

A Successful Regeneration from Shoot Tips of *Chrysanthemum morifolium* (Ramat.) following Cryopreservation by Droplet-vitrification

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Abstract - This study describes an efficient and widely applicable droplet-vitrification following cryopreservation for shoot tips of *Chrysanthemum morifolium* (Ramat.) cvs. 'Borami' and 'Yes morning'. The shoot tips of *Chrysanthemum* were precultured in Murashige and Skoog (MS) liquid medium supplemented with sucrose (0.3-0.7 M). Precultured explants were treated with loading solution (LS, C6) containing glycerol 20% and sucrose 20% for 30 min and exposed to dehydration solution (B5) containing 40% of glycerol and 40% of sucrose for 60 min at 25 °C, and then transferred onto droplets containing 2.5 µl PVS3 on sterilized aluminum foils (4 cm × 0.5 cm) prior to direct immersion in liquid nitrogen (LN) for 1 h. The highest regeneration rate (%) was obtained when shoot tips were precultured with treatment-2 (exposing of shoot tips to MS + 0.3M sucrose for 30 h and then treated with MS+0.5 M sucrose for 16 h) at 25 °C in both the cultivars. The viability of cooled samples, followed by culturing on NH₄NO₃-free MS medium for first 5 days was increased to two-fold (80.7%) regrowth rate over those cultured on normal MS medium or MS medium containing plant growth regulators. This result shows droplet-vitrification would be a promising method for cryobanking chrysanthemum germplasm.

Key words – Chrysanthemum, Cryopreservation, Liquid nitrogen, Regrowth, Vitrification

Introduction

Chrysanthemum (*Chrysanthemum morifolium* (Ramat.) from Asteraceae family is one of the most important ornamental crops in the world (Teixeira da Silva, 2004). Chrysanthemum is being used for different purposes such as using these flowers in decorations during various festivals, making flower gardens, and making bouquets for honoring people. It is an important ornamental pot plant or cut flower in Korea (Liang *et al.*, 2014). In Asian countries like China, Japan and Korea, chrysanthemum has been used as medicines since a long time, mainly due to the presence of various biologically active compounds and essential oils (Schwinn *et al.*, 1994; Luyen *et al.*, 2015).

Characterization of chrysanthemums is vital for their conservation for understanding their genetic relationships (Khaing *et al.*, 2013). In addition to ornamental effects, chrysanthemums are well known for their medicinal values, especially, its flowers have numerous antibiotic activities, and therefore chrysanthemum flowers have been used in traditional Chinese medicine for centuries (Yeung, 1983). In addition to its medicinal properties, chrysanthemum extract can also be used as raw material in dye and tea production. Due to over exploitation of these species, the protection of genetic resources are gaining importance to conserve the plant material through various means to meet the future demand (Wang *et al.*, 2014). Therefore, storage of explants at ultra-low temperatures, under liquid nitrogen (LN, -196 °C), is being recognized as an ideal means for the long-term conservation of plant germplasm, including ornamental

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species (Kulus and Zalewska, 2014; Teixeira da Silva *et al.*, 2015).

Previously, various cryopreservation protocols such as preculture-desiccation (Hitmi *et al.*, 2000), encapsulation-dehydration (Martín *et al.*, 2011; Sakai and Engelmann 2007; Yi *et al.* 2017), vitrification (Martín and González-Benito, 2005), and droplet-vitrification (Kim *et al.*, 2010; Lee *et al.*, 2011) have been reported. Chrysanthemum has been cryopreserved by controlled-freezing rate long before by Fukai *et al.* (1991). In general, meristematic organs are used for the long-term storage due to their higher genetic stability (Vidal *et al.*, 2005). Previous studies have reported the successful cryopreservation of shoot tips derived from greenhouse grown chrysanthemum plants (Fukai *et al.*, 1990, 1991). On the other hand, studies with *C. morifolium* by encapsulation-dehydration were rather unsatisfactory, resulting in a low survival and regrowth through callus (Halmagyi *et al.*, 2004; Martín and Gonzalez-Benito, 2009).

