

Wide applicability of cryopreservation with vitrification method for seeds of some *Cymbidium* species

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Abstract The mature seeds of 7 *Cymbidium* species, including those from different climatic regions (temperate or tropical), with different life-forms (terrestrial or epiphytic), and different nutritional modes (autotrophic or mycoheterotrophic) were cryopreserved by vitrification to demonstrate the effectiveness of this method for germplasm preservation. The period of exposure to vitrification solution was initially optimized for seeds of 3 species, and the optimum periods were found to be 60 min for *C. goeringii* and *C. macrorhizon* seeds and 30 min for *C. finlaysonianum* seeds. Cryopreservation under optimum conditions did not have any deleterious effect on the seeds, and the germination rates of the cryopreserved seeds were as follows (these rates were almost identical to those of untreated control seeds): 32% for *C. goeringii*, 82% for *C. macrorhizon*, and 76% for *C. finlaysonianum*. The cryopreservation method was also successfully applied to the seeds of other members of the genus *Cymbidium*, although the period of exposure to the vitrification solution varied with life-form. The periods were optimized to 60 and 30 min for the terrestrial and epiphytic species, respectively. These results suggest that cryopreservation by vitrification has wide applicability for the seeds of several orchid species with varying ecological and physiological adaptations.

Key words: Cryopreservation, *Cymbidium*, germplasm, mature seeds, vitrification.

The orchid genus *Cymbidium* comprises approximately 52 species native to regions of Asia ranging from the northwestern Himalayas to Japan and from Indochina and Malaysia to northern and eastern Australia (Du Puy and Cribb 2007). *Cymbidium*s are quite diversified ecologically, and they exhibit terrestrial, epiphytic, and lithophytic life-forms. For example, *Cymbidium macrorhizon* lacks foliage leaves and has a strongly mycoparasitic nature (Yukawa and Stern 2002). However, many *Cymbidium* species are in danger of extinction because of the extensive disturbance of their natural habitat and indiscriminate harvesting of naturally growing plants. Therefore, it is extremely important to conserve the germplasms of these orchids.

Cryopreservation, i.e., the storage of biological material at ultralow temperatures (−196°C) usually using liquid nitrogen (LN), is the only technique currently available to ensure the safe, cost-efficient, and long-term conservation of different types of germplasms (reviewed by Sakai and Engelman 2007). Vitrification, a cryopreservation technique, has been successfully applied to

seeds of several orchid species. Mature seeds of *Bletilla striata* (Ishikawa et al. 1997), *Dendrobium* (Vendrame et al. 2007), *Doritis pulcherrima* (Thammasiri 2000), *Phaius tankervilleae* (Hirano et al. 2009), and *Vanda coerulea* (Thammasiri and Soamkul 2007) and immature seeds of *B. striata* (Hirano et al. 2005a) and *Ponerorchis graminifolia* var. *suzukiana* (Hirano et al. 2005b) have been preserved by this technique. However, thus far, no study has compared the differences among species of a single Orchidaceae genus with different life-forms in terms of the response of seeds to vitrification. In the present study, we aimed to demonstrate the wide applicability of vitrification for conserving the seeds of various *Cymbidium* species with different ecological habitats, including both terrestrial and epiphytic life-forms from temperate and tropical regions.

Mature seeds of the 7 species listed in Table 1 were used in this study. All seeds were surface sterilized with sodium hypochlorite solution containing 0.05% Tween-20. However, optimum concentrations of chlorine and sterilization period were varied according to the species

Abbreviations: LN, liquid nitrogen; ND, New Dogashima; TTC, 2, 3, 5-triphenyltetrazolium chloride

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and was chosen based on our preliminary experiments as follows: 1% of available chlorine for 10 min in *C. goeringii*, *C. kanran*, and *C. macrorhizon*; 0.8% for 7 min in *C. finlaysonianum* and *C. maguanense*; and 0.5% for 5 min in *C. iridioides* and *C. tracyanum*. The seeds were surface sterilized, washed 3 times with sterilized water, transferred to 2.0-ml cryotubes, and treated with 1.5 ml of cryoprotective solution (2 M glycerol and 0.4 M sucrose) for 15 min at 25°C. After discarding the cryoprotective solution, the seeds were dehydrated at 0°C for various periods using 2.0 ml PVS2 (Sakai et al. 1990) containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide, and 0.4 M sucrose. The seeds were then directly immersed in LN for 30 min. The cryotubes were then thawed in a water bath at 38°C for 2 min. After PVS2 was drained off the cryotubes, the seeds were treated with 1.0 ml of 1.2 M sucrose solution for 10 min. This solution was then drained off, and the seeds were treated with 1.0 ml of 0.4 M sucrose solution for 10 min. The experiment was repeated 3 times.

