Efficient Cryopreservation of *Lilium* spp. Shoot Tips using Droplet-vitrification

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ABSTRACT Newly developed shoot tips from adventitious buds induced by tissue cultured bulb-scale segments of five accessions of *Lilium* spp. were successfully cryopreserved by a droplet-vitrification method. Bulb-scale segments cultured on Murashige and Skoog (MS) medium with 0.1 mg·L⁻¹ IAA and 0.1 mg·L⁻¹ zeatin were then cold-hardened at 4°C for 7 days. The excised shoot tips were pre-cultured on solidified MS medium containing 0.3 M sucrose for 1 day at 23°C and then soaked in a mixture of 0.7 M sucrose for a day at 23°C. Pre-cultured shoot tips were cryoprotected with two loading solutions, LD1 and LD2, which included 35% and 40% plant vitrification solution (PVS3), respectively, for 40~60 min at 23°C. The cryoprotected shoot tips were then soaked in PVS2, modified PVS2 and PVS3 for 90~120 min at 23°C. The shoot tips, frozen in microdroplets of vitrification solution, were wrapped with aluminum foil strips, which were immersed rapidly in liquid nitrogen. The shoot tips were then rapidly warmed using unloading solution, transferred to a regeneration medium, stored in the dark for two weeks at 23°C, and then cultured under white fluorescent light at an intensity of 2000 lux with a 16-h photoperiod at 23°C. The average post-cryo regeneration rates of five accessions ranged from 52.7% to 87.5%.

Keywords Cryopreservation, Droplet-vitrification, *Lilium*, Vitrification, Shoot-tips

INTRODUCTION

The genus *Lilium* comprises about 100 species distributed in the northern hemisphere and extending through to the Asian tropics (latitude 10~60°). In Korea, 15 species of lily have been reported, but their exact number varies on the researchers (Lee 2002).

As with other plant species, *Lilium* are also facing the threat of genetic erosion, therefore increasing attention is being paid on the conservation of *Lilium* germplasm. This is because the genus *Lilium* represents one of the most important crops of cut flowers and pot plants worldwide, and it is also used as a vegetable and medicine in some parts of the world.

The germplasm resources of lily cannot be preserved in low-temperature seed banks because they are vegetatively propagated. Furthermore, *in vivo* field preservation is labor intensive, and there is considerable risk of loss due to

disease or extreme weather. Additionally, *in vitro* tissue culture conservation is susceptible to contamination and somaclonal variation. Thus, cryopreservation of the lily meristem or shoot tip may represent a suitable method for long-term preservation. Several research results have indicated that the meristems of lily have been cryopreserved successfully using techniques of vitrification (Bouman and De Klerk 1990). Other researchers have reported that lilies have been cryopreserved by vitrification and encapsulation-dehydration (Bouman et al. 2003; Chen et al. 2007; Matsumoto et al. 1995; Zhang et al. 2004; Kaviani et al. 2008). Chen et al (2011) reported the first successful cryopreservation of *Lilium* spp. by droplet-vitrification using meristems from adventitious buds. Droplet-vitrification combines droplet freezing with vitrification protocols and is a very efficient cryopreservation method (Kim et al. 2006; Yoon JW et al. 2006).

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In this study, to improve the droplet-vitrification cryopreservation method for lily shoot tips, we tested two kinds of loading solution and three types of dehydration solutions on five *Lilium* spp. germplasms. We determined the most efficient loading solution and the optimum application time, after which we tested three types of dehydration solutions and treatment times.

MATERIALS AND METHODS

Preparation of plant material

The basal medium used for all the trials was Murashige and Skoog (MS) containing 3% sucrose and adjusted to a pH of 5.8 prior to autoclaving at 121°C for 15 min. Adventitious buds were formed on the surface of bulb-scale segments after 30 days on MS medium with 0.1 mg·L⁻¹ indole-3-acetic acid (IAA), 0.1 mg·L⁻¹ zeatin, 3% sucrose, and 2.2 g·L⁻¹ phytagel. Bulb-scale segments were sub-cultured to conserve or multiply on MS medium with 0.15 mg·L⁻¹ IAA, 0.2 mg·L⁻¹ zeatin, 1 g·L⁻¹ charcoal, 30 g·L⁻¹ sucrose, and 2.7 g·L⁻¹ phytagel. For forming *in vitro* bulb, we used MS medium with 0.15 mg·L⁻¹ IAA, 0.2 mg·L⁻¹ zeatin, 1 g·L⁻¹ charcoal, 7.5% sucrose, and 2.7 g·L⁻¹ phytagel. All the cultures were carried out under white fluorescent light (2000 lux) with a 16-h day photoperiod at 23°C.

