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Effect of capsule maturity and desiccation time on viability of Taiwan native orchid, *Bletilla formosana* seeds (Orchidaceae) after cryopreservation

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ABSTRACT: Desiccation method has been successfully applied to the cryopreservation of *Bletilla formosana* seeds (Orchidaceae). This study investigated the effects of capsule maturity and desiccation time on the viability of *B. formosana* seeds after cryopreservation. Seeds with various degrees of maturity (60, 70, 80, 90, 100, and 110 days after pollination; DAP) were used as the test samples and dried for 0 and 24 h prior to cryopreservation. The results demonstrated that the germination percentage of the 110 DAP seeds with 24 h desiccation pretreatment was the highest (96.7%). The 110 DAP seeds were then used to further test the effects of various desiccation time (0, 2, 4, 6, 8, and 24 h) on the viability of the seeds after cryopreservation. The results demonstrated that when seeds were dried for 4-24 h prior to preservation, the water content was reduced to 10.8-0.6% (w/w), and the germination percentage (86.7-95.5%) were significant difference higher than un-desiccation treatment after cryopreservation and thawing to room temperature. The seeds of *B. formosana* were highly tolerant to desiccation, thus, they should be orthodox seeds and are suitable for long-term storage through cryopreservation.

KEY WORDS: *Bletilla formosana,* Days after pollination, Long-term preservation, Orchid, Orthodox seed, Preservation.

INTRODUCTION

Cryopreservation is one of the most effective methods for the long-term conservation of plant germplasm at ultra-low temperatures (–196°C), because almost all biological activities of cells cease at this temperature (Engelmann, 2000; Gonzalez-Arnao *et al.*, 2008). Intracellular freezing should be avoided for successful cryopreservation (Sakai, 2000). Therefore, the water content of tissue is a critical point. Three pretreatments prior to cryopreservation are typically applied for orchids, namely desiccation, vitrification, and encapsulation-dehydration (Hirano *et al.*, 2006; Jitsopakul *et al.*, 2012; Khoddamzadeh *et al.*, 2011; Luza and Polito, 1988; Popov *et al.*, 2004). The purpose of these pretreatments is to reduce the water content of tissue and prevent tissue from harm. Reducing the seed water content through desiccation in conjunction with low temperatures (3–4°C) and ultralow temperatures (–196°C) has been applied in the storage of *Phaius tankervilleae* and *Bletilla formosana* (Hayata) Schltr. seeds (Chang *et al.*, 2006; Hirano *et al.*, 2009; Wu *et al.*, 2013). However, adequate water content for successful cryopreservation varies according to species. The seeds of *P. tankervilleae* are difficult to preserve with low water content (2%–5%) at 4°C and 25°C (Hirano *et al.*, 2009). Numerous terrestrial and epiphytic species are not damaged by storage in liquid nitrogen (LN) if the water content in the seed is below a critical point (Batty *et al.*, 2001; Nikishina *et al.*, 2001; Pritchard, 1984; Wang *et al.*, 1998). The pollen of English walnut lost viability after being stored at –196°C, because the pollen water content was above 7.5%. However, pollen remained viable after cryopreservation when the pollen water content was reduced to 3.2%–7.5% (Luza and Polito, 1988). Freshly harvested seeds of *B. formosana* can be desiccated for 2 h by using silica gel and through air-drying for 24 h under laboratory conditions prior to cryopreservation and remain highly viable (Wu *et al.*, 2013). The desiccation tolerance of *B. formosana* seeds is high because they exhibit high viability when water content is reduced to 1.9%. However, successful cryopreservation of fresh *Encyclia cochleata* seeds with 24% water content has been reported (Nikishina *et al.*, 2001). Thus, the appropriate water content of orchid seeds for successful cryopreservation depends on the species. Water content of tissue is also affected by desiccation time and maturity of preserved material (Hirano *et al.*, 2005). An inappropriate desiccation time reduces the viability of tissue. Moreover, the water content of seeds gradually decreases with increasing time after pollination (Hirano *et al.*, 2005). Seeds collected 3 to 4 months after pollination might be able to survive ultralow temperatures.

