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Embryo Development of *Cypripedium formosanum* in Relation to Seed Germination In Vitro

Yung-I Lee and Nean Lee
Department of Horticulture, National Taiwan University, No 1, Sec. 4, Roosevelt Road, 106, Taipei, Taiwan, Republic of China

Edward C. Yeung
Department of Biological Science, University of Calgary, Calgary, Alberta T2N 1N4, Canada

Mei-Chu Chung[1]
Institute of Plant and Microbial Biology, Academia Sinica, 11529, Taipei, Taiwan, Republic of China

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Abstract. **This investigation documents the key anatomical features in embryo development of *Cypripedium formosanum* Hayata, in association with the ability of embryos to germinate in vitro, and examines the effects of culture media and seed pretreatments on seed germination. A better understanding of zygotic embryogenesis for the *Cypripedium* L. species would provide insights into subsequent germination events and aid in the in vitro propagation of these endangered species. In seeds collected at 60 days after pollination (DAP), soon after fertilization, no germination was recorded. The best overall germination was found at 90 DAP (≈70%), at which time early globular to globular embryos with a single-celled suspensors can be observed. After 135 DAP, the seeds germinated poorly. At this time the inner integument shrinks and forms a tight layer, which encloses the embryo, the so-called "carapace." Using Nile red stain, a cuticular substance was detected in the carapace, which may play a role in the impermeability of the mature seed and may help the seeds survive in the stringent environment. At maturity (after 210 DAP), the embryo proper has an average size of eight cells along its length and six cells across the width. Lipids and proteins are the main storage products within the embryo. To improve seed germination, experiments were conducted to test the suitability of various media and pretreatments of seeds. When different media were used, except for the Harvais medium at 120 DAP, there was no significant difference in seed germination at three different developmental stages tested. Soaking mature seeds in 1% NaOCl or treating them with ultrasound may slightly increase the germination percentage. For seed germination, our results indicate that the timing of seed collection outweighs the composition of medium and the seed pretreatments.**

The genus *Cypripedium* comprises ≈45 species, found over an extensive latitudinal range, extending from north of the Arctic Circle to southwestern China and even further south to Central America (Cribb, 1997). The North American *Cypripedium* species are usually found in lowlands, whereas the south Asiatic species inhabit high mountainous areas. *Cypripedium formosanum*, a representative terrestrial orchid species, is found in the high altitude mountains of Taiwan (Lin, 1987). Its delicate flowers make it a desirable candidate for the horticultural trade. Unfortunately, the wild populations are becoming increasingly scarce, and are under threat of extinction due to over-collection and habitat destruction. Hence, there is an urgent need to conserve this endangered species.

Asymbiotic germination is a practical and useful technique to re-establish plants in the wild and also for commercial propagation (Arditti, 1967). Although many orchids have been propagated successfully, using this technique, the *Cypripedium* species is still considered difficult (Arditti and Ernst, 1993; Oliva and Arditti, 1984; St-Arnaud et al., 1992). According to De Pauw and Remphrey (1993), there is an optimum time, which is crucial to collect seed to maximize germination. It has also been reported that fully mature seeds of the *Cypripedium* species are more difficult to germinate in vitro than immature seeds (De Pauw and Remphrey, 1993; St. Arnaud et al., 1992; Withner, 1953). In spite of the fact that the timing of seed collection plays a critical role in germination, knowledge of the time frame for embryo ontogeny, in the *Cypripedium* species, is lacking. A better understanding of zygotic embryogenesis for the *Cypripedium* species would provide insights into subsequent germination events, and aid in the in vitro propagation of these endangered species. Furthermore, previous studies have revealed that the North American *Cypripedium* species have stringent germination requirements for nutrients (Harvais, 1973; Henrich et al., 1981), cultural conditions (Chu and Mudge, 1994) and plant growth regulators (De Pauw et al., 1995; Harvais, 1982). Although the seed germination of the North American *Cypripedium* species has been studied in detail, little is known about the Asiatic *Cypripedium* species (Masanori and Tomita, 1997; Yoshikazu et al., 1994).

Seed pretreatments may overcome dormancy and improve germination by altering the physical integrity of testa, making the integument tissues more permeable (Linden, 1980; Miyoshi and Mii, 1988; Van Waes and Debergh, 1986a, 1986b). Soaking in NaOCl solution between 10 and 45 min can also increase germination of some European terrestrial orchids (Linden, 1980; Van Waes and Debergh, 1986a, 1986b). Miyoshi and Mii (1988) suggested that ultrasonic treatment might enhance seed germination of *Calanthe* R. Brown species by breaking the testa and increasing the permeability of the integument tissues.

Thus, this paper has attempted to document the key anatomical events in the embryo development of *C. formosanum* from

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[1]Corresponding author: E-mail: bomchung@gate.sinica.edu.tw

fertilization to seed maturity, within a defined time scale [15 days after pollination (DAP)]. Our goal was to determine the optimum time for seed collection, in order to maximize germination percentage, and to examine the influence of culture media and seed pretreatments on germination in vitro.

Material and Methods

Pollination and capsule collection. The plants of *C. formosanum* were maintained at Mei-Feng farm (2100 m above sea level) in Taiwan. Anthesis usually takes place in March, each year. When the flowers had fully opened, they were pollinated manually by transferring pollinia onto the stigma of the same flower in the third week of March. Hand pollination ensured good capsule sets, seed quantity, and seed viability. We usually got ≈60,000 seeds per capsule by hand pollination. In the end of November or early December, capsules begin to ripen and split after 240 DAP. In each experiment, at least five capsules were collected, at regular intervals, after pollination.