After the vitrification treatment or surface sterilization, the seeds were sown on 0.2% gellan gum-solidified New Dogashima (ND) medium (Tokuhara and Mii 1993) supplemented with 58 mM sucrose and incubated at 25°C in the dark for 16 or 8 weeks if the seeds were of a terrestrial or an epiphytic species, respectively. The embryos that emerged from the seed coat were defined as germinated.

The survival rate of the seeds was estimated by the 2,3,5-triphenyltetrazolium chloride (TTC) stainability test according to the protocol described by Ishikawa et al. (1997) with some modifications. After cryoprotective treatment and 3-day culture on 0.2% gellan gum-solidified ND medium supplemented with 58 mM sucrose, approximately 100 seeds were then incubated in 1% (w/v) TTC solution for 1 day at 25°C in the dark; the TTC stainability (%) of the cryopreserved and untreated control seeds was determined by calculating the percentage of seeds with TTC-stained embryos.

The relative germination rates (%) calculated as the ratio of the germination rate to TTC stainability, which is an indicator of the percentage of viable seeds, were compared by Student's *t* test ($P=0.05$) between cryopreserved and untreated control seeds.

Since the optimum period of exposure to the vitrification solution usually differs depending on the species, the treatment conditions were initially optimized for seeds of *C. goeringii*, *C. macrorhizon*, and *C. finlaysonianum*. In these 3 species, the survival rate of seeds that were not immersed in LN after PVS2 treatment (treated controls) did not decrease after PVS2 treatment (Figure 1A–C). In the case of *C. goeringii*, almost none of the PVS2-untreated seeds survived after cryopreservation; however, the survival rate of seeds that

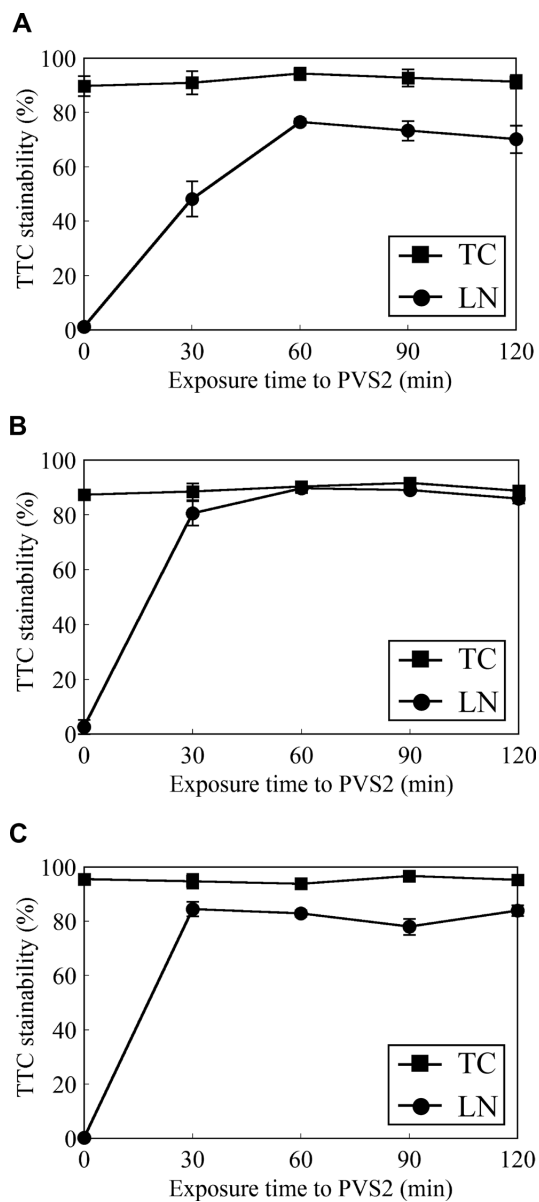


Figure 1. Effect of PVS2 treatment period on the survival rate of *Cymbidium* seeds after cryopreservation. The seeds of *C. goeringii* (A), *C. macrorhizon* (B), and *C. finlaysonianum* (C) were treated by vitrification procedure, and then plunging into liquid nitrogen (LN). TC: no immersion in LN after vitrification treatment. The survival rates of the seeds were estimated by the TTC stainability test. Data represent mean \pm standard error.