Previously, the seeds of *B. formosana*, have ornamental and medicinal value and have been successfully cryopreserved using desiccation. The purpose of this study was to investigate the correlations among maturity, drying time, and water content of *B. formosana* seeds and to determine the best condition of capsule maturity and water content of seeds for cryopreservation.

MATERIALS AND METHODS

Plant materials.

Artificial self-pollination was performed in full bloom. The capsules were harvested on 60, 70, 80, 90, 100, and 110 days after pollination (DAP) before cracking, and surface-sterilized with 70% ethanol, followed by treatment with 1.0% sodium hypochlorite solution for 10 min, and rinsed three times with sterile distilled water. The capsules were air-dried in laminar flow and then cut open; next, the seeds were scooped out and subsequently used as the material for cryopreservation.

Water content analysis of seeds exhibiting various degrees of maturity.

The seeds of *B. formosana* were obtained from capsules 60, 70, 80, 90, 100, 110 DAP and used as the test materials. Fresh seeds collected from same DAP were mixed for testing and subsequently weighed after drying in a sealed container with silica gel for both 0 and 24 h during pretreatment. The seed water content was calculated on a fresh weight basis.

Viability and germination percentage analysis of seeds with various degrees of maturity after cryopreservation.

The seeds dried for 0 and 24 h with silica gel described above were subsequently placed into 2-mL cryotubes and immersed in liquid nitrogen (LN). After 24 h LN storage, the cryotubes were thawed in a water bath at 40°C for 1 min. To determine viability of the seeds, more than 600 seeds were conducted for 2,3,5-triphenyltetrazolium chloride (TTC) staining tests, and 200 seeds were estimated for each replicated. The seeds were incubated in 1% (w/v) TTC solution for 1 day at 25°C in the dark. The percentage of viable TTC-stained embryo seeds was calculated as the viability percentage of the seeds. More than 600 seeds were placed in a petri dish containing germination medium to measure seed germination (200 seeds were counted for each replicate) by microscope. The germination medium consisted of half-strength macroelements and full-strength microelements of MS medium (Murashige and Skoog, 1962) containing 0.7% Bacto agar (Difco Laboratories, Detroit, MI, USA), 2% sucrose, and 0.1% activated charcoal. The pH value of the medium was adjusted to 6 prior to autoclaving at 121°C of 1.05 kg/cm^2 for 20 min. The seeds were incubated at 25 ± 1°C with a 14 h photoperiod at an irradiance level of 40 μmol m^{-2}s^{-1} provided by daylight with cool white fluorescent light.

Water content and germination of seeds from 110 DAP according to various desiccation time.

The seeds of *B. formosana* obtained from 110 DAP capsules were used as the materials. Fresh seeds from capsules were mixed and subsequently weighed after being dried in a sealed container, using silica gel for 0, 2, 4, 6, 8, and 24 h. The seed water content was calculated on a fresh weight basis. Next, the seeds each dry treatment were placed into 2-mL cryotubes and immersed in LN. After 24 h incubation, the cryotubes were thawed in a water bath at 40°C for 1 min. More than 600 seeds were placed in a petri dish containing germination medium to measure seed germination (200 seeds were counted for each replicate) by microscope. The medium ingredients and incubation condition of the seeds was as described above. For each experiment, approximately 0.05g of seeds was weight for each replicate and 3 replicates for each treatment.

Statistical analysis.

The germination and viability percentage of the seeds were subjected to an analysis of variance following arcsine transformation using CoHort Software 6.4 (USA), and the mean values were analyzed using a least significant difference (LSD) test.

RESULTS AND DISCUSSION

Water content analysis according to seeds with various degrees of maturity.