Cytological study. Developing capsules were collected and fixed in a solution of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 6.8, at 4 °C overnight. After three 15-min buffer rinses, the materials were postfixed in 1% OsO_4 in the same buffer for 4 h at room temperature, then rinsed in three 15-min changes of buffer. Following fixation, the materials were dehydrated in an acetone series, and embedded in Spurr's resin (Electron Microscope Sciences, Washington, Pa.). Serial sections 1 μm thick were cut, using a diamond knife, on an ultramicrotome (Ultracut E; Reichert-Jung, Vienna, Austria). Some of the other fixed tissues were dehydrated through a methyl cellosolve, 100% ethanol series. These tissues were then infiltrated with Historesin (Leica, Toronto, Canada) and subsequently embedded in the same material (Yeung, 1999). Sections, 2–3 μm thick, were cut using a glass knife on a Reichert 2040 Autocut microtome. These sections were stained with the periodic acid–Schiff (PAS) procedure and counterstained with toluidine blue O (TBO; Merck KGaA, Darmstadt, Germany). Histochemical staining of protein and total carbohydrates was performed according to Yeung (1984). The sections were stained with the PAS reaction for total carbohydrates, and counterstained with amido black 10B (Merck KGaA) for proteins. Red coloration indicated carbohydrates, while blue coloration indicated proteins. These preparations were examined and photographed with a Nikon E-600 light microscope (Nikon USA, Stamford, Conn.).

The presence of the cuticle was detected using Nile red 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) 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 a Nikon E-600 epifluorescence microscope.

Timing of seed collection. In this experiment, five capsules were collected at intervals of 15 d, 60–240 DAP from year 1999 to 2001. The capsules were taken to the laboratory, surface sterilized with a 1% sodium hypochlorite solution for 20 min, and rinsed three times with sterile distilled water. After surface sterilization, the capsules were cut open, the seeds were scooped out with forceps and placed onto the Thomale GD medium (Thomale, 1957), supplemented with 20 g·L^{-1} sucrose (Sigma Chemical Co.), 100 mL·L^{-1} coconut water and solidified with 2.2 g·L^{-1} Phytagel (Sigma Chemical Co.). The pH value was adjusted to 5.7 before autoclaving.

Media for in vitro germination. For this study, in order to investigate the interaction of the timing of seed collection and culture medium effects on seeds germination, seeds collected at 90, 120, and 150 DAP were inoculated on four different media compositions: Harvais medium (Harvais, 1982); modified MS medium (Murashige and Skoog, 1962); modified Norstog medium (Norstog, 1973); and Thomale GD medium (Thomale, 1957). Seeds from each capsule were evenly distributed into four replicates. Each treatment had 20 replicates which were composed of seeds from those five capsules. The modified MS medium contained macro-elements at 1/4 strength and microelements at full strength. Norstog medium was modified as detailed in De Pauw and Remphrey (1993). These four media were supplemented with 20 g·L^{-1} sucrose, 100 mL·L^{-1} coconut water and solidified with 2.2 g·L^{-1} Phytagel. The pH value was adjusted to 5.7 before autoclaving.

Seed pretreatments and TTC staining. Seeds at 180 DAP were used for the pretreatments and 2,3,5 triphenyl tetrazolium chloride [TTC (Merck KGaA)] staining. There were five capsules collected for each pretreatment and TTC staining. Each treatment had 20 replicates. After surface sterilization, the capsules were dissected and the seeds were scooped out. Both chemical and physical pretreatment methods used in this study followed previous descriptions (Linden, 1980; Van Waes and Debergh, 1986a, 1986b). Chemical methods included soaking the seeds in three different solutions, 1% NaOCl, 1 N NaOH, and 1 N HCl, with two drops of a wetting agent (Tween 20; Sigma Chemical Co.) each, for 15, 30, 45, and 60 min, respectively. Ultrasonic pretreatment was the physical method used. Seeds were scooped into the tubes with distilled water, and treated with the ultrasonicator (Branson 8210; Branson Ultrasonic Corp., Danbury, Conn.) for 15, 30, 45, and 60 min. After the pretreatments, the seeds were rinsed three times with sterilized water and either placed on the germination medium or subjected to TTC staining. In this experiment, Thomale GD medium was used as the basal medium and supplemented with 20 g·L^{-1} sucrose, 100 mL·L^{-1} coconut water and solidified with 2.2 g·L^{-1} Phytagel. The pH value was adjusted to 5.7 before autoclaving. TTC staining was carried out in 60-mm-diameter petri dishes containing 15 mL of a 1% TTC solution, at pH 6.5 (Lauzer et al., 1994). Staining experiment was performed a minimum of three times with 100–150 seeds in each petri dish. Seeds were incubated in darkness at 30 ± 1 °C for 4 d. Embryos remaining yellow were considered as unstained, and those turning orange to red were classified as stained.

Inoculation and culture conditions for germination. The capsules were surface sterilized, in a laminar flow hood, for 30 min in a 1% sodium hypochlorite solution containing two drops of a wetting agent (Tween 20), and then rinsed in sterile distilled water. They were then dissected and the seeds were scooped onto the surface of gelled medium in 20 × 100-mm culture tubes dispensed with 10 mL of medium. Seeds from each individual capsule were assigned to all treatments. There were ≈100 seeds per tube. After sowing, the culture tubes were placed in a growth room at 25 ± 1 °C, in constant darkness.

Germination ratio calculation and data analysis. Each tube was examined and recorded at 15-d intervals during the 120 d, using a Nikon stereomicroscope (10× magnification). Germination was defined as emergence of the embryo from the testa. The germination ratio was calculated as the percentage of the number of seeds that germinated, out of the total number of seeds.

At each sampling date, five capsules were harvested for inoculation. In all experiments, 20 replicates per treatment were arranged

in a completely randomized design. Data were statistically analyzed by using analysis of variance (ANOVA) method followed by Duncan's multiple range test (Duncan, 1955). Percentage data were arcsine transformed before performing ANOVA.

