydrated in methyl cellosolve for 24 h followed by two changes of 100% ethanol for 24 h each at 4 ºC. The samples were infiltrated gradually (3:1, 1:1, and 1:3 100% ethanol: Historesin, 24 h each) with Historesin (Leica Canada, Markham, Ontario, Canada) followed by two changes of pure Historesin. The tissues were then embedded according to Yeung (1999). Median longitudinal serial sections, 3 μm thick, were cut using a Ralf knife on a Reichert Jung 2040 Autocut rotary microtome. Sections were stained with periodic acid-Schiff's reaction for total carbohydrate and counterstained with either 0.05% (w/v) toluidine blue O (TBO) in benzene buffer for general histology or 1% (w/v) amido black 10B in 7% acetic acid for protein. The accumulation of cuticular material was detected by Nile red staining as detailed in Yeung et al. (1996). The Historesin-embedded tissues 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. The fluorescence pattern 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 viewed and the images were captured digitally using a CCD camera attached to a light microscope (Axioskop 2; Carl Zeiss AG).

Effect of seed collection timing and cultural medium on seed germination. Capsules of various developing stages were collected for asymbiotic germination experiments. The capsules were taken to the laboratory and were surface-sterilized with a 1% sodium hypochlorite solution for 15 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). Two cultural media used in this experiment were the modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and 1/4 Murashige and Skoog basal medium (Murashige and Skoog, 1962).
and Thomale GD medium (Thomale, 1957). The modified MS medium contained 1/4 strength of macroelements with full strength of microelements, 2 mg L⁻¹ glycine, 0.5 mg L⁻¹ nicotin, 0.5 mg L⁻¹ pyridoxine HCl, 0.1 mg L⁻¹ thiamine, and 100 mg L⁻¹ myo-inositol. Both the modified MS medium and Thomale GD medium were supplemented with 1 g L⁻¹ tryptone, 1 g L⁻¹ yeast extract, 20 g L⁻¹ sucrose (Sigma Chemical Co.), and 100 mL L⁻¹ coconut water. Fresh coconut water was taken from a green coconut and was added into the culture medium immediately. The pH value was adjusted to 5.6 before autoclaving. Twenty milliliters of medium were placed into each 10-cm diameter plate (90 × 15 mm; Scientific Corp., Taiwan) in the laminar flow cabinet. After sowing, the plastic plates were sealed with parafilm and incubated in a growth room at 25 ± 1 °C in constant darkness.

Seedling growth medium for developing protocorms. After 5 months of seed culture in vitro, the young protocorms were transferred to the seedling growth medium: 1/4 MS medium supplemented with 20 g L⁻¹ sucrose, 1 g L⁻¹ activated charcoal, 20 g L⁻¹ potato homogenate, and 7 g L⁻¹ agar for future growth as reported by Lee (2010). The pH value was adjusted to 5.6 before autoclaving. One hundred milliliters of medium was placed into a 500-mL culture flask. After transferring the young protocorms, the flasks were incubated in a growth room at 25 ± 1 °C in constant darkness.

Germination percentage calculation and data analysis. Each plate or flask was examined at 1-month intervals for 5 months in culture by using a Carl Zeiss stereomicroscope (10 × magnification). Germination was defined as emergence of the embryo from the testa. The germination percentage was calculated as the percentage of the number of seeds germinated among the total countered number of seeds with embryos. Experiments were performed in a randomized design. Twelve replicates were taken for each treatment in the experiments of seed germination in vitro. The data were statistically analyzed using analysis of variance followed by Fisher’s protected least significant difference test.

Results

Structural and histochemical study. After fertilization (∼2 months after anthesis), the zygotes and proembryos could be observed within the capsules. The zygote was highly polarized with a large vacuole located toward the micropylar end (Fig. 1A). The cytoplasm at the zygote tip contained several tiny starch granules congregating around the nucleus. In the six-celled proembryo, the cells of embryo proper had dense cytoplasm, whereas the basal cell was highly vacuolated (Fig. 1B). By the early globular stage (∼3 months after anthesis), protoderm had formed (Fig. 1C). Numerous starch granules were found to accumulate in the cytoplasm of the developing embryo proper and the suspensor. In this species, the suspensor consisted of a single cell that was highly vacuolated. At this stage, the suspensor cell had protruded beyond the micropyle opening of the inner seedcoat and grew into the lumen enclosed by the outer seedcoat. As the embryo reached the globular stage (∼4 months after anthesis), mitotic activity had ceased and the suspensor cell began to degenerate (Fig. 1D). At this stage, the cytoplasm of embryo proper still contained numerous starch granules. At maturity (∼5 months after anthesis), the embryo was only eight cells long and five cells wide (Fig. 1E). The embryo cells had abundant storage products in the form of lipid and protein bodies in the cytoplasm, whereas the starch granules became absent as the embryo approached maturity.

The seedcoat of C. debile has two layers: the inner and outer seedcoats that were derived from the inner and outer integuments of the ovule separately. Both of them were composed of two layers of highly vacuolated parenchyma cells (Fig. 1A). As the seeds...
approached maturity, the cells of the seedcoat dehydrated and constricted into a thin layer, which enclosed the embryo (Fig. 1E). At maturity, staining by using Nile red demonstrated that cuticular materials were present over the surface wall of embryo proper and the radial wall of outer seedcoat, whereas the fluorescence was less intensive in the inner seedcoat (Figs. 1F and 1H). Moreover, only the radial wall of the outer seedcoat stained greenish blue with the TBO stain, indicating the presence of phenolic compounds in the radial wall.