The seeds of *B. formosana* that were obtained from capsules 60, 70, 80, 90, 100, and 110 DAP were used as the test materials. An analysis revealed high water content in all fresh seeds from capsules with various degrees of maturity (Fig. 1). Among all degrees of maturity, the water content of 60 DAP seeds was the highest 63.7%, followed by that of 70 DAP seeds. The seed water content decreased with increasing maturity. 100 DAP and 110 DAP seeds exhibited the most stable and the lowest water content among all harvested seeds, at 28.8% and 27.2%, respectively. The water content of seeds from all degrees of maturity was reduced to close to 0% (0.36-1.34%) after 24 h of desiccation, regardless of their prior water content (Fig. 1).

Viability and germination percentage analysis of seeds with various degrees of maturity after cryopreservation.

The viability percentages of freshly harvested *B. formosana* seeds that were subjected to TTC staining tests were higher than 90% regardless of the degrees of maturity (Fig. 2). The viability percentage of fresh seeds that were not subjected to desiccation prior to treatment with LN were below 6%. The viability percentage of seeds desiccated prior to treatment with LN were considerably high except 60 DAP seeds. The germination percentage of fresh, immature 60 DAP seeds was only 12.3%, the lowest among all degrees of maturity (Fig. 3). With increasing maturity, the seed germination percentage gradually increased. The highest germination percentage of fresh 110 DAP seeds reached 94%. The germination percentage of fresh 110 DAP seeds that were not subjected to desiccation prior to immersion in LN were considerably low (Fig. 3),

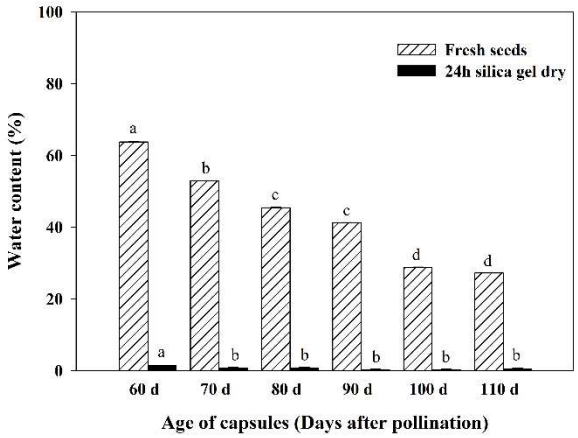

Fig. 1. Water content of different maturity ***Bletilla formosana*** fresh seeds and dried seeds by silica gel. Different letters indicate significant differences among the individual means for each treatment by LSD test (P ≤ 0.05).

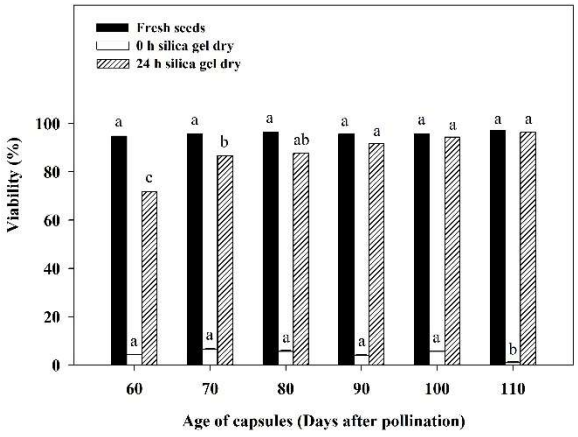

Fig. 2. Effect of capsule maturity (days after pollination) and silica gel desiccation on seed viability by TTC staining of ***Bletilla formosana*** after cryopreservation for 24 h. Different letters indicate significant differences among the individual means for each treatment by LSD test (P ≤ 0.05).

