Chapter 12

Cryopreservation of Orchid Genetic Resources by Desiccation: A Case Study of *Bletilla formosana*

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Abstract

Many native orchid populations declined yearly due to economic development and climate change. This resulted in some wild orchids being threatened. In order to maintain the orchid genetic resources, development of proper methods for the long-term preservation is urgent. Low temperature or dry storage methods for the preservation of orchid genetic resources have been implemented but are not effective in maintaining high viability of certain orchids for long periods. Cryopreservation is one of the most acceptable methods for long-term conservation of plant germplasm. Orchid seeds and pollens are ideal materials for long-term preservation (seed banking) in liquid nitrogen (LN) as the seeds and pollens are minute, enabling the storage of many hundreds of thousands of seeds or pollens in a small vial, and as most species germinate readily, making the technique very economical. This article describes cryopreservation of orchid genetic resources by desiccation and a case study of *Bletilla formosana.* We hope to provide a more practical potential cryopreservation method for future research needs.

Keywords: long-term conservation, *Bletilla formosana*, Desiccation, Dry, Orchid, Seed, Pollen, genetic resources

1. Introduction

Germplasm conservation is mostly applied for breeding purpose. Four methods are usually used in orchid preservation. The first method is more easy to preserve whole plant. It preserves the whole plant in the net-house or greenhouse, most orchid breeders follow this method, but

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the orchid plants are often lost due to natural disasters, pests, diseases, and physiological disorders during cultivation process. The second method is to preserve orchid cells or tissues by tissue culture. Besides much labor requirements, a lot of problems may occur, such as genetic variation, germplasm pollution, and somatic cell clone variation during the continuous subculture process. The third method, dry storage or low temperature method has been carried out for the preservation of orchid genetic resources [1, 2]. In order to achieve a successful hybridization or a special breeding purpose, orchid breeders must preserve pollens from different flowering parents. Moreover, seeds of some important, economic value, particularly endangered species also need to be preserved. Depending on the equipment, cost, and convenience, orchid breeders often preserve pollens or seeds at 4°C in a refrigerator. However, this method does not get an acceptable result in keeping high viability of certain orchids for long period [3–5]. In addition, dry storage and low temperature methods used in case of many orchid seeds are only for short-term preservation for 1–6 months. Viability of most orchid seeds is significantly reduced after less than 1 year for preservation. Furthermore, the seeds of certain orchid species lose their viability quickly upon desiccation [6, 7]. Therefore, the last method, cryopreservation which is a long-term preservation technique has been researched and developed intensively for the need of orchid genetic resources preservation and the orchid industry. Cryopreservation is one of the most reliable methods for long-term conservation of plant genetic resources, because all metabolic processes and physicochemical changes are arrested at the cryogenic temperature (-196°C) [8, 9]. However, it is usually lethal to expose biological specimens to such low temperatures without any pretreatment because of intracellular freezing [4]. Vitrification and desiccation methods have been often used to preserve seeds by removing water from the cells [9–11] because the water content of plant materials may affect cryopreservation success. Orchid PLB (protocorm like body) conservation by combining encapsulation and dehydration has been suggested [12–14].

Bletilla formosana belongs to genus *Bletilla* in the family Orchidaceae. The species is distributed widely in Taiwan and is renowned for its ornamental value [5, 15, 16]. *B. formosana* is endangered due to the destruction of its habitat and over collection for ornamental use. Therefore, preservation of *B. formosana* is urgent to be proceeded. The purpose of this article is to review the cryopreservation of orchid germplasm, describe a practical method of long-term preservation for *Bletilla formosana* seeds, and to provide potential cryopreservation methods for other orchid species.

2. Cryopreservation

The process of cryopreservation preserves structurally intact living cells and tissues by cooling them to very low temperatures [17]. Cryopreservation is one of the most effective methods for the long-term conservation of plant germplasm at ultra-low temperatures (–196°C) because through it, the vitality of cells is preserved despite the cessation of almost all of their biological activities [8, 9]. During cryopreservation, degradation or somatic mutation phenomenon rarely occurs [4, 8, 9, 18, 19].