Results

Capsule development. After successful pollination, the ovary began to enlarge as the ovular tissue proliferated and ovule development and seed formation proceeded within the ovary. The main structural changes occurring within the ovary from 0 DAP until seed maturity at 240 DAP are summarized in Table 1. The length of the ovary extended steadily (≈1 mm/d) at 20 DAP, and then grew slowly, until the maximum size (≈51 mm) was reached at 40 DAP (Fig. 1). The diameter of the ovary also increased steadily until the maximum size (≈13 mm) was reached at 60 DAP.

Embryo development. At 60 DAP, most of the ovules had been fertilized and embryo development commenced. The zygote took on an elongated shape (Fig. 2A). The nucleus was located toward the chalazal end and a prominent vacuole was found toward the micropylar end. Endosperm failed to develop in this species. The nuclei within the endosperm cavity formed a polar chalazal complex (Fig. 2A); these nuclei would eventually disintegrate as the embryo developed. The first zygote division was unequal, producing a larger basal cell and a smaller terminal cell (Fig. 2B). Derivatives of the basal cell gave rise to the suspensor, and the terminal cell gave rise to the embryo proper. The suspensor of this species consisted of a single cell that was highly vacuolated (Fig. 2F). No further divisions or enlargement of the suspensor could be seen through the early globular stage (Figs 3A and B). Once the embryo developed to a late globular stage (Fig. 3C), the single-celled suspensor became highly compressed and degenerated.

At 75 DAP, two- to three-celled embryos were commonly observed (Fig. 2B and C). At 85 DAP, the cell at the terminus divided anticlinally, giving rise to a four-celled embryo (Fig. 2D). Soon after, the middle cell of the four-celled embryo divided periclinally, resulting in the formation of a six-celled embryo (Fig. 2E). As development progressed (90 DAP), cell divisions occurred within each of the embryos proper, resulting in an increase in size of the proembryo (Fig. 2F). By 105 DAP, anticlinal divisions in the outer-most layer clearly delineated the protoderm while allowing for increasing the volume of the embryo as cell division occurred in the inner cells, forming a globular embryo (Fig. 3A).

At 120 DAP, mitotic activity ceased within the embryo proper (Fig. 3C). The cells of the embryo proper had a dense cytoplasm throughout their development, and the cell expanded as storage products accumulated. At maturity (after 210 DAP), the embryo was only eight cells long and six cells wide (Fig. 3E). Nile red staining indicated that the surface layer of the embryos was covered by cuticular substance (Fig. 3F).

Storage products. Starch was the first product to accumulate within the embryo. Starch granules were detectable in small quantities in the upper region of the embryo proper (Fig. 3B). After the cells had ceased to divide, the large vacuoles began to breakdown. Protein and lipid accumulation were greatest at 120 DAP (Fig. 3C). At maturity, a large quantity of protein bodies and lipid bodies had been deposited within the cells of the embryo proper.

Changes in the integumentary tissues. The seedcoat developed from the outer integument, which in the young ovule consists of two-celled layers. Initially, the outer integuments were highly vacuolated and contained chloroplast starch granules (Fig. 3A and B). As the seed matured, a secondary wall was added to cells of the seedcoat. At maturity, the cells became dehydrated and compressed into a thin layer (Fig. 3E). The dehydrated seedcoat was stained blue green with the TBO stain and reacted positively to the Nile red stain.

The inner integument was two cells thick. After fertilization, mitotic activity is not detected within the inner integument. It is composed of vacuolated parenchyma cells and plastids and starch granules are not apparent within the cytoplasm of the cells (Fig. 3B). By 150 DAP, the cells of the inner integument began to dehydrate and compress into a tight and thin layer, known as the carapace (Fig. 3D). The carapace was stained blue with the TBO stain also reacted positively to Nile red stain (Fig. 3E). At maturity, the embryo was enveloped by such a carapace.

Timing of seed collection. The experiments were repeated in three consecutive years (1999–2001) to confirm the validity of findings (Fig. 4). The work, presented here, shows that the timing of seed collection had as a significant effect on the germination of *C. formosanum*. The best overall germination rate was found

Table 1. Major microscopic structural events occurring in the developing fruits of *C. formosanum* after pollination.

DAP[z]	Developmental stage	Seed color
0	Ovule primordia	---
15	Archesporial cell	---
30	Megasporogenesis and megagametogenesis	---
45	Fertilization	---
60	Zygote	White
75	Proembryo	White
90	Early globular embryo	Yellowish white
105	Globular embryo	Yellowish white
120	Late globular embryo, and the suspensor starts to degenerate	A mixture of yellow and light brown seeds
150	Suspensor degeneration	Light brown
180	Seed desiccation	Brown
210	Dry, mature seed	Dark brown
240	Fruit ripe and split	Dark brown

[z]DAP = days after pollination.

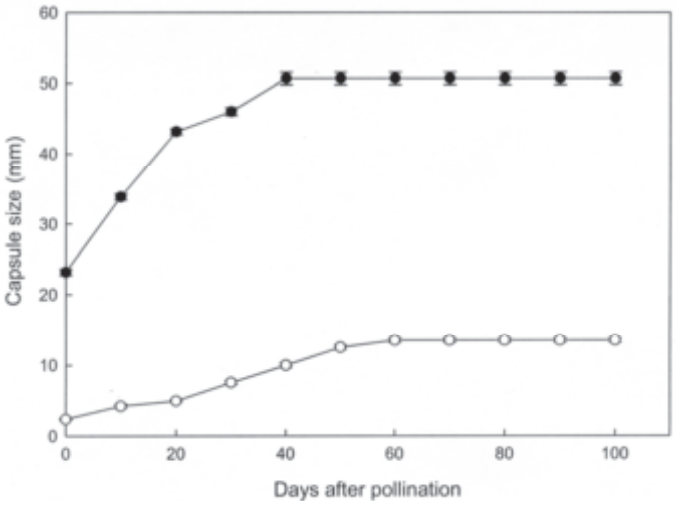

Fig. 1. Changes in capsule length (●) and width (○) of *C. formosanum* during development. Error bars represent SE (n = 3).