Cold hardening and pre-culture

Bulb-scales cultured on MS medium with 0.1 mg·L⁻¹ IAA, 0.1 mg·L⁻¹ zeatin, 3% sucrose, and 2.2 g·L⁻¹ phytagel at 23°C for 2 weeks were cold-hardened at 4°C for 7 days under white fluorescent light (2000 lux) with a 16-h day

photoperiod at 23°C. The apical shoot tips were pre-cultured in liquid MS basal medium supplemented with 0.3 M sucrose overnight. They were then placed in 0.7 M sucrose overnight under the same light conditions as indicated previously.

Loading and dehydration procedure

Pre-cultured shoot tips were osmoprotected in loading solution for 40 and 60 min at 23°C. The loading solutions, designated LD1 and LD2, contained MS basal medium supplemented with 35% and 40% plant vitrification solution 3 (PVS3), respectively (Table 1). The shoot tips were then soaked in three types of vitrification solution: PVS2, modified PVS2, and PVS3, for 90~360 min. For the droplet-vitrification treatment, five shoot tips were transferred to one droplet of vitrification solution on thin strips of sterile aluminum foil. The aluminum foil strips were then carefully immersed into liquid nitrogen using fine forceps. After immersion, the strips were quickly transferred to 2 mL cryotubes, which were immediately plunged into liquid nitrogen.

Thawing and plant regeneration

Samples were maintained in liquid nitrogen for at least 1 day. For warming, foil strips were taken out of the cryovials and immediately plunged into a pre-heated (40°C) unloading solution containing 0.8 M sucrose for 30 s, after which another 5 mL of room temperature unloading solution was added. The shoot tips were then incubated at room temperature for 30 min to facilitate unloading. This step helps to rinse the highly concentrated vitrification solution from shoot tips. After thawing and unloading, shoot tips were placed onto regeneration medium containing MS

Table 1. Compositions of the vitrification and loading solutions used in this study.

Solution	Composition	Reference
PVS2	30%G ²⁾ + 15%DMSO ³⁾ + 15%EG ⁴⁾ + 13.7%Suc ⁵⁾ in MS	Sakai et al. 1990
Modified PVS2	37.5%G + 15%DMSO + 15%EG + 22.5%Suc in MS	-
PVS3	50%G + 50%Suc in MS	Nishizawa et al. 1993
LD1	35% of PVS3	
LD2	40% of PVS3	

²⁾ G, glycerol; ³⁾ DMSO, dimethyl sulfoxide; ⁴⁾ EG, ethylene glycol; ⁵⁾ Suc, sucrose

Fig. 1. Cryopreservation of lily shoot tips using droplet-vitrification. A) bulb-scale culture for producing shoot tips for cryopreservation; B) dehydration of shoot tips in vitrification solution droplets on aluminum foil strips; and C) plant regeneration from cryopreserved shoot tips 2 weeks after cryopreservation.

supplemented with $0.15 \text{ mg}\cdot\text{L}^{-1}$ IAA, $0.2 \text{ mg}\cdot\text{L}^{-1}$ zeatin, $0.05 \text{ mg}\cdot\text{L}^{-1}$ GA₃, $15 \text{ mg}\cdot\text{L}^{-1}$ putrescine, $30 \text{ g}\cdot\text{L}^{-1}$ sucrose, and $2.2 \text{ g}\cdot\text{L}^{-1}$ phytigel and cultured in the dark for 2 weeks at 23°C . They were then cultured under white fluorescent light at an intensity of 2000 lux with a 16-h photoperiod at 23°C .

Assessment of survival and regeneration rates

Survival rates were evaluated 14 days after cryopreservation by counting the number of shoot tips that were green and swollen ($>3 \text{ mm}$). Approximately 18 to 20 shoot tips were used per treatment, and each experiment was replicated three times. Regeneration rates were estimated at 7 to 8 weeks after cryopreservation by counting the number of shoot tips that were differentially swollen and green.

Data analysis and statistical procedures

The results were obtained as average percentages with standard deviations. Each experiment consisted of three replicates per treatment, and each cryovial held 10 samples. The results were analyzed by ANOVA, and the means were separated using Duncan's multiple-range test ($p < 0.05$).

RESULTS AND DISCUSSION

Effects of cold hardening

Table 2 showed the effects of cold hardening on the survival and regeneration rates of cryopreserved lily germ-plasms. In all five accessions, the survival and regeneration rates of cold-hardened shoot tips were considerably higher than those of controls, implying a requirement of cold hardening for the cryopreservation of shoot tips in *Lilium* spp. Cold hardening of donor plants tends to induce an intrinsic tolerance to low temperature and desiccation by triggering genes responsible for cold stress (Takagi 2000). Tahtamouni and Shibli (1999) demonstrated that cold hardening of the mother stock for 3 weeks at 4°C under dark conditions followed by vitrification treatment improved the survival and re-growth of cryopreserved shoot tips of wild pear. When the shoot tips of strawberry were cryopreserved by encapsulation-vitrification, those shoot tips that were cold-hardened at 4°C for 2 weeks showed higher levels of shoot formation when compared with non-hardened shoot tips (Hirai et al. 1998).

Table 2. Effect of cold-hardening on the survival and regeneration percentage of shoot tips of the five accessions of *Lilium* spp.

		Accession No.				
		GBL0089	GBL0099	GBL0202	GBL0474	GBL0518
Survival rate (%)	Cold-hardened	$89.5 \pm 5.83^2)$	67.9 ± 5.31	83.3 ± 4.33	57.7 ± 3.67	67.9 ± 4.87
	Control	43.3 ± 2.21	31.3 ± 2.87	43.3 ± 1.22	24.3 ± 1.13	40.2 ± 3.25
Regeneration rate(%)	Cold-hardened	87.5 ± 3.32	64.3 ± 4.34	77.8 ± 2.41	52.7 ± 2.83	60.7 ± 4.63
	Control	33.3 ± 1.11	28.5 ± 2.17	29.5 ± 3.33	19.9 ± 1.82	28.2 ± 2.17

²⁾ Mean $\pm$ standard deviation

Effects of loading solution and application time

Pre-cultured shoot tips were osmoprotected with a mixture of glycerol and sucrose. Many papers have reported that osmotic loading treatment (or loading treatment) increases the osmotic level of the cell and minimizes osmotic damage caused by the vitrification solution (Volk et al. 2004). The effects of loading solution and application time on the survival and shoot formation rates of vitrified shoot tips are summarized in Table 3. Two types of loading solutions tested were both very effective at improving the survival and regeneration rates of vitrified shoot tips cooled to -196°C .

As shown in Table 3, the highest survival (89.5%) and regeneration rates (87.5%) occurred with the treatment with LD1 for 60 min. Thus, LD1 treatment for 60 min is considered optimal in the osmoprotectant solution to achieve plant survival and regeneration after cryopreservation. In the paper by Matsumoto et al (1994), a mixture of 2 M glycerol plus 0.4 M sucrose provided the highest rate of shoot formation in wasabi meristems. Therefore, a mixture

of 2 M glycerol plus 0.4 M sucrose was adopted as the loading solution for *Lilium* spp. meristems in subsequent experiments. The selection of cryoprotectant is one of the most important factors for successful cryopreservation, because osmoprotection before vitrification is based on the ability of highly concentrated cryoprotectant solutions to function without causing plant injury (Rall and Fahy 1985; Benson et al. 1996).

Effects of vitrification solution and application time on recovery

To determine the optimal vitrification solution and application time, pre-cultured and loaded shoot tips were dehydrated using PVS2, modified PVS2 and PVS3 for 90~360 min prior to a plunge into liquid nitrogen (LN). The highest rate of shoot formation was obtained from shoot tips treated with PVS3 for 240 min at 23°C (Table 4). Shoot tips treated with PVS2, modified PVS2, and PVS3 for 90~360 min at 23°C without cooling in LN (treated control)

Table 3. The effect of different loading solutions and treatment times on the survival and regeneration rates of the control (-LN) and cryopreserved (+LN) lily shoot tips of the *Lilium* hybrid Carmina.

Loading solution	-LN		+LN	
	Survival (%)	Regeneration (%)	Survival (%)	Regeneration (%)
Non-loading	98.3	97.3	45.3d ²⁾	38.3d
LD1 40 min	99.3	97.8	80.3a	78.5ab
LD1 60 min	98.2	97.7	89.5a	87.5a
LD2 40 min	97.9	96.9	66.3b	63.5b
LD2 60 min	99.1	98.3	53.2c	50.3bc

²⁾ Mean separation within columns by Duncan's multiple range test at 5% level

Table 4. The effect of different vitrification solutions and treatment times on the survival and regeneration rates of the control (-LN) and the cryopreserved (+LN) shoot tips of the *Lilium* hybrid Carmina.

Vitrification solution & time	-LN		+LN	
	Survival (%)	Regeneration (%)	Survival (%)	Regeneration (%)
PVS2 90 min	99.9	95.3	25.5c	18.3d
Modified PVS2 90 min	97.1	96.9	49.8bc	32.3c
Modified PVS2 150 min	95.3	93.7	51.3bc	33.3c
PVS3 180 min	99.3	98.1	63.3b	56.8bc
PVS3 240 min	98.2	97.7	89.5a	87.5a
PVS3 360 min	95.7	92.5	73.1ab	67.8b

z; Mean separation within columns by Duncan's multiple range test at 5% level

Table 5. Survival and regeneration rates of the shoot tips of the five lily accessions cryopreserved using droplet-vitrification.

Accession no.	Scientific name	Accession name	Survival rate (%)	Regeneration rate (%)
GBL0089	<i>Lilium</i> hybrid	Carmina	89.5 ± 4.43 ²⁾	87.5 ± 5.89
GBL0099	<i>Lilium</i> hybrid	Crystal Light	67.9 ± 5.05	64.3 ± 0.01
GBL0202	<i>Lilium</i> callosum	Sinomartagon section	83.3 ± 7.86	77.8 ± 5.46
GBL0474	<i>Lilium</i> hybrid	Santander	57.7 ± 7.36	52.7 ± 5.33
GBL0518	<i>Lilium</i> hybrid	Marrero	67.9 ± 5.05	60.7 ± 3.15

²⁾ Mean ± standard deviation

retained high levels of shoot formation (>90%). Bouman and De Klerk (1990) reported a survival of 8% of meristems of *L. speciosum* using a vitrification method, which was declared significant as it was the first trial of cryopreservation of *Lilium* spp. ever conducted. Vitrification refers to a physical process whereby a concentrated aqueous solution solidifies into metastable glass at sufficiently low temperatures. Highly concentrated cryoprotective solutions become so viscous that they solidify into a metastable glass at a practical cooling rate (Fahy et al. 1984). A vitrification procedure eliminates the need for controlled slow freezing and permits cells and meristems to be cryopreserved by a direct transfer to liquid nitrogen (Sakai et al. 1990, 1991). A few years after Bouman and De Klerk (1990) research, the vitrification method was successfully applied to five other lily genotypes (Matsumoto et al. 1995), resulting in the first successful cryopreservation of lily. The droplet-vitrification method is based on the droplet-freezing method developed for potato (Schäfer-Menuhr et al. 1997). This method has also been successfully used for the cryopreservation of *Prunus* (De Boucaud et al. 2002), *Carica papaya* (Ashmore et al. 2001), *Chrysanthemum* (Halmagyi et al. 2004), and *Musa* (Panis et al. 2005). Chen et al (2011) reported the first successful cryopreservation of *in vitro*-grown apical meristems of *Lilium* by a droplet-vitrification method. The highest regeneration rate was 67.6%, which is much higher than the result of Bouman and De Klerk (1990) suggesting that droplet-vitrification is more efficient than vitrification as data on survival and regeneration rates of certain lily cultivars suggest.

In this study, regeneration rate (87.5%) was higher than that reported by Chen et al (2011). In a similar study, droplet-vitrification method was reported to improve the recovery rate of *Musa* shoot tips compared to cryovial-

vitrification (Panis et al. 2005). Survival and shoot formation rates of the five accessions of *Lilium* spp. studied here were compared using droplet-vitrification (Table 5) where *Lilium* hybrid Carmina, exhibited the highest survival (89.5%) and regeneration rates (87.5%). Moreover, we analyzed several factors affecting survival and regeneration to provide the foundation for the long-term cryopreservation of *Lilium* spp. shoot tips. We consider the droplet-vitrification method an optimum means of shoot tip or meristem cryopreservation for additional plant species because of the higher cooling rate promoted by this method. Further research is required using cytological and molecular analyses to confirm the morphological and genetic stability of regenerated plantlets produced by this type of germplasm preservation.

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