The advantages of cryopreservation are as follows:

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1. The ability to preserve the vitality and regenerative potential of cells.
2. A requirement for minimal tissue to be effective, resulting in minimal space being used for operation.
3. The prevention of genetic variation and germplasm pollution, and the reduction of somatic cell clone variation rates.
4. The protection against damage from natural disasters, pests, and diseases by using liquid nitrogen (LN) as the storage material.
5. The reduction in labor requirements to accomplish the complicated process of subculture.
6. The possibility of being applied to vegetative propagation plants, nonseed propagation plants, transgenic plants, and gene banks.

3. Cryopreservation of orchid genetic resources: seed and pollen

The main purpose of the long-term preservation of orchid seeds and pollens is to preserve endangered or economically crucial species. Since orchid seeds and pollens are minute, storing many hundreds of thousands of them in a single small vial is possible, making them ideal materials for long-term preservation in LN. Furthermore, most species of orchids germinate readily. Thus, for both of these reasons, cryopreservation is economical and convenient [11]. As reported in [20], maintaining the proper water content (WC) of seeds is critical for successful cryopreservation because excess moisture can result in 'free' water in tissues forming damaging ice crystals during freezing. In most species, exposing biological samples to such low temperatures without any WC pretreatment is typically lethal because of intracellular freezing [4]. Therefore, pretreatment technologies, for example the vitrification and desiccation methods, have been developed [21] to use dehydration for the reduction of the WC of cells and avoid the formation of ice crystals from ultra-low temperature preservation. Prior to ultra-low temperature preservation, suitable pretreatment methods are used to increase the survival rate of the materials to be preserved. Three pretreatments, namely desiccation, vitrification, and encapsulation–dehydration, are typically applied for orchids [13, 21–24]. Pretreatment technologies prior to cryopreservation are still a fancy work to investigate now.

According to the aforementioned reports, three cryopreservation methods are available for orchids.

3.1. Vitrification method

The vitrification technique was introduced by Sakai et al. and is typically used to preserve immature and mature seeds with a higher than average WC for extended periods. Preserved materials are sufficiently dehydrated osmotically by being placed in a high osmolarity vitrification solution (glycerol, dimethyl sulfoxide, and ethylene glycol), which alters their intracellular WC so as to vitrify them through the penetration of cryoprotectants. The chemicals used in this process are toxic. The functions of cryoprotectants are to reduce the amount of freezable

water in seed tissue, reduce the freezing temperatures of the intracellular solutes, and inhibit ice nucleation and growth [24–27].

The seeds of some orchids cannot survive, when preserved at cryogenic temperatures even with relatively low WC. For example, mature seeds of *Oncidium flexuosum* (11% WC) and a *Dendrobium* hybrid (13% WC) were unable to germinate or exhibited a low germination percentage after direct immersion in LN [28, 29]. The seeds of these species should be cryopreserved through a vitrification method combined with rapid cooling and rewarming [28–30].

Vitrification has been applied in the cryopreservation of immature and highly hydrated seeds of orchid species from several genera including *Bletilla*, *Cymbidium*, *Cyrtopodium*, *Dendrobium*, *Doritis*, *Encyclia*, *Phaius*, *Ponerorchis* and *Vanda* [24]. The vitrification method is effective for all types of in vitro material and for not fully dehydrated materials, but the procedure is more complex and high concentrations of cryoprotectants may be toxic to plant tissues [10, 11].

3.2. Desiccation method

The desiccation method is more suitable for mature seeds than for immature seeds. Narrow desiccation refers to when seeds are first dehydrated through slow drying with a controlled desiccation rate under a constant relative humidity (RH) or through rapid drying under a laminar flow chamber or with silica gel or a saturated salt solution of $CaCl_2{\cdot}6H_2O$ (approximately 33% RH) to reduce seed WC before LN preservation. For example, *Bletilla formosana*, *Caladenia arenicola*, *Caladenia flava*, *Caladenia huegelii*, *Dactylorhiza fuchsii*, *Dactylorhiza majalis* spp. *praetermissa*, *Diuris fragrantissima*, *Diuris laxiflora*, *Diuris magnifica*, *Eulophia gonychlia*, *Eulophia stenophylla*, *Gymnadenia conopsea*, *Microtis media* spp. *media*, *Orchis coriophora* (*Anacamptis coriophora*), *Orchis morio* (*Anacamptis morio*), *Paphiopedilum rothschildianum*, *Pterostylis recurva*, *Pterostylis sanguinea*, *Thelymitra crinita*, *Thelymitra macrophylla*, and *Dendrobium candidum* (*Dendrobium moniliforme*) are all most successfully preserved through the desiccation method [21, 31–35] (**Table 1)**.