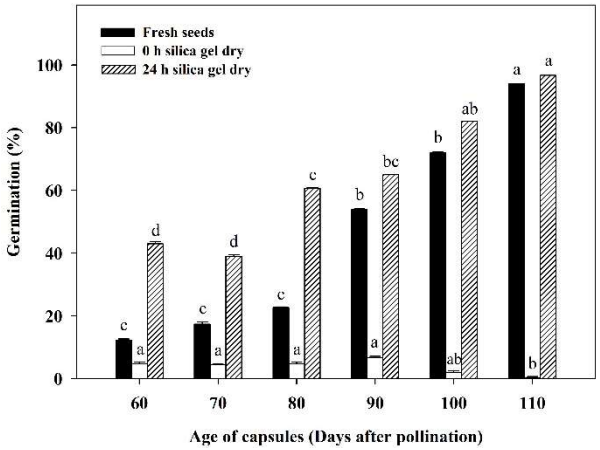

Fig. 3. Effect of capsule maturity and silica gel desiccation on germination of ***Bletilla formosana*** seeds after cryopreservation for 24 h. Different letters indicate significant differences among the individual means for each treatment by LSD test (P ≤ 0.05).

however, as long as 24 h desiccation with LN treatments, high seed germination percentage of 96.7% was measured. The similar result was observed in seeds of all degrees of maturity. The germination percentage of certain low-maturity seeds was increased than that of fresh seeds after desiccation and cryopreservation. Two possible reasons might explain the increasing germination percentage; one is that immersing seeds in LN induces breakage in the seed coat, and another one is due to the lipid body dissociation of seed tissue. Patanè and Gresta (2006) indicated that the seed coats of *Medicago orbicularis* were broken by exposing them to LN, and the affected seeds germinated quickly and in large amounts. Cracks in the seed coat provide channels that facilitate hydration and may increase germination. The testa of orchids may be a barrier to expansive growth. Using LN caused several cracks in the testa and increased the germination percentage of *Phalaenopsis* seeds (Mweetwa and Welbaum, 2007). Pritchard (1984) reported that lipid body dissociation occurred after exposing seeds to LN and suggested that this increased germination rather than broken testa. Batty *et al.* (2001) observed seed surfaces of *Caladenia arenicola, Diuris magnifica, Pterostylis sanguine, Thelymitra crinite* and did not discover any physical damage to the testa; however, they observed natural pits on the surface of the testa. These pits may contribute to the regulation of seed coat permeability. The physiological and ecological functions of these pits warrant further research.

Different results of the TTC staining tests and germination test suggested that the viability percentage of low-maturity seeds were high (Fig. 2), but the germination percentage was low (Fig. 3). A high viability percentage of embryos was observed under the microscope (Fig. 4A), but the embryo in immature seeds was not developed adequately; therefore, the seeds did not necessarily germinate (Fig. 4B). Generally, orchid seeds lack endosperm and have an undifferentiated embryo. On the other hand, the embryo of fresh seed is a group of living cells, which will be stained by TTC. Thus, the viability test with TTC staining was not suitable for immature seeds. Hirano *et al.* (2005) reported that the viability test based on TTC staining was not an accurate predictor of germination for immature orchid seeds. Because embryogenesis is completed immediately 3 months after pollination in *B. striata* (Nagashima 1982), immature seeds of this species are considered to exhibit a low germination percentage and acquire an increasingly high germination percentage when embryogenesis is completed. In most orchids with mature embryos, the germination percentages are high (Lee *et al.*, 2005; Nagashima, 1993). The results of this study are identical to those of Nagashima (1993) and Hirano *et al.* (2005). Therefore, the seed vigor test adopting a TTC staining method was suitable for seeds with high maturity and relatively close to those of the real seed germination test.