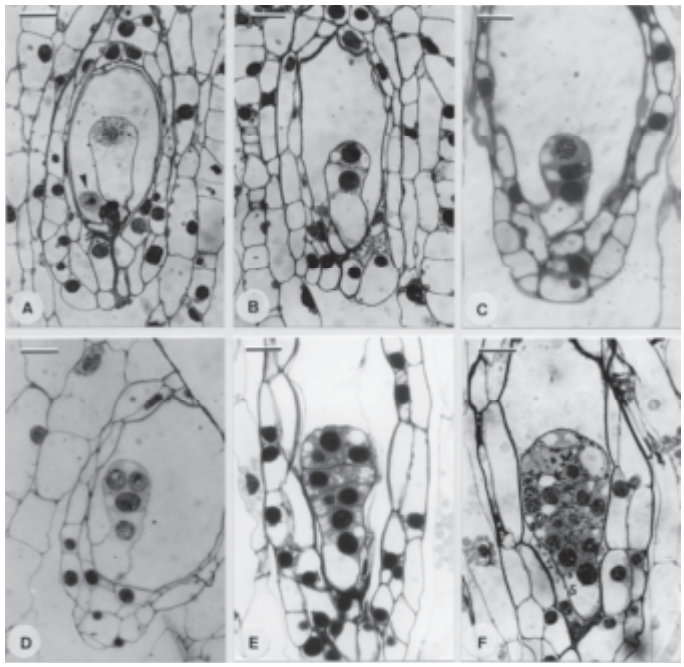

Fig. 2. Early embryo development of *C. formosanum*. (**A**) A median longitudinal section showing a zygote at 60 d after pollination. The nucleus is located toward the chalazal end, while a large vacuole is located at the micropylar end of the cell. Scale bar = 30 μm. (**B**) Light micrograph showing a two-celled embryo at 60 d after pollination. The cell division is unequal as judged from the location of the newly formed cell plate. Scale bar = 30 μm. (**C**) Light micrograph showing a three-celled embryo at 75 d after pollination. Scale bar = 25 μm. (**D**) At 75 d after pollination, a T-shape, four-celled embryo is observed, which is the product of an anticlinal division occurring in the terminal cell of a three-celled embryo. Scale bar = 30 μm. (**E**) Light micrograph, showing a periclinal division occurring within the middle cell, resulting in the formation of a six-celled embryo at 75 d after pollination. Scale bar = 30 μm. (**F**) Light micrograph showing a longitudinal section through an early globular embryo with a single-celled suspensor (S) at 90 d after pollination. The suspensor cell is highly vacuolated. Scale bar = 20 μm.

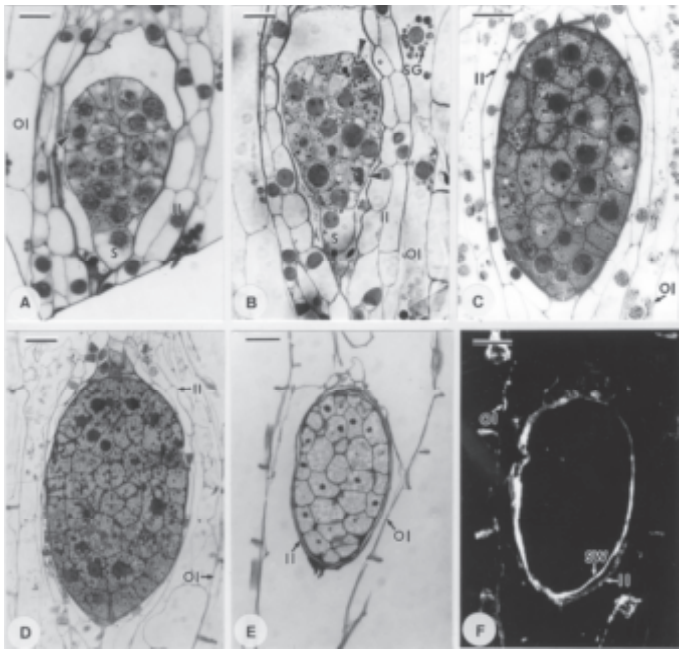

Fig. 3. Late embryo development of *C. formosanum*. (**A**) At 105 d after pollination, anticlinal divisions (arrowhead), occurring in the outer layer of cells of a globular embryo, clearly delineating the protoderm. Scale bar = 25 μm, II = inner integument, OI = outer integument, S = suspensor. (**B**) Light micrograph showing the occurrence of periclinal division (arrowhead) within the embryo proper at 105 d after pollination. Starch grains (double arrowhead) can be seen in the cells of embryo proper. The cell of outer integument (OI) contains many starch grains (SG), whereas no starch grains are observed in the inner integument (II). Scale bar = 30 μm, S = suspensor. (**C**) Light micrograph showing a globular embryo at the stage of suspensor degeneration at 120 d after pollination. Scale bar = 38 μm, II = inner integument, OI = outer integument. (**D**) As the seed matured, large vacuoles began to be replaced by small ones. Small protein bodies began to appear within the cells of the embryo proper at 150 d after pollination. The cells of both inner (II) and outer integuments (OI) became dehydrated and gradually compressed into a thin layer. Scale bar = 45 μm. (**E**) Light micrograph showing a longitudinal section through a mature seed at 210 d after pollination. The embryo is enveloped by the shriveled inner integument (II) and outer integument (OI). Scale bar = 35 μm. (**F**) Light micrograph showing the fluorescence pattern of a mature seed after Nile red staining. The shriveled inner integument (II) and the outermost layer of outer integument (OI) react positively to the stain. The surface wall (SW) of the embryo proper also fluoresces brightly. Scale bar = 60 μm.

at 90 DAP. At this stage, part of the placenta remained attached to the seeds, which were yellowish white and moist. Seeds collected at 75 DAP also germinated well, a noticeable increase in germination being observed at this time. After 135 DAP, the seeds germinated poorly. During this period, the seeds were beginning to turn light brown, and became dry and loose, a condition that allowed them to be readily shaken onto the media.