A generalized drying method should be included as the storage condition before LN, such as seeds stored in a low RH environment for a period or fresh seeds stored in a refrigerator, then directly immersed in LN. For example, harvested seeds of *Calanthe gorey*, *Calanthe vestita* var. *rubro oculata* Paxt., *Encyclia cochleata*, *Angraecum magdalenae*, *Miltonia flavescens* × *Brassia longissima*, and *Trichopilia tortilis* were stored in a refrigerator for 7 days to 7 years prior to cryopreservation [36]. Harvested seeds of *Dactylorhiza balitica*, *D. fuchsii*, *Dactylorhiza incarnata*, and *Dactylorhiza maculata* were stored at 5–6°C for 2–3 weeks, then directly immersed in LN [37]. Harvested seeds of *Disa uniflora*, *Eulophia Alta*, *Eulophia streptopetala*, *Satyrium nepalense* var. *ciliatum*, *S. nepalense* var. *nepalense*, and *Phalaenopsis equestris* are stored at 2°C without silica gel for 6–12 weeks prior to immersion in LN [34] (**Table 1**).

The critical factor for successful cryopreservation through desiccation is the existence of the proper WC of tissue, which varies among species [38]. However, because the orchid seed is tiny and light, accurately measuring its moisture context is technically difficult [21]. Therefore, numerous orchid seeds are required to determine their moisture content (MC) prior to desiccation [4, 7, 11]. Moreover, seed viability after cryopreservation by desiccation varies among

Species	Preserved material	Pretreatment before LN	Storage duration	Germination%		Reference
				control [z]	cryo.[z]	
Angraecum magdalenae	Mature seed	At refrigerator for 7 days-7 years	1 month	55	80	[36]
Bletilla formosana	MAP[y]mature seed	Direct LN	1 day	77	2	[35]
Bletilla formosana	3MAP mature seed	Air dry dry over silica gel for 24 h	1 day 1 day	77 77	69 69	[35]
Bletilla striata	2, 3, 4MAP immature mature seed	Direct LN	30 min	2 MAP:0.2 3 MAP:25 4 MAP:99	0 12 33	[43]
Bratonia × *Miltonia flavescens*	Mature seed	At 8°C for 403 days	1 month	100	100	[55]
Caladenia arenicola	Mature seed	Dry over silica gel for 24 h	1 week	31	37	[31]
Caladenia arenicola	Mature seed	Air dry for 1week →20°C	3-24 months	70-83	55-85	[33]
Caladenia flava	Mature seed	Air dry for 1week →20°C	3-24 months	35-80	0-83	[33]
Caladenia huegelii	Mature seed	Air dry for 1week →20°C	3-24 months	63-95	80-97	[33]
Calanthe Gorey	Mature seed	At refrigerator for 7 days-7 years	1 month	59	90	[36]
Calanthe vestita var. rubrooculata Paxt.	Mature seed	At refrigerator for 7 days-7 years	1 month	56	69	[36]
Dactylorhiza balitica	Mature seed	At 5-6°C for 2-3 weeks	1 month	33	39	[37]
Dactylorhiza fuchsii	Mature seed	Dry over a saturated salt solution of $CaCl_26H_2O$ (ca. 33% RH)at 16°C	1-12 months	74	51	[6]
Dactylorhiza fuchsii	Mature seed	At 5-6°C for 2-3 weeks	1 month	36	44	[37]
Dactylorhiza incarnata	Mature seed	At 5-6°C for 2-3 weeks	1 month	14	27	[37]
Dactylorhiza maculata	Mature seed	At 5-6°C for 2-3 weeks	1 month	71	42	[37]
Disa uniflora	Mature seed	No wit silica gel at 2°Cfor 6-12 weeks	15 min	61	56	[34]
Diuris fragrantissima	Mature seed	Air dry for 1 week →20°C	3-24 months	<20	<20	[33]
Diuris laxiflora	Mature seed	Air dry for 1 week →20°C	3-24 months	55-85	20-55	[33]
Diuris magnifica	Mature seed	Dry over silica gel for 24 h	1 week	37	44	[31]