Droplet-vitrification was developed more recently and proved to be a suitable protocol for cryopreservation of a wide-range of genotypes within the same species (Panis *et al.*, 2005; Kim *et al.*, 2006; Kim *et al.*, 2012). It is considered very promising for implementing large-scale cryopreservation (Teixeira da Silva *et al.*, 2015). Similarly, regrowth following cryopreservation is most concerned issues in cryopreservation. A droplet-vitrification procedure produced the highest post-cryopreservation regeneration in comparison with other procedures applied for chrysanthemum shoot tips (Lee *et al.*, 2011a,b). However, a droplet-vitrification cryopreservation for diverse Chrysanthemum genotypes has not yet been reported for the establishment of cryo-banking of germplasm (Martín and González-Benito, 2009). The main objective of the present study was to develop an easy and efficient droplet-vitrification cryopreservation for shoot tips of the two cultivars: 'Borami' and 'Yes morning' of *C. morifolium*, and to achieve a high recovery of regrowth following cryopreservation of explants.

Materials and Methods

Preparation of plant material

In vitro grown *Chrysanthemum* (*Chrysanthemum morifolium*

(Ramat.) cvs., 'Borami' and 'Yes morning' were used in this study. *In vitro* stock shoots were maintained on shoot multiplication medium composed of Murashige and Skoog (MS, 1962) medium supplemented with 1.0 mg/L BA + 0.1 mg/L NAA, 30 g/L sucrose + 2.6 g/L phytigel (Duchefa Biochemie B.V., The Netherlands). The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C for 15 min. The stock cultures were maintained at 24 ± 1 °C under a 16-h photoperiod with a light intensity of 50 μmol s⁻¹m⁻² provided by cool-white fluorescent tubes. Sub-culturing was done for every 6-7 weeks intervals. Explants used for experiments were excised from 6 week-old plants (Fig. 2a).

Cryopreservation using droplet-vitrification

Nodal segments (~0.5 cm in length), were excised from 4-week-old stock shoots and cultured on shoot multiplication medium (Fig. 2b) composed of Murashige & Skoog (MS) medium supplemented with 1.0 mg/L BA + 0.1 mg/L NAA, 30 g/L sucrose + 2.6 g/L phytigel. The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C for 15 min. The cultures were cold-hardened in the dark at 4 °C for various time periods ranging from 0 to 6 weeks. Shoot tips (1.5–2.0 mm in length) containing 4~5 leaf primordia (Fig. 2c and d) were excised from the shoots that had been cold-hardened for 3 weeks, and precultured on MS medium containing sucrose at 0.3 - 0.7 M concentrations and cultures were incubated for different time durations at 25 °C. Droplet-vitrification protocol is explained very clearly in a schematic diagram (Fig. 1). The various preculture treatments (PCT) are as follows: MS + 0.3 M Sucrose for 30 h (PCT-1), MS + 0.3 M Sucrose for 30 h and then treated in MS + 0.5 M sucrose for 16 h (PCT-2), MS + 0.3 M Sucrose for 30 h, and treated in MS + 0.5 M sucrose for 16 h and then treated in MS + 0.7 M Sucrose for 16 h (PCT-3). Precultured shoot tips were osmoprotected with a loading solution (LS) for different duration of time ranging from 0 to 60 min. Loading solution (LS, C6) (Kim *et al.*, 2009a) is composed of MS medium containing 40% of PVS3 (20% of Glycerol + 20% of sucrose) for different time durations ranging from 0 to 60 min. Thus loaded shoot tips were exposed to dehydration solution (B5) (Kim *et al.*, 2009b) containing 80% of PVS3 (40% of Glycerol + 40% of sucrose) for time durations ranging from 0 to 90 min at 25 °C. The

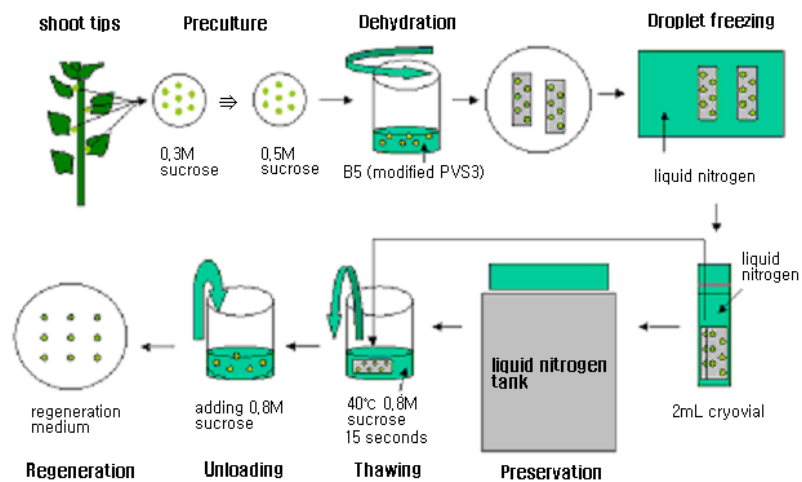


Fig. 1. Schematic diagram showing the process of cryopreservation of shoot tips of *C. morifolium* by droplet-vitrification and regeneration under *in vitro* conditions.