No germination occurred in the 60 DAP seeds. Figure 5 shows the progression of germination over 120 d in culture, for seed collected at regular intervals after pollination on Thomale GD medium. Seeds collected at 90 and 105 DAP showed a dramatic increase in germination (from 5% to 60%) between 45 and 90 d in culture. In general, the germination reached the maximum (≈65% to 70%) after 105 d in culture.

Media effect on germination. Results in this investigation showed that the timing of seed collection, but not the culture medium, affected germination. Seeds collected at 90 DAP germinated well (ranged from 48.6% to 59.5%) among the four media tested, no significant differences in germination were observed. At 120 DAP, even if germination was higher on Harvais medium (7.3%), when compared with the other three media; seed germination declined (ranged from 2.7% to 7.3%). Percentage of germination was poor (0% to 0.04%) when seeds collected at 150 DAP.

Seed pretreatments for germination and TTC staining for viability testing. The percentage of embryo staining with TTC ranged from 28.4% to 47.4% that was much higher than the germination percentage (<0.8%). In compared with the control (0.1%), 30 min of ultrasonic treatment caused an increase in germination (0.8%). However, longer periods of ultrasonic treatment (45 or 60 min) resulted in the lower germination (0.14% and 0.05%, respectively). Soaking seeds with sodium hypochlorite also enhanced germination. Increasing the duration of soaking from 30 min, 45 min to 60 min resulted in the better germination (0.1%, 0.6%, and 0.8%, respectively). Soaking the seeds with 1 N NaOH and 1 N HCl did not prompt germination; in both of these treatments, germination was poor and varied between 0.02% and 0.1%. Although mature seeds germinated poorly, pretreated with ultrasound and sodium hypochlorite showed a higher percentage of germination than the control.

Discussion

In the present study, we have shown a time frame for embryogenesis and a general overview with emphasis on key structures in the embryogenesis of *C. formosanum*. Based on the defined time frame, experiments were initiated to investigate the effects

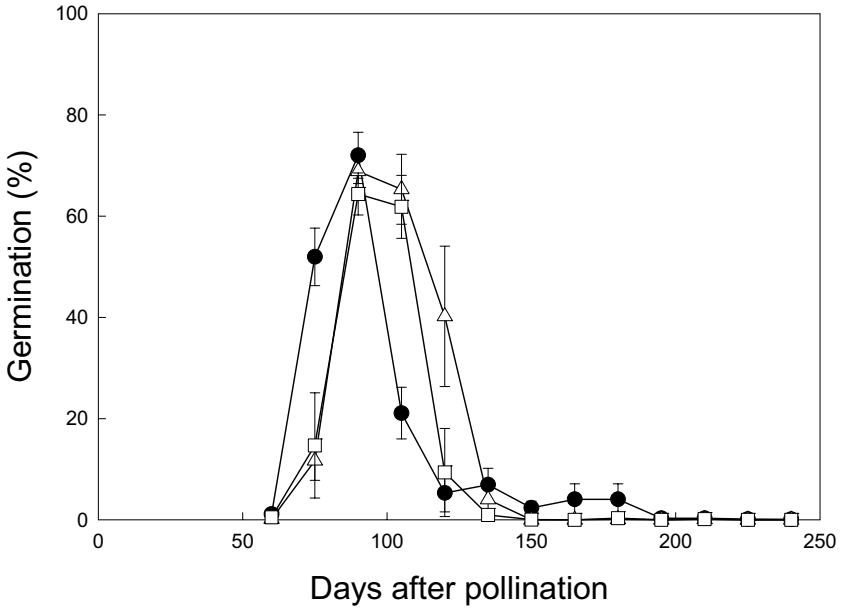

Fig. 4. Mean percent germination of *C. formosanum* seed collected in 1999 (●), 2000 (❒), and 2001 (Δ) at each successive 15 d after pollination on Thomale GD medium. Data were scored after 120 d of culture. Error bars represent SE (n = 3).