Species	Preserved material	Pretreatment before LN	Storage duration	Germination%		Reference
				control [z]	cryo.[z]	
Encyclia cochleata (*Prosthechea cochleata*)	Mature seed	At refrigerator for 7 days-7 years	1 month	100	100	[36]
Eulophia alta	Mature seed	No wit silica gel at 2°C for 6-12 weeks	15 min	52	47	[34]
Eulophia stenophylla	Mature seed	In wit silica gel at 2°C for 8 months	15 min	68	70	[34]
Eulophia streptopetala	Mature seed	No wit silica gel at 2°C for 6-12 weeks	15 min	63	58	[34]
Gymnadenia conopsea	Mature seed	In wit silica gel at 2°C for 6-12 weeks	15 min	45	46	[34]
Juglans regia (English walnut)	Pollen	1.at -1°Cmin^{-1} by a controlled-rate freezer 2. air dry 24 h 3. air dry 72 h	12 months 12 months 12 months	1. 82-96 2. 60-88 3. 5-54	1. 0-70 2. 48-81 3. 2-40	[23]
Luisia macrantha	Pollen	1. air dry: 0-30 min 2. dry over silica gel:120 min	48 h	1. 65-67 2. ca.67	1. 54 2. 51-52	[44]
Microtis media spp. media	Mature seed	Air dry for 1 week→20°C	3-24 months	30-67	5-63	[33]
Miltonia flavescens×*Brassia longissima*	Mature seed	At refrigerator for 7 days-7 years	1 month	100	100	[36]
Narcissus St. Keverne	Pollen	AF, AN, PN [x]	3 days 351 days	AF:27 AN:27 PN:27	AF:16(3 days), 0.1(351 days) AN:15(3 days), 13(351 days) PN:16(3 days), 13(351 days)	[56]
Orchis coriophora (*Anacamptis coriophora*)	Mature seed	In wit silica gel at 2°C for 6-12 weeks	15 min	43	43	[34]
Orchis morio (*Anacamptis morio*)	Mature seed	In wit silica gel at 2°C for 6-12 weeks	10 cycle(5 min each)	62	75-82	[34]
Phalaenopsis equestris	Mature seed	No wit silica gel at 2°C for 6-12 weeks	15 min	84	79	[34]
Platanthera bifolia	Mature seed	At 5-6°C for 2-3 weeks	1 month	49	24	[37]
Pterostylis recurva	Mature seed	Air dry for 1week→20°C	3-24 months	70-85	0-90	[33]
Pterostylis sanguinea	Mature seed	Dry over silica gel for 24 h	1 week	49	50	[31]
Pterostylis sanguinea	Mature seed	Air dry for 1 week→20°C	3-24 months	85-90	25-75	[33]

Species	Preserved material	Pretreatment before LN	Storage duration	Germination%		Reference
				control [z]	cryo.[z]	
Satyrium nepalense var. ciliatum	Mature seed	No wit silica gel at 2°C for 6-12 weeks	15 min	58	55	[34]
Satyrium nepalense var. nepalense	Mature seed	No wit silica gel at 2°C for 6-12 weeks	15 min	62	62	[34]
Thelymitra crinita	Mature seed	Dry over silica gel for 24 h	1 week	59	67	[31]
Thelymitra crinita	Mature seed	Air dry for 1week→20°C	3-24 months	25-85	0-80	[33]
Thelymitra macrophylla	Mature seed	Air dry for 1week→20°C	3-24 months	20	3-37	[33]
Trichopilia tortilis	Mature seed	At refrigerator for 7 days-7 years	1 month	83	90	[36]
Vanda pumilla	Mature seed	Not given	15 min	94	97	[34]
Vanda tricolor	Mature seed	Direct LN	1 day	11	1	[22]
Vanda tricolor	Immature seed	Direct LN	1 day	26	10	[22]

[z] Germination of control and cryopreserved seeds, control indicates germination of seeds before cryopreservation.
[y] MAP means months after pollination.
[x] AF: anthers held in a desiccator with calcium chloride at 2°C; AN: anther kept overnight in a desiccator at room temperature→-130°C 1h; PN: anther kept overnight in a desiccator at room temperature→ shake, pollen transferred to straw→-130°C 1h.