were treated with PVS2 for extended periods from 0 to 60 min increased after cryopreservation, with seeds treated for the latter period showing the highest survival rate of 77% (Figure 1A). In the case of *C. macrorhizon* and *C. finlaysonianum*, the survival rate of the cryopreserved seeds showed a similar pattern to that of *C. goeringii* seeds, and the highest survival rates obtained were 90% for 60-min treatment of the *C. macrorhizon* seeds and 85% for 30-min treatment of the *C. finlaysonianum* seeds (Figure 1B, C). For cryopreservation of orchids by vitrification, preculture in a medium containing a high concentration of sucrose or

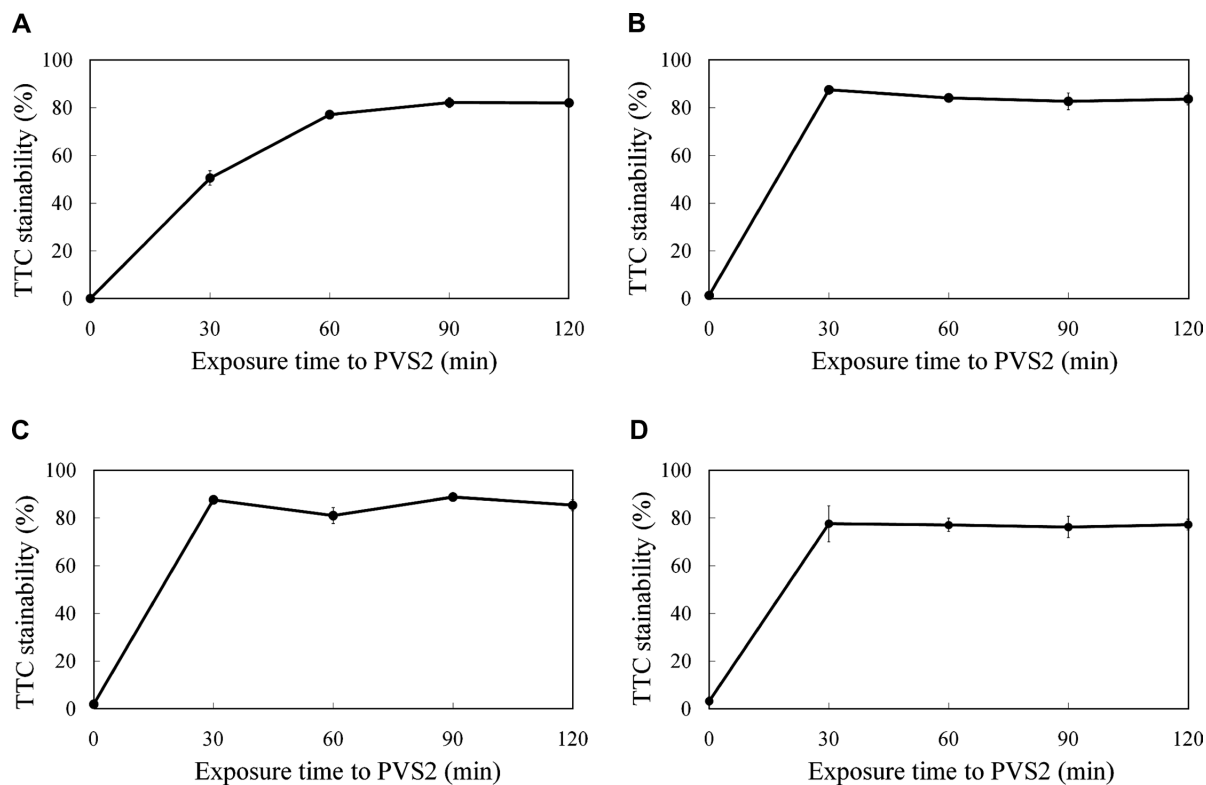


Figure 2. Survival rates of *Cymbidium* seeds after cryopreservation. The seeds of *C. kanran* (A), *C. iridioides* (B), *C. maguanense* (C), and *C. tracyanum* (D) were cryopreserved by vitrification, and their survival rates were then determined by the TTC-stainability test for all PVS2 exposure periods. Data represent mean \pm standard error.

abscisic acid has been reported to be useful for improving the survival of cryopreserved tissues or organs (Hirano et al. 2006). In these 3 *Cymbidium* species, however, such treatments were not required for obtaining high survival rates probably because of their intrinsic trait of high dehydration tolerance.

The vitrification method was also applied to the mature seeds of 4 other *Cymbidium* species. The PVS2 treatment periods were optimized to 60 min for seeds of the terrestrial species *C. kanran*, and 30 min for seeds of the epiphytic species *C. iridioides*, *C. maguanense*, and *C. tracyanum* (Figure 2). The seed coats of epiphytic species are known to exhibit higher water permeability than those of terrestrial species (Yorder et al. 2002), and similar differences in the seed coat properties were reported among species of *Cymbidium* (Yukawa et al. 2003). The different degree of penetration of the PVS2 solution into the seeds may have resulted in the differences in the optimum PVS2 treatment period between the terrestrial and epiphytic species.