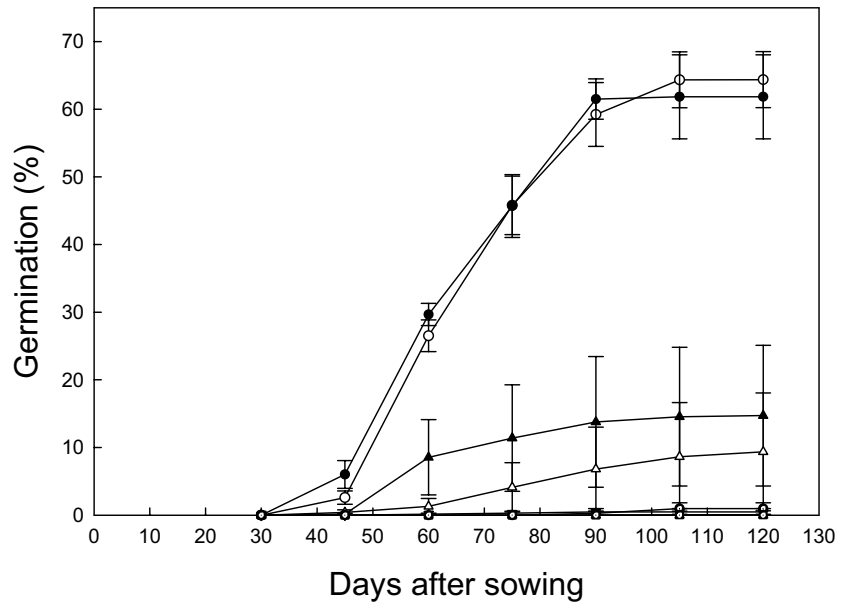

Fig. 5. Mean percent germination of *C. formosanum* on Thomale GD medium in relation to the time in culture for different seed maturity (DAP = days after pollination): 60 DAP (❒), 75 DAP (▲), 90 DAP (○), 105 DAP (●), 120 DAP (Δ), 135 DAP (ϴ). Since the germination percentage of 150 to 240 DAP are close to 135 DAP, the data are not shown here. Error bars represent SE (n = 3).

of the timing of seed collection, culture media, and seed pretreatments on germination in vitro, in order to provide information about the interrelation between germination rate and the structural changes of seeds, at the time of collection for inoculation.

An interesting feature of this investigation demonstrated that there was a dramatic increase in seed germination with a peak at 90 DAP (Fig. 4), while after 105 DAP, seed germination dropped off sharply. Earlier works have indicated that immature seeds of the *Cypripedium* species germinated more readily and better than mature seeds (Light, 1989; Withner, 1953). Our results showed that seeds collected from 90 to 105 DAP were the most suitable for culture in vitro. This finding agrees with the view that the optimum timing of seed collection is essential in maximizing germination (De Pauw and Remphrey, 1993). It is noteworthy that the seeds of most North American *Cypripedium* species approach maturity after 90 DAP (De Pauw and Remphrey, 1993; Rasmussen, 1995), whereas the early globular embryo occurs at ≈90 DAP in *C. formosanum*. According to Cribb (1997), the flowering of *C. formosanum* generally occurs in March, about three months earlier than that of the North American *Cypripedium* species. In *C. formosanum*, the prolonged development of seeds may correspond to the habit of plant growth, since the growing season is relatively longer in the subtropical high mountains, when compared to those of temperate regions.

In order to test the effect of medium compositions on germination, seeds collected at 90, 120, and 150 DAP were also subject to different media. The present data has shown that the timing of seed collection outweighs the medium composition. Our results support previous observations on *C. reginae* Walter (De Pauw and Remphrey, 1993), and suggest that the composition of the medium may not be a critical factor affecting germination in *C. formosanum*.

Although the timing of seed collection for germination in vitro was found to be crucial, little information was available about the embryo development stage, while the seed had been excised for culture. According to Light (1989), a three-celled embryo was present at 43 DAP in *C. calceolus* L. var. *pubescens* (Willd.) Correll. In the present study, the initiation of seed germination occurred at 75 DAP; at this time, most embryos consisted of only two to three cells. These results indicate that the proembryos are capable of continuous development and germination, when placed on culture media.

The seeds collected from 90 to 105 DAP, were yellowish white and moist, which is the best stage to maximize germination. During this period, the early globular to globular stages of embryos could be observed. As shown in Fig. 2H, a single-celled suspensor was found, while the periclinal division occurred within the embryo proper, leading to the formation of the protoderm. This result generally concurred with the suggestion published by Rasmussen (1995, p. 25): "If orchid seeds are excised while the suspensor is still functioning, and the seed envelope has not yet acquired its moisture-repellent qualities, there are few obstacles to rapid rehydration. The stage of metabolic quiescence that follows desiccation can thus be bypassed." Similarly, in *Calypso* Salisb. orchids, Yeung and Law (1992) suggested that if mitotic activity is not arrested, continued development might be feasible under in vitro culture. Furthermore, the suspensor has been proposed as a channel for the conduction of nutrients and as a food storage site for the developing embryo (Yeung and Law, 1992; Yeung et al., 1996). In *C. formosanum*, the wall of the suspensor cell was stained purple-blue with TBO, whereas the thickened wall of the inner integument was stained blue-green. This indicates that the suspensor cell wall remained primary in

nature. As suggested in this study, the suspensor may serve as a channel of nutrient uptake for the developing embryo, while immature seeds are excised and cultured.