Table 1. Cryopreservation of orchid seed or pollen by desiccation prior to LN.

different species. The seeds of some species lose viability after drying to a specific level of WC. Some seeds remain highly viable after being air-dried or exposed to a controlled desiccation rate under a constant RH or to rapid desiccation.

The advantages of the desiccation method are simple, practical, nontoxic, and low cost. Also, the method is more suitable for orchid seed with low WC [24]. Therefore, it can be used for long-term preservation of orchid seed by international plant germplasm centers and private companies.

3.3. Encapsulation-dehydration method

The third cryopreservation method for orchid materials is encapsulation-dehydration. This procedure is based on the technology developed for the production of artificial seeds [39]. Preserved tissues encapsulated in alginate beads, pregrown in a liquid medium with enriched sucrose, partially desiccated in the air current of a laminar airflow cabinet or with silica gel to reduce the WC to a suitable level, and then directly immersed in LN [21].

Among the three aforementioned techniques, the encapsulation-dehydration method is applied only for particular orchid species. For example, pretreatments prior to LN only improved germination of *Cyrtopodium hatschbachii* immature seeds (control/cryopreservation 50/64%), and *Oncidium bifolium* (*Gomes Bifolium*) mature seeds (control/cryopreservation 88/89%) [40, 41] (**Table 1**).

4. Factors affecting cryopreservation

For successful cryopreservation, the required WC of tissue is a critical factor, and varies among species [21, 38]. However, studies have revealed that the survival of orchid seeds in storage is also affected by other factors [7, 42]. For example, the WC of preserved tissue is also affected by the desiccation time and the maturity of the preserved material [43, 44]. An inappropriate desiccation time also reduces the viability of the tissue. Furthermore, the WC of seeds gradually decreases after pollination [43]. The impact of these factors is described as follows.

4.1. Water content

For most orchid species, for example, *Bletilla striata*, *B. formosana*, *D. candidum* (*D. moniliforme*), and *Vanda tricolor* [22, 35, 43, 45], preserving tissue immersed directly in LN without any pretreatment results in high mortality rates because of formation of intracellular ice nucleation and growth in the cells [4, 20, 21, 38]. Only a few examples were exceptions, for example, fresh pollens of English walnut (*Juglans regia* L.) described above [23]. As revealed in related studies, various terrestrial and epiphytic species have not been damaged by storage in LN when seed WC was below a critical point [6, 21, 34, 36]. For example, in seeds of *D. candidum*, a high survival rate (approximately 95%) was obtained when the WC of seeds decreased to 8–19% prior to cryopreservation [33]. *E. cochleata* seeds sealed in cryovials with a 24% WC could be cryopreserved without any loss in viability [36]. Fresh mature seeds of *B. formosana*, an endangered Taiwanese orchid, reached a 68% germination rate after being air-dried for 24 h to a 25% WC and cryopreserved through direct immersion in LN [35]. These studies reveal that the WC of orchid seeds required for successful cryopreservation clearly varies among the species.

Pretreatment techniques must be performed before preserving seeds in LN for most orchids. Previous research on plants such as *B. striata*, *Phaius tancarvilleae*, and *Vanda coerulea* has revealed that preserved tissue must be pretreated prior to cryopreservation to yield high survival rates [4, 11, 18, 43, 46]. Pretreatment is critical and should be applied to most plant tissues before cryopreservation, as stated in the preceding paragraph. Therefore, although cryopreservation has been employed for the long-term preservation of plant tissues for a number of decades, investigation into pretreatment techniques has continued to contribute to improved tissue viability [14, 34, 47].

Some seeds of orchid species have exhibited sensitivity toward extreme desiccation and a reduced viability after short periods of storage under dry conditions (3–5% WC) at high subzero temperatures (e.g., −5°C to −20°C) generally applied in conventional seed banking

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regimes [4, 24, 36, 45, 48, 49]. The seeds of *P. tancarvilleae* are difficult to preserve with low WC (2–5%) and at 4°C and 25°C [4]. Pollinia of *Luisia macrantha* subjected to 0–30 min of dehydration exhibited maximum germination rates of 65–67% [44]; their germination rates decreased with increasing desiccation time. A high survival rate (approximately 95%) of *Dendrobium candidum* (*Dendrobium moniliforme*) seeds can be achieved by drying seeds to WC of 8–19%; however, seed growth is slower in samples dried to WC less than 12%. This reveals that an optimal WC of 12–19% exists for cryopreservation. Species with such desiccation sensitivity have a different optimal cryopreservation method than the general orchid species.