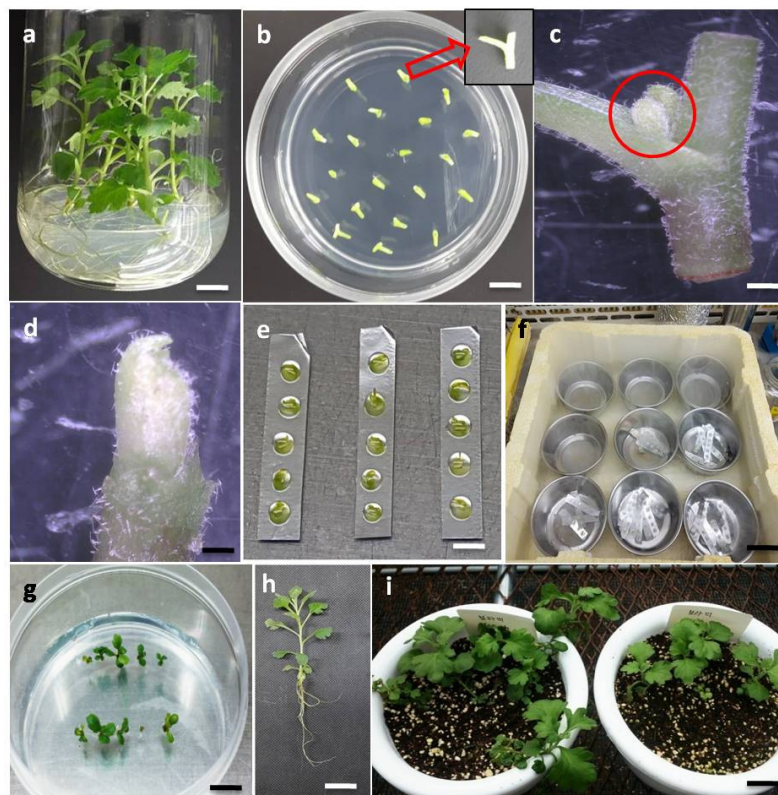


Fig. 2. Cryopreservation of shoot tips of *C. morifolium* L. cv. 'Borami' by droplet-vitrification. (a) *In vitro* grown plantlets of *Chrysanthemum* used for cryopreservation experiments. (b) Nodal explants cultured on MS medium for the emergence of shoot tips. Enlarged form of nodal explant (indicated by arrow). (c) Appearance of shoot tip from nodal axially bud (shoot tip is rounded by circle). (d) dissected shoot tip used for cryopreservation. (e) Dehydration of shoot tips in vitrification solution (B5) droplets on sterilized aluminum foil strips. (f) Immersion of aluminum foil along with shoot tips into the liquid nitrogen (LN) for 1 h. (g) Plant regeneration from cryopreserved shoot tips 8 weeks after inoculation. (h) A shoot regenerated into whole plant from cryopreserved shoot tips that had been cultured on post-culture medium (PCM-2) containing NH_4NO_3 -free MS medium. (i) Acclimatized plantlet from cryopreserved (+LN) shoot tips (right), and treated control (-LN) shoot tips (left) under greenhouse conditions. c, d, e) Bar = 0.5 cm ; a, b, f, g, h, i) Bar = 1.0 cm.

shoot tips were transferred onto droplets containing 2.5 μ l PVS3, each carried on sterilized aluminum foil strip (4.0 cm \times 0.7 cm) (Fig. 2e). PVS3 is composed of MS supplemented with 50% Glycerol and 50% Sucrose, followed by direct immersion in LN (Fig. 2f). The shoot tips were taken out from the LN after a few minutes and transferred into a 2 ml cryotube filled with LN for cryostorage for 1 h. Frozen foils with shoot tips were taken out from LN and immediately placed into an unloading solution containing liquid MS with 1.2 M sucrose at room temperature for 20 min.