For the seeds collected after 120 DAP, the germination rate sharply decreased. Difficulties encountered in the germination of fully mature seeds may be due to various factors, such as the low permeability of the seedcoat (Van Waes and Debergh, 1986a, 1986b) and the presence of inhibitors in mature seeds (Burgeff, 1936; Linden, 1980; Van der Kinderen, 1987). In legumes, many species have seeds with a high proportion of physical exogenous dormancy, caused by the tight packing of testa (Ballard, 1973). Moreover, various chemical constituents of the testa have been proposed as being responsible for causing the impermeability (Bhalla and Slattery, 1984; Werker et al., 1979; White, 1908). In orchids, it has been shown that seeds with a firm carapace, such as *Cephalanthera* Rich. and *Epipactis* Zinn., are more difficult to germinate than those with an incomplete carapace (Veyret, 1969). In *C. formosanum*, the timing of carapace formation coincided with the decline of germination percentage. As the inner integument gradually shriveled after 105 DAP, the seed germination percentage began to decrease. After 150 DAP, the inner integument was not crushed, but compressed into a rigid, thin layer enveloping the embryo (Fig. 3D). The work, presented here, provides evidence that the carapace is actually the remnant of the inner integument that persists at maturity, supporting the observations of Carlson (1940) and Veyret (1969). The carapace, forming an impermeable container, may make it difficult for the embryo to obtain water for germination. However, the chemical basis of the impermeability has not been clearly identified. According to the histological and histochemical study of seed development in *C. parviflorum* Salisb. by Carlson (1940), lignin and cellulose could be detected in the walls of the seedcoat, but no cuticular material. In *C. reginae*, Harvais (1980) suggested that the accumulation of suberin in the seedcoat might contribute to the hydrophobic nature of the mature seed. In the present investigation, one of the most noteworthy findings was the Nile red staining outline. Fluorescence microscopy, using Nile red stain for lipids, indicated that cuticular substance was present in the walls of the seedcoat, the carapace, and the embryo surface. Cuticular substance had been observed on the surface cells of the embryo proper as the protoderm became determined (Lackie and Yeung, 1996; Yeung et al., 1996). The occurrence of this cuticular substance may provide a covering for the embryo, to protect it from early desiccation. In addition, our results clearly showed that, at seed maturity, the cuticular substance was deposited in the walls of the inner integument, enclosing the embryo of *C. formosanum*. These findings contradict those of Carlson (1940), who pointed out that no cuticular material was found in the integument tissues. The role of cuticular material in the integument tissues is not clear. We suggest that the presence of cuticular substance in the inner integument may block the supply of metabolites to the embryo sac, providing a unique environment for the developing embryo. Since the cuticular material is hydrophobic in nature, it is not surprising that in the seedcoat and the carapace, cuticular substance may bring about impermeability as seeds mature. Moreover, in *C. formosanum*, the carapace and the outermost layer of the seedcoat was also stained greenish blue with TBO, indicating the presence of polyphenols and lignin in the wall. We can deduce, from these findings, that the low germinability in the mature seed of *C. formosanum* may be due to the hydrophobic nature of the integument tissues, forming an important barrier to the uptake of water and nutrients.

To dissolve this barrier, previous studies have pretreated the mature seeds of terrestrial orchids with chemical or mechanical scarifications for an increased germination percentage (Linden, 1980; Miyoshi and Mii, 1988; Steele, 1995). According to Van Waes and Debergh (1986 a and 1986b), longer pretreatments bring about higher permeability, which result in better germination of some European terrestrial orchids. In the previous study, ultrasonic treatments were used to rupture the seedcoat of *Paphiopedilum* Pfitzer species and raise the germination percentage (Chen, 1996). Increased germination in the *Paphiopedilum* species was also obtained by soaking the mature seeds in 1% NaOCl until the seeds lost their dark color (our unpublished data). In *C. formosanum*, the strong hydrophobic nature of mature seeds persisted, when pretreated with 1% NaOCl for 1 h, whereas the seeds of *Paphiopedilum* species became submerged in the NaOCl solution after a 10-min pretreatment. In the present investigation, while mature seeds of *C. formosanum* were subject to different pretreatments, in order to increase germination, none of these pretreatments improved germination (<1%).

Since the germination percentage of mature seeds was much lower than the percentage of embryo staining with TTC, the low germination was not caused by low viability. Moreover, the germination percentage did not increase after the 1 h or longer pretreatments (our unpublished data) in 1% NaOCl, the reason for the low germination of mature seeds may be caused not only by the physical constraint of the carapace, but also by the existence of the germination inhibitor. A similar result was found with pretreatments on the mature seeds of *Dactylorhiza maculata* L. which led to the proposition of the influence of ABA on low germination (Van Waes and Debergh, 1986b). Other findings also reveal that the presence of ABA in the seeds of terrestrial orchids may be responsible for low germination (Van der Kinderen, 1987). According to Lee (2003), endogenous ABA content of *C. formosanum* is low at 60 DAP, and increases rapidly during 120 to 150 DAP, a time that coincided with a quick decrease in seed germination. Using an immunohistochemical technique, a high level of ABA was found to accumulate on the surface wall of the embryo proper and the shriveled inner integument at seed maturity (Lee, 2003). Thus, both the physical barrier and the chemical inhibitor may contribute to the low germination of the mature seeds of *C. formosanum*.

How do the hydrophobic materials function in relation to the germination strategy of *C. formosanum*? As the seed development progresses, the cuticular substance, polyphenols, and lignin may deposit in the integument tissues, forming the hydrophobic nature of mature seeds. Since it is crucial to prevent the seeds from germinating right away, in a severe cold environment, ABA and these hydrophobic substances (the cuticular substance, polyphenols, and lignin) may help the seeds survive in the stringent climate. Under natural conditions, seasonal changes in temperature, wetting and drying, or mechanical abrasion and mycorrhizal fungi digestion may be important factors in damaging the seedcoat and carapace of the seeds of *C. formosanum*. These conditions enable the seeds to be permeated by water and nutrients, thus facilitating germination.

Literature Cited

Arditti, J. 1967. Factors affecting the germination of orchid seeds. Bot. Rev. 33:1–97.

Arditti, J. and R. Ernst. 1993. Micropropagation of orchids. Wiley, New York.

Ballard, L.A.T. 1973. Physical barriers to germination. Seed Sci. Technol. 1:285–303.