4.2. Maturity

Successful cryopreservation is closely related to the WC of plant tissue [49]. Furthermore, the WC of seeds is related to seed maturity. For example, the seeds of *B. striata* revealed a steady reduction in WC with an increase in time after pollination: 84, 57, and 33% for 2, 3, and 4 months after pollination (MAP), respectively [43]. The WC of the developing seeds decreased with time after pollination [43]. When these immature seeds were directly immersed in LN without any pretreatment, there was a tendency toward an improved germination rate with an increase in seed age: 0, 11.6, and 32.8% for 2, 3, and 4 MAP, respectively. Those of the three or four MAP samples might have developed an ability to survive the frozen state. Vacuolation has been observed in young embryo cells of orchids [50, 51]. However, it disappears as the orchid tissue approaches maturity. In the aforementioned study of *B. striata*, reduced vacuolation and, consequently, a lower level of bulk water in the embryo cells that accompany seed maturation may have prevented intracellular freezing in immature seeds [43].

The cryopreservation methods also differ depending on the seed maturity level. The desiccation method is more suitable for mature seeds as a cryopreservation pretreatment. The vitrification method or the encapsulation-dehydration method may be used for many immature and highly hydrated orchid seeds. The pretreatment in concentrated cryoprotectant solutions before rapid immersion in LN is critical for post-cryopreservation survival of orchid seeds, for example, *Bletilla*, *Cyrtopodium*, *Doritis*, *Phaius*, *Ponerorchis*, and *Vanda* [4, 10, 11, 22, 24, 41, 43, 52].

4.3. Other factors

In addition to WC and seed maturity, successful cryopreservation is related to the type of orchid species [21]. Studies have shown that the survival of orchid seeds in storage is affected by factors, such as desiccation time [7, 31, 42]. Desiccation time, by directly affecting the level of seed WC, is crucial for the survival of preserved tissue. Inappropriate desiccation time reduces tissue viability. For example, seed germination of *Coelogyne foerstermannii*, *C. rumphii*, and *D. stratiotes* decreased to 1–5% from initial values of 65–96% after drying in equilibrium to a 15% RH and storage at −20°C for 9–12 months. By contrast, *X. undulatum* seeds lost 13% of their germination rate during the same interval [53]. Such varying results to conventional banking conditions have strengthened the requirement for research into the cryostorage behavior of orchid seeds and the seeds of other species [54]. According to Seaton and Hailes [7], silica gel reduces *Cattleya aurantiaca* seed moisture to a level that results in a rapid loss of

viability. This contrasts with results that have indicated that desiccating orchid seeds over silica gel for 24 h induces a significant increase in seed germination. The effect of longer seed desiccation durations remains to be determined. For example, the seeds of *P. tancarvilleae* are difficult to preserve with a low WC (2–5%) at 4 and 25°C [4]; however, the germination rates of *B. formosana* dried seeds with a low WC are 68.5% after cryopreservation [35]. Thus, the appropriate WC that orchid seeds require for successful cryopreservation varies among the species.

The values for the pollen germination and WC of the English walnut (*Juglans regia L.*) were the highest before storage. Subsequently, the values decreased along with an increase in storage time at room temperature. However, they did not decrease linearly with time. The ability to germinate was significantly reduced during drying, though some pollen retained some viability until a 3.2% MC [23]. Pollinia of *L. macrantha* subjected to 0–30 min dehydration within a laminar air flow cabinet showed maximum germination of 65–67% in desiccated controls and 54% in LN treated samples. The germination rate decreased with prolonged desiccation time [44].

Long-term preservation of plant genetic resources is not easy. Orchid seeds and pollens are ideal materials suitable for cryopreservation because of the characteristics of tiny volume and low water content. This article describes common pretreatment techniques used in orchid cryopreservation and the factors affecting material viability. Besides, this describes Taiwan's endangered native medicinal and ornamental plants, and the desiccation method applied in a case study of *Bletilla formosana*. The genetic resources of other economic orchids will continue to be tested by the desiccation method in the future. The ultimate object of our study is to provide a more practical potential cryopreservation method and apply in the other economic orchids for sustainable development of orchid industry.