Regrowth assessment and plant regeneration

Based on our preliminary studies, cryopreserved, unloaded shoot tips were then post-thaw cultured in Petri dishes (90 mm in diameter) for shoot recovery on various post-cultured media (PCM) under standard conditions, as described by Lee *et al.* (2011a) and Wang *et al.* (2014). Various PCM are as follows: PCM-1: Normal MS medium; PCM-2: NH_4NO_3 -free MS medium; PCM-3: MS + 1.0 mg/L 6-benzylaminopurine (BA) + 0.1 mg/L α -naphthalene acetic acid (NAA); PCM-4: MS + 1.0 mg/L BA + 0.2 mg/L NAA; PCM-5: MS + 0.1 mg/L indole-3-acetic acid (IAA) + 0.2 mg/L zeatin + 0.05 mg/L gibberellic acid (GA_3). Filter-sterilized GA_3 was added to the medium after autoclaving, while BA and NAA were directly added to the medium before autoclaving. The cultures were placed at $24 \pm 1^\circ\text{C}$ in the dark for 1 day and then transferred to light conditions for recovery. After two weeks of growth, all the plantlets were transferred to normal MS media. Shoot regrowth was defined as percentage of shoot tips regenerating into shoots with development of leaves after 4~6 weeks of post-thaw culture. Shoots developed with roots after 4 weeks onto hormone-free $\frac{1}{2}$ MS medium. Thus, fully *in vitro* grown plantlets were transferred to soil under greenhouse conditions for acclimatization.

Statistical analysis

In cryopreservation experiments, shoot tips receiving all treatments but without freezing in LN served as the treated control ($-$ LN), while cryopreserved ($+$ LN) shoot tips served as test material. At least 10~12 shoot tips were used in each treatment of three replicates. All experiments were conducted twice. Results are presented as means with their standard

error (SE). The data were analyzed (mean \pm SE) from two experiments, and least significant differences (LSD) were calculated at $P < 0.05$.

Results and Discussion

Effects of preculture conditions on the regeneration rate (%) of cryopreserved *Chrysanthemum* cultivars

The sucrose concentration and time duration in preculture treatments did not significantly affect the regeneration rate (%) of treated control ($-$ LN) of the shoot tips in both the cultivars ('Borami' and 'Yes morning'), while did affect significantly in cryopreserved ($+$ LN) shoot tips of the two cultivars of *C. morifolium* (Table 1). The regeneration rate (%) was higher from the shoot tips of treated control ($-$ LN) than from cryopreserved ($+$ LN) shoot tips in both the cultivars. For cryopreserved ($+$ LN) shoot tips, the highest regeneration rate (%) was obtained when shoot tips were exposed to the PCT-2 (where exposing shoot tips to MS + 0.3 M Sucrose for 30 h and then treated with MS + 0.5 M sucrose for 16 h) at 25°C in both the cultivars. On the other hand, when exposing the shoot tips to increased concentration of sucrose (0.7 M), the regeneration rate (%) started decreasing, which showed the standardization of sucrose concentration plays an important role during preculture of the explants for cryopreservation protocol. The highest regeneration rates (%) were 42.1% and 38.8% for cryopreserved shoot tips of 'Borami' and 'Yes morning', respectively. For cryopreserved ($+$ LN) shoot tips without preculture treatment, the regeneration rate (%) was drastically lower in comparison with the regeneration rate after preculture treatments. However, there was no effect of preculture treatments on regeneration rate (%) in treated control ($-$ LN) in both the cultivars.

Exposure of *in vitro* grown explants with preculture treatment containing various sucrose concentrations is necessary step to obtain the best shoot regeneration rate (%) from cryopreserved shoot tips. Previous reports by various researchers have displayed the effect of sucrose concentrations on the regeneration rate (%) during preculturing which are supported to our research. Halmagyi *et al.* (2004) used 0.5 M sucrose in preculture treatment for 24 h, and similarly, Lee *et al.* (2011a) reported that the highest shoot regrowth was