Bhalla, P.L. and H.D. Slattery. 1984. Callose deposits make clover seeds impermeable to water. Ann. Bot. 53:125–128.
Burgeff, H. 1936. Samenkeimung der Orchideen. Gustav Fischer, Jena, Germany.
Carlson, M.C. 1940. Formation of the seed of *Cypripedium parviflorum*. Bot. Gaz. 102:295–301.
Chen, M.H. 1996. Effect of capsule maturity, seed pretreatments and medium on seed germination and seedling growth in *Paphiopedilum* spp. MS Thesis. Natl. Taiwan Univ., Taipei, Taiwan, Republic of China.
Chu, C.C. and K.W. Mudge. 1994. Effects of prechilling and liquid suspension culture on seed germination of the yellow lady's slipper orchid (*Cypripedium calceolus var. pubescens*). Lindleyana 9:153–159.
Cribb, P.J. 1997. The genus *Cypripedium*. Timber Press, Portland, Ore.
De Pauw, M.A. and W.R. Remphrey. 1993. *In vitro* germination of three *Cypripedium* species in relation to time of seed collection, media and cold treatment. Can. J. Bot. 71:879–885.
De Pauw, M.A., W.R. Remphrey, and C.E. Palmer. 1995. The cytokinin preference for in vitro germination and protocorm growth of *Cypripedium candidium*. Ann. Bot. 75:267–275.
Duncan, D.B. 1955. Multiple range and multiple F test. Biometrics 11:1–42.
Harvais, G. 1973. Growth requirements and development of *Cypripedium reginae* in axenic culture. Can. J. Bot. 51:327–332.
Harvais, G. 1980. Scientific notes on a *Cypripedium reginae* of Northwestern Ontario. Amer. Orchid Soc. Bul. 49:237–244.
Harvais, G. 1982. An improved medium for growing the orchid *Cypripedium reginae* axenically. Can. J. Bot. 60:2547–2555.
Henrich, J.E., D.P. Simart, and P.D. Ascher. 1981. Terrestrial seed germination in vitro on an defined medium. J. Amer. Soc. Hort. Sci. 106:193–196.
Lackie, S. and E.C. Yeung. 1996. Zygotic embryo development in *Daucus carota*. Can. J. Bot. 74:990–998.
Lauzer, D., M. St-Arnaud, and D. Barabe. 1994. Tetrazolium staining and *in vitro* germination of mature seeds of *Cypripedium acaule* (Orchidaceae). Lindleyana 9:197–204.
Lee, Y.I. 2003. Growth periodicity, changes of endogenous abscisic acid during embryogenesis, and *in vitro* propagation of *Cypripedium formosanum* Hay. PhD Diss., National Taiwan Univ., Taipei, Taiwan, Republic of China.
Light, M.H.S. 1989. Germination in the *Cypripedium*/*Paphiopedilum* alliance. Can. Orchid J. 5:11–19.
Lin, T.P. 1987. Native orchids of Taiwan. vol. 3. Southern Materials Ctr., Taipei, Republic of China.
Linden, B. 1980. Aseptic germination of seeds of northern terrestrial orchids. Ann. Bot. Fennici 17:174–182.
Masanori, T. and M. Tomita. 1997. Effect of culture media and cold treatment on germination in asymbiotic culture of *Cypripedium macranthos* and *Cypripedium japonicum*. Lindleyana 12:208–213.
Miyoshi, K. and M. Mii. 1988. Ultrasonic treatment for enhancing seed germination of terrestrial orchid, *Calanthe discolor*, in asymbiotic culture. Scientia Hort. 35:127–130.
Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–479.
Norstog, K. 1973. New synthetic medium for the culture of premature barley embryos. In Vitro 8:307–308.
Oliva, A.P. and J. Arditti. 1984. Seed germination of North American orchids. II. Native California and related species of *Aplectrum*, *Cypripedium* and *Spiranthes*. Bot. Gaz. 145:495–501.
Rasmussen, H. N. 1995. Terrestrial orchids from seed to mycotrophic plants. Cambridge University Press.
St. Arnaud, M., D. Lauzer, and D. Barabe. 1992. In vitro germination and early growth of seedling of *Cypripedium acaule* (Orchidaceae). Lindleyana 7:22–27.
Steele, W.K. 1995. Growing *Cypripedium reginae* from seed. Amer. Orchid Soc. Bul. 64:382–391.
Thomale, H. 1957. Die Orchideen. Einfuhrung in die Kultur und Vermehrung tropischen und einheimischen Orchideen. 2nd ed. Eugen Ulmer, Stuttgart, Germany.
Van der Kinderen, G. 1987. Abscisic acid in terrestrial orchid seeds: A possible impact on their germination. Lindleyana 2:84–87.
Van Waes, J.M. and P.C. Debergh. 1986a. Adaption of the tetrazolium method fot testing the seed viability and scanning electron microscopy of some western European orchids. Physiol. Plant. 66:435–442.
Van Waes, J.M. and P.C. Debergh. 1986b. *In vitro* germination of some western European orchids. Physiol. Plant. 67:253–261.
Veyret, Y. 1969. La structure des semences des orchidaceae et leur aptitude a la germination *in vitro* en cultures pures. Musee d'Histoire Naturelle de Paris, Travaux du Laboratoire La Jaysinia 3:89–98.
Werker, E., I. Marbach, and A.M. Mayer. 1979. Relation between the anatomy of the testa, water permeability and the presence of phenolics in the genus *Pisum*. Ann. Bot. 43:765–771.
White, J. 1908. The occurrence of an impermeable cuticle on the exterior of certain seed. Proc. Royal Soc. of Victoria 21:203–210.
Withner, C.L. 1953. Germination of "Cyps." Orchid J. 2:473–477.
Yeung, E.C. 1984. Histological and histochemical staining procedures, p. 689–697. In: I.K. Vasil (ed.). Cell culture and somatic cell genetics of plants. Vol. 1. Laboratory procedures and their applications. Academic, Orlando, Fla.
Yeung, E.C. 1999. The use of histology in the study of plant tissue culture systems—Some practical comments. In Vitro Cell. Dev. Biol. Plant 35:137–143.
Yeung, E.C. and S.K. Law. 1992. Embryology of *Calypso bulbosa*. II. Embryo development. Can. J. Bot. 70:461–468.
Yeung, E.C., S.Y. Zee, and X.L. Ye. 1996. Embryology of *Cymbidium sinense*: Embryo development. Ann. Bot. 78:105–110.
Yoshikazu, H., K. Kondo, and S. Hamatani. 1994. In vitro seed germination of four taxa of *Cypripedium* and notes on the nodal micropropagation of American *Cypripedium monotanum*. Lindleyana 9:93–97.