To evaluate the effect of cryopreservation treatment on seed germination, the relative germination rate, i.e., the ratio of germination rate to TTC stainability, was compared between untreated and cryopreserved seeds of each species. In the case of the terrestrial species *C. goeringii* and *C. macrorhizon* and the 4 epiphytic species examined, the relative germination rates did not differ

significantly between the untreated and cryopreserved seeds (Table 1). In contrast, neither untreated nor cryopreserved seeds of the terrestrial species *C. kanran* germinated (Table 1), even though a high TTC stainability of 89% and 78% was obtained for the untreated and cryopreserved seeds, respectively. In addition, a slight but significant decrease in the TTC stainability of the seeds was noted to have been induced by cryopreservation ($P=0.05$; data not shown). Generally, in vitro seed germination of terrestrial *Cymbidium* species, including *C. kanran*, that are native to temperate regions is known to be difficult (Paek and Murthy 2002). This difficulty was partially solved in the case of *C. kanran* by the use of immature seeds (Sawa and Nanba 1976), and potassium hydroxide or ultrasonic treatment before surface sterilization (Paek and Murthy 2002). Therefore, it is considered that seeds of recalcitrant species, such as *C. kanran*, could be cryopreserved with ensured germinable condition by combination of the pretreatments and vitrification method although effectiveness of the pretreatments mentioned above needs to be confirmed in further studies. Since cryopreservation was successfully employed for the immature seeds of *Ponerorchis graminifolia* var. *suzukiana* (Hirano et al. 2005b), whose mature seeds are recalcitrant, this preservation technique may also be applied to *C. kanran*. In the case of *Phaius*

Table 1. TTC stainability and germination rate of cryopreserved seeds of the 7 *Cymbidium* species

Species	Untreated control seeds			Cryopreserved seeds ^a		
	TTC stainability (%)	Germination rate (%)	Relative germination rate (%)	TTC stainability (%)	Germination rate (%)	Relative germination rate (%) ^b
Terrestrial						
<i>C. goeringii</i>	79.0 ± 2.8	32.1 ± 3.3	40.6 ± 4.0	76.5 ± 1.5	31.5 ± 4.0	41.2 ± 5.7NS
<i>C. kanran</i>	88.6 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	78.1 ± 1.5	0.0 ± 0.0	0.0 ± 0.0NS
<i>C. macrorhizon</i>	86.3 ± 4.8	77.7 ± 1.8	91.9 ± 4.8	89.6 ± 2.0	81.8 ± 3.3	91.2 ± 2.6NS
Epiphytic						
<i>C. finlaysonianum</i>	96.2 ± 1.7	91.9 ± 2.0	95.6 ± 3.3	84.5 ± 3.4	75.9 ± 0.5	89.9 ± 3.1NS
<i>C. iridioides</i>	87.9 ± 1.8	85.0 ± 3.0	96.7 ± 2.8	83.9 ± 0.6	81.0 ± 4.2	96.5 ± 5.6NS
<i>C. maguanense</i>	86.4 ± 2.9	70.1 ± 1.8	81.2 ± 2.1	87.6 ± 1.1	73.3 ± 2.0	83.7 ± 2.2NS
<i>C. tracyanum</i>	89.5 ± 4.1	89.2 ± 4.8	99.7 ± 2.6	77.6 ± 9.2	69.1 ± 2.7	90.4 ± 8.2NS

Data represent mean ± standard error. ^aThe cryopreserved seeds of the terrestrial species were treated with PVS2 for 60 min and those of the epiphytic species for 30 min. ^bStudent's *t* test was used to compare the relative germination rates between untreated and cryopreserved seeds of each species. NS indicates not significant.

tankervilleae, no apparent differences in the deterioration of seed viability and germination were noted between storage periods of 30 min and 12 months at -196°C (Hirano et al. 2009). Therefore, we expect that the *Cymbidium* seeds used in the present study can be stored over a long time by cryopreservation.

In the present study, the mature seeds of seven species of *Cymbidium* with various life-forms were successfully cryopreserved by vitrification. Thus, cryopreservation of seeds by vitrification will be applied successfully for other members in Orchidaceae with wide range of biodiversity. In particular, *C. macrorhizon* is a myco-heterotrophic orchid and difficult to maintain under artificial cultivation conditions. Therefore, cryopreservation of the seeds of such plants can be a useful method for their long-term conservation.

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