Seed germination in vitro and seedlings development. In C. debile, high germination (72.6% to 75.0%) was obtained with the seeds collected in the globular and mature stages (≥4 to 5 months after anthesis). On the contrary, no germination was obtained with the immature seeds collected from the zygote to early globular stages. In addition, the germination of mature seeds was higher in 1/4 MS medium than in Thomale GD medium (75.0% and 72.6%, respectively; Table 1). In the liquid culture, the germination rate was higher and more uniform than those in the solid culture (Figs. 2 and 3A–B). For both liquid and solid cultures, the germination increased sharply after 2 months in culture and reached the maximum after 5 months in culture. The roots began to develop from young protocorms after 1 month of culture in the seedling growth medium (Fig. 3C). After 2 months of culture, the formation of shoots from seedling could be observed (Fig. 3D).

Discussion

In the present study, the optimum germination of C. debile was found in mature seeds (Table 1). This feature is contrary to previous reports of most Cypripedium species, in which mature seeds are more difficult to germinate in vitro than immature seeds (De Pauw and Remphrey, 1993; Lee et al., 2005). From the results of histological and histochemical studies, two notable cytological changes were observed during the course of seed formation. First was the protruding suspensor cell beyond the micropyle opening of the inner seedcoat at the globular stage. Second was the fragmentary deposition of cuticular material on the seedcoat.

Most terrestrial orchids have firm seedcoats protecting their embryos at maturity (Arditti, 1967; Rasmussen, 1995). In the difficult-to-germinate species, besides the outer seedcoat, there is one more layer of seedcoat: the inner seedcoat (also known as “carapace”) that formed a rigid envelope enclosing the embryo at seed maturity (Carlson, 1940; Lee et al., 2005; Veryet, 1969; Yamazaki and Miyoshi, 2006). In C. debile, during the early stages of embryo development (Fig. 1A–B), the proembryo was growing in the lumen enveloped by the inner seedcoat. At the early globular stage, the suspensor cell protruded outside the micropylar opening of the inner seedcoat (Fig. 1C). On the contrary, in the more difficult-to-germinate species such as C. formosanum Hayata (Lee et al., 2005) and Cephalanthera falcate (Thunb.) Blume (Yamazaki and Miyoshi, 2006), their suspensor cells never grew beyond the inner seedcoat, and consequently their embryos were enveloped more tightly by the inner seedcoat as the seed approached maturity. In C. debile,
because the suspensor cell wall is primary in nature (Fig. 1E–F), the micropylar site of the inner seedcoat may remain the character of the primary cell wall as the protruded suspensor cell and the inner seedcoat compressed into a thin layer at seed maturity. This flexible micropylar site of the inner seedcoat may imply that there are fewer obstacles for the embryos to absorb water and nutrients when sowing the mature seeds on culture media.

The accumulation of cuticular material is commonly observed in the epidermal tissue, forming a vital hydrophobic barrier over the aerial surfaces, preventing water loss and gaseous exchanges (Esau, 1977). In orchid seeds, because their embryos are rudimentary and are enveloped by thin seedcoats, the presence of cuticular material in the protoderm of globular embryos and seedcoat may help to protect the embryos from early desiccation (Lee et al., 2006; Yeung et al., 1996). The thick cuticular layer covering the surface of the inner and outer seedcoats has been detected in several difficult-to-germinate orchid species. In *C. falcata*, the accumulation of lignin and cuticular material in the inner seedcoat has been reported (Yamazaki and Miyoshi, 2006). Additionally, in *C. formosanum* (Lee et al., 2005), the intense staining of Nile red in both the inner and outer seedcoats indicates the heavy accumulation of cuticular material. The results of these studies suggest that the cutinization and lignification of inner seedcoat may play a regulatory role in controlling the seedcoat-imposed dormancy. On the contrary, in the easy-to-germinate epiphytic orchids, i.e., *Phalaenopsis* Blume (Lee et al., 2008) and *Bulbophyllum* Thouars (Lee and Yeung, 2010), the seedcoat cells with discontinuous thickened walls in the radial direction may not block the uptake of water and nutrients for germination. From the results of Nile red staining in *C. debile* seeds (Figs. 1F and 1H), it is worthy to note that the cuticular material is primarily present in the embryo surface, and it is weak and discontinuous in the radial wall of the outer seedcoat. This feature suggests the seedcoat of *C. debile* has a less hydrophobic nature than the seedcoat of *C. formosanum*, which makes the mature seeds of *C. debile* easier to germinate. In the previous report by Tomita and Tomita (2002), a decrease of germination percentage was found in mature seeds of *C. debile*. It is possible that the populations of *C. debile* in the north temperate area have deeper seedcoat-imposed dormancy. In the future study, it would be interesting to investigate if the seeds of *C. debile* in Japan have a more substantial seedcoat and hydrophobic nature.

In this study, the increased germination percentage and germination rate were found in liquid culture (Figs. 2 and 3A). Liquid culture has been shown to improve the seed germination of slipper orchids such as *C. calceolus* (L.) var. pubescens (Willd.) Correll and Mudge (1946) and *Paphiopedilum primulinum* M. Wood & P. Taylor (Lee and Lee, 2001). Liquid culture may facilitate the entrance of water and nutrients into seeds and help diffuse exudates from seeds. Unlike most *Cypripedium* species, *C. debile* is not perennial. Under the natural condition, the plant of *C. debile* can survive no longer than three years (our unpublished data). As compared with most *Cypripedium* species, the seedcoat of *C. debile* does not look substantial and is less hydrophobic, which makes the mature seeds easier to obtain water and nutrients for germination. The speedy generation of numerous young seedlings annually may provide another strategy for maintaining the populations in natural habitats.

**Literature Cited**