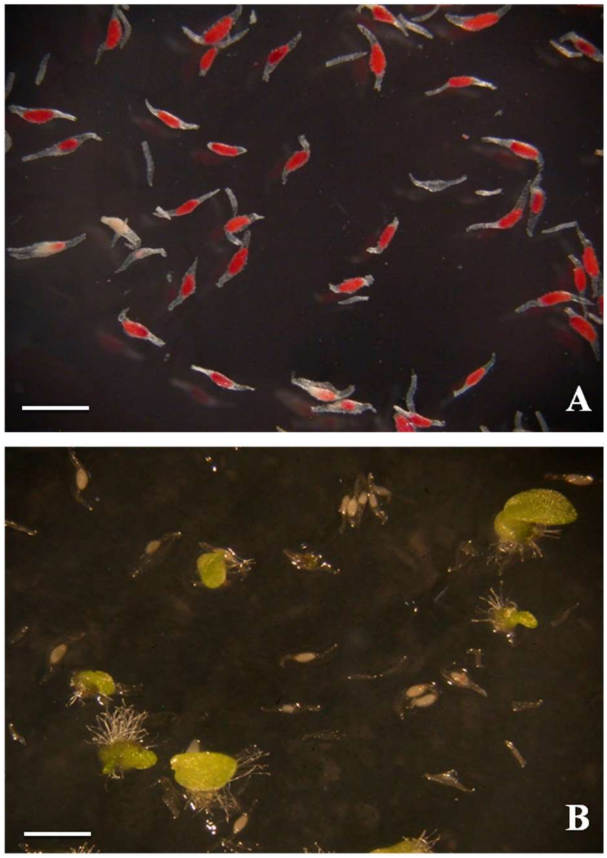

Fig. 4. Seeds germination of ***Bletilla formosana*** 80 DAP seeds observed 45 days after sowing. **A**: High viability by TTC staining and **B**: low germination. Scale bar: A = 1 mm; B = 1.5 mm.

Seed water content and germination according to various desiccation times.

To determine the effect of the desiccation time prior to cryopreservation on seed germination, the seed water content was analyzed. The seeds of *B. formosana* obtained from 110 DAP capsules were used as the test materials. The seed germination percentage after storage in LN for 24 h was used as an indicator of the viability for long-term preservation (Hirano *et al.*, 2009). Harvested fresh seeds exhibited a water content of 29.8% (Fig. 5); this decreased rapidly to 15.4% and 10.8% when dried over silica gel for 2 and 4 h, respectively. The seed water content continued to decrease slowly after 6 h and decreased to 0.6% after 24 h.

The germination percentage of fresh seeds that were not subjected to desiccation prior to placement in LN was 0.3% after thawing and incubation (Fig. 5). However, the germination percentage increased to 47.3% and 86.7% for seeds dried with silica gel for 2 and 4 h prior to treatment with LN, respectively. The seed germination percentage increased with prolonged desiccation time. The germination percentage of seeds dried for 24 h prior to cryopreservation was the highest,

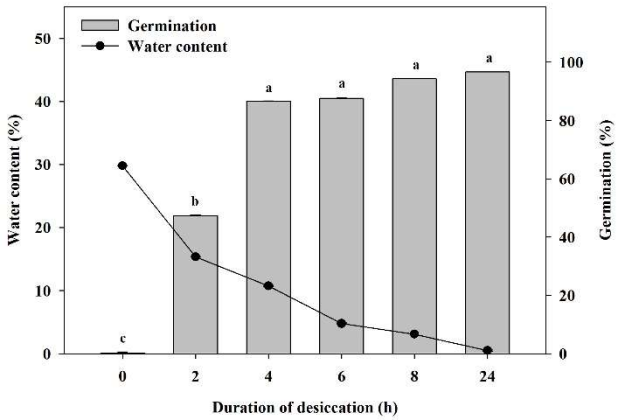

Fig. 5. Effect of desiccation time on water content and germination of ***Bletilla formosana*** seeds after cryopreservation for 24 h. Bars represent standard error of the mean and are not visible if smaller than the symbol.

but no significant difference among 4-24 hrs. Generally, three cryopreservation methods are used for orchids, namely desiccation, vitrification, and encapsulation-dehydration (Hirano *et al.*, 2006; Jitsopakul *et al.*, 2012; Khoddamzadeh *et al.*, 2011; Luza and Polito, 1988; Popov *et al.*, 2004). The purpose of these pretreatments is to reduce the water content in tissue. The water content of harvested fresh seeds that were not dried was 29.8% (Fig. 5). Therefore, the water content of seeds must be reduced to ensure high viability (Wu *et al.*, 2013). The results of this study showed that the germination percentage was as low as in previous studies in which seeds were not subjected to desiccation prior to treatment with LN (Hirano *et al.*, 2006; Touchell *et al.*, 2002; Wu *et al.*, 2013). In this study, water content decreased 50% after 2 h desiccation. The germination percentage increased with prolonged desiccation. Water content and germination percentage of seeds exhibit an inverse relationship. The seed tolerance to desiccation depends on the species. The germination percentage of four western Australian terrestrial orchid seeds that were dried for 24 h prior to cryopreservation was higher than that achieved through other methods (Batty *et al.*, 2001). Whereas the seed water contents of *Thelymitra crinita* before and after desiccation were 16.7% and 5.45%, the germination percentage after storage were 0% and 67.2%, respectively. The harvested fresh seeds of *Encyclia cochleata* with 24% water content were stored successfully without desiccation prior to cryopreservation (Nikishina *et al.*, 2001). However, the seeds of *P. tankervilleae* were difficult to preserve with water content below 5% and at 4°C storage temperature (Hirano *et al.*, 2009). Therefore, *P. tankervilleae* has been described as recalcitrant regarding seed storage. In this study, the seeds of *B. formosana* were dried for 4-24 h prior to cryopreservation; the water content was reduced to 10.8-0.6%, and the germination percentage

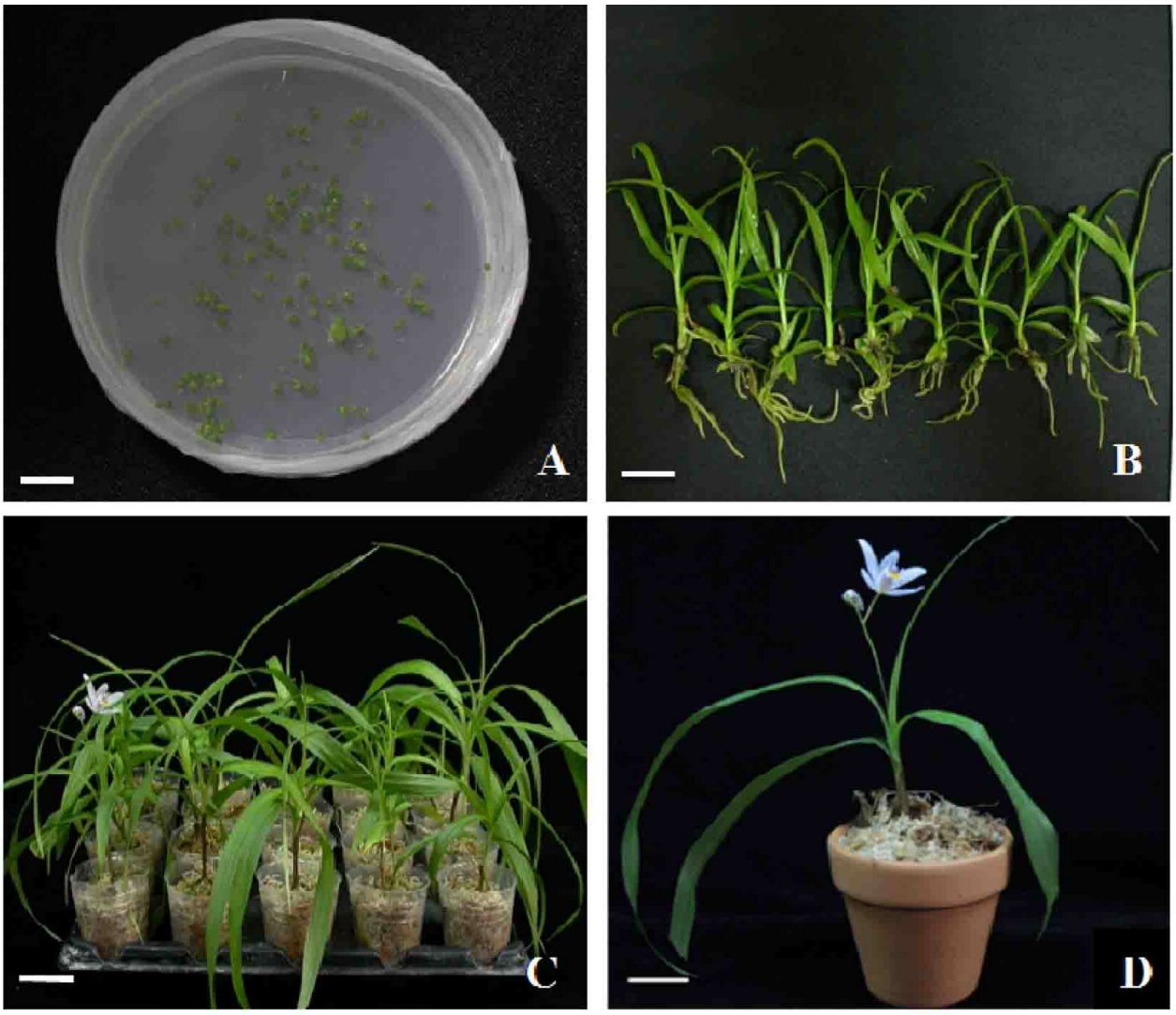

Fig. 6. The plants developed from cryopreserved seeds of ***Bletilla formosana*** **A**: one-month-old protocorm. **B**. six-month-old plantlets. **C** & **D** fifteen-month-old plants after sowing. Scale bar: **A** = 1.3 cm; **B** = 2 cm **C** =2 cm; **D** = 2.5 cm.

remained high (86.7-95.5%) after cryopreservation and thawing to room temperature. These findings demonstrate that the seeds of *B. formosana* exhibit a high tolerance toward desiccation and are suitable for long-term storage, and can be considered as orthodox in seed storage, as suggested in previous studies (Dickie *et al.*, 1991; Grout *et al.*, 1983) The seedlings derived from the desiccated seeds that were preserved in LN and exhibited favorable growth 4 weeks after being sown (Fig. 6).

CONCLUSION

The long-term preservation technology of orchid germplasm need to be established urgently. It's also an extremely important topic for sustainable development of orchid industry in Taiwan on biodiversity maintenance. The purpose of this study was to improve the traditional pretreatment process of LN, to develop a simple, convenient, practical, and non-toxic technology for plant tissues conservation (or LN). The seeds of *Bletilla formosana,* a native orchid in Taiwan with high economical value, were used as the material for platform establishment. Desiccation method has been proved to be successfully applied for the cryopreservation of *B. formosana* seeds. The other factors worthy to been discussed further in order to fully establish the protocol for cryopreservation. The results shown those seeds from 110 DAP, yellow-green capsules, are proper material for preservation. The highest germination percentage (96.7%) after cryopreservation for 24 h was found on 24 h silica gel treatment to reduce water content below 0.5% (w/w) prior to –196℃ LN preservation. Also, the data shown that the germination percentage of seed increased after drying and LN storage. Furthermore, the seed

germinability after long term conservation will be another issue that worthy to study in future. The LN preservation technology process established in this research can be further applied to other orchid plant, and it's a simplified and powerful method for conserving endangered or precious orchid genetic resources of seeds to assist the orchid industry persistently.

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