Table 1. Effect of preculture treatment on regeneration rates of chrysanthemum shoot tips before (–LN) and after cryopreservation (+LN) following liquid nitrogen immersion^z

| Preculture treatment | Regeneration rate (%) | | | |
|----------------------|------------------------|------------------------|-----------------------|-----------------------|
| | ‘Borami’ | | ‘Yes morning’ | |
| | –LN | +LN | –LN | +LN |
| No preculture | 92.4±2.5 ^{ay} | 10.3±2.9 ^c | 89.3±1.7 ^a | 9.2±1.3 ^d |
| PCT-1 | 90.3±3.2 ^a | 28.9±4.2 ^{bc} | 95.2±4.1 ^a | 18.7±2.6 ^c |
| PCT-2 | 90.0±1.9 ^a | 42.1±1.9 ^a | 91.3±3.7 ^a | 38.8±3.1 ^a |
| PCT-3 | 88.4±4.1 ^a | 32.3±3.2 ^b | 93.5±1.8 ^a | 29.8±1.7 ^b |

^zShoot tips were precultured on MS medium containing sucrose at 0.3 – 0.7 M concentrations and incubating the cultures for different time durations at 25°C. The various preculture treatments (PCT) are as follows: MS + 0.3M Sucrose for 30 h (PCT-1), MS + 0.3M Sucrose for 30 h and then treated in MS + 0.5 M sucrose for 16 h (PCT-2), MS + 0.3M Sucrose for 30 h and then treated in MS + 0.5 M sucrose for 16 h and then treated in MS + 0.7M Sucrose for 16 h (PCT-3), followed by loaded and dehydrated with PVS2 at 0°C, prior to direct immersion in LN for 1 h.

^yThe results are presented as means ± SE. Values in the columns followed by the same letter(s) are not significantly different from each other according to the least significant difference (LSD) ($P < 0.05$).

obtained when shoot tips were precultured with 0.3 M sucrose for 31 h. But studies with some other species like gentian (Suzuki *et al.*, 1998) and Lily (Yi *et al.*, 2013) have reported that exposure of explants to sucrose concentration has been also effective for shoot regeneration. The regenerate rates were 42 and 38% in ‘Borami’ and ‘Yes morning’, respectively. In contrast, Halmagyi *et al.* (2004) reported the plant regeneration rate as 55% after a 10-min incubation in 100% PVS2. On the other hand, our results of preculture treatments in both the cultivars were in corroborative with the results developed by Lee *et al.* (2011) in *Chrysanthemum*, and in addition, displays that the explants from non-cold-hardened plants could withstand cryopreservation after preculture in media with increased sucrose concentration.

Effects of loading and dehydration conditions on regeneration rate (%) from cryopreserved chrysanthemum cultivars

The percentage of regeneration rate was observed at a minimum level from cryopreserved (+LN) shoot tips when they were not treated with loading solution, but the same was high in treated control (–LN) in both the cultivars of *C. morifolium* (Table 2). The range of regeneration rate (%) was from 77 to 85 in treated control (–LN). When the shoot tips were treated with loading solution (LS, C6) containing 40% of PVS3 (20% of Glycerol + 20% of sucrose) for 30 min, and followed by exposure to dehydration solution (B5) containing 80% of PVS3 (40% of Glycerol + 40% of sucrose) for 90 min

at 25°C, prior to direct immersion in LN for 1 h, the regeneration rates (%) were recorded as 48.1 and 42.8 for the cultivars ‘Borami’ and ‘Yes morning’, respectively. Regeneration rates started decreasing when the exposure time exceeded 90 min in dehydration solution (B5). The optimal time durations of exposure to loading solution (C6) and dehydration solution (B5) were 30 min and 90 min, respectively for regrowth of cryopreserved (+LN) shoot tips from both the cultivars of *C. morifolium*.

Previously, Kim *et al.* (2009a, b) developed alternative loading solutions (LSs) and vitrification solutions (VSs) and applied those to *Chrysanthemum* cv. ‘Peak’, which was sensitive to the cytotoxicity of cryoprotectants used in the droplet-vitrification method. This method has been successfully used for the cryopreservation of certain plant species such as *Prunus* (De Boucaud *et al.*, 2002), *Musa* (Panis *et al.*, 2005), including *Chrysanthemum* (Halmagyi *et al.*, 2004). In studies with alternative LSs, chrysanthemum axillary shoot tips were progressively precultured with various concentrations of sucrose ranging from 0.3 M – 0.7 M with various time durations (Kim *et al.*, 2009a). Interestingly, in our study