5. Case study for cryopreservation of *Bletilla formosana* seeds (Orchidaceae) by desiccation

B. formosana belongs to genus *Bletilla* in the family Orchidaceae. The species is distributed widely in Taiwan and is known for its medicinal and ornamental value [5, 15, 16]. *B. formosana* is endangered during the destruction of habitat and over collection for ornamental use. Therefore, preservation of *B. formosana* is urgent to be proceeded.

Seeds of *Bletilla* genus had been studied in the pretreatment method prior to cryopreservation. However, the procedure of common vitrification method is more complex and high concentrations of cryoprotectants may be toxic to plant tissues [10, 11]. Therefore, the purpose of this study is to establish a practical method of long-term preservation for *B. formosana* seeds and to provide potential cryopreservation methods for other orchid species.

Mature seeds of *B. formosana* obtained from capsules 3 months after hand-pollination (3 MAP) were collected as the test materials, then dried at room temperature (27 ± 1°C) for 24 h in the

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laboratory with air conditioning and dried in a sealed container using silica gel for 0–24 h, respectively. Seeds were then stored in LN for 24 h, to investigate the viability rate and the germination rate of seeds after warming.

The viability and germination rates of fresh seeds of *B. formosana* are 89.9 and 76.8%, respectively. The initial water content of fresh seeds is 49.5% but decreasing significantly within 1–4 h by silica gel desiccation and then getting stable to 1.9%, 1 day after desiccation. When fresh seeds placed directly into LN for cryopreservation, the viability and germination rates are suddenly went down to 2.3 and 1.8%, respectively. However, for the fresh seeds pretreated by silica gel desiccation for 24 h or air-dried for 24 h at room temperature before putting in LN, the viability rates dramatically increased to 86.8 and 84.9%, respectively, and germination rates are up to 68.5 and 68.6%, respectively (**Figure 1**). These data show that the seed viability of *B. formosana* after cryopreservation is affected by the water content of storage seeds. When the water content of orchid seeds decreases to 24.8% by 24 h air drying or to 21.9–31.2% by 1–2 h silica gel drying, high viability and germination rates still remain after long-term

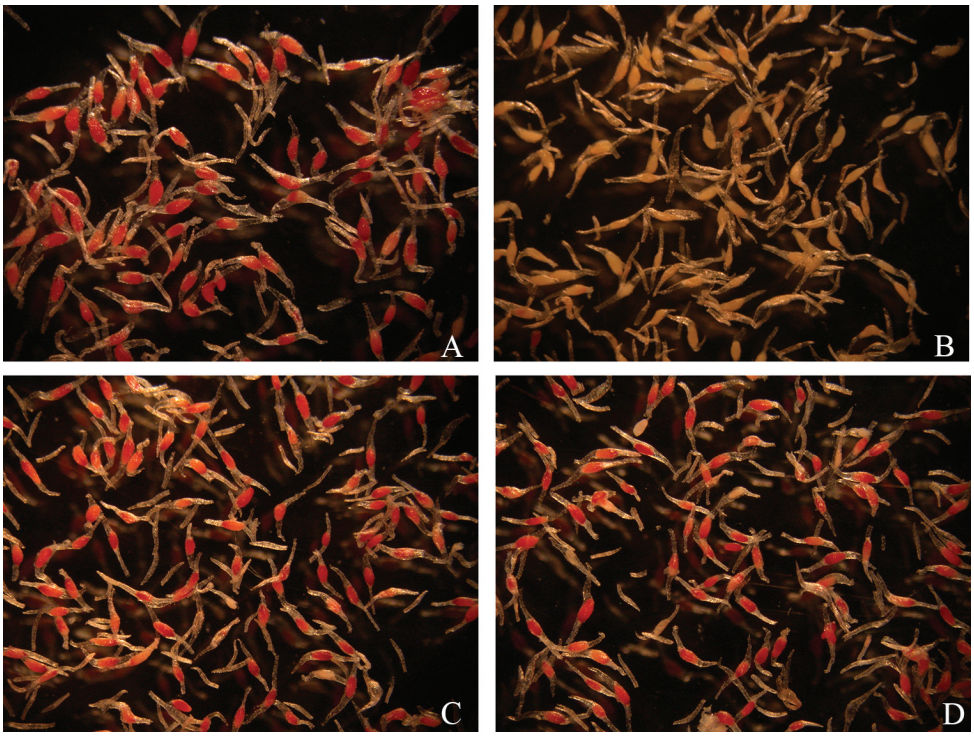


Figure 1. Effect of desiccation on viability of *Bletilla formosana* seeds after cryopreservation by TTC staining method. (A) Harvested fresh seeds; (B) fresh seeds plunged into liquid nitrogen (LN, -196°C); (C) fresh seeds dried for 24 h with silica gel prior to LN; (D) fresh seeds dried for 24 h with air-drying in laboratory conditions prior to LN. Bar =1 mm [49].

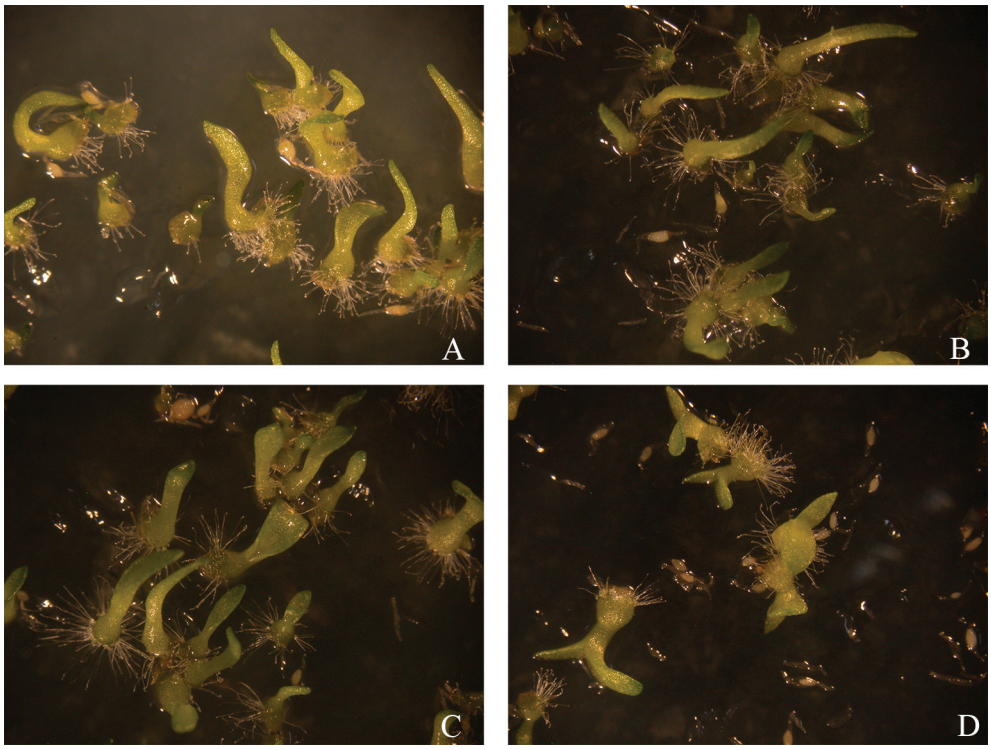


Figure 2. Seedling growth of *Bletilla formosana* for 6 wk after sowing on MS medium. (A) Fresh seeds; (B) fresh seeds were dried for 24 h with air-drying in laboratory conditions prior to LN; (C) fresh seeds were dried for 24 h with silica gel prior to LN; (D) fresh seeds were dried for 24 h with silica gel, vitrification (0.06 M sucrose solution+LS+PVS2 1 h) prior to LN. Bar =1 mm [49].

cryopreservation. Therefore, those desiccation methods are recommended for long term storage of *B. formosana*. Fresh seeds of orchid pretreated in sucrose solution or vitrification before LN treatment are unsuitable for cryopreservation in our previous study [35]. Seedlings derived from the seeds desiccated by these two methods and then preserved in LN grew well 6 weeks after seed sowing (**Figure 2**). In addition, *B. formosana* seeds with 1.9–24.8% water content were found to be suitable for cryopreservation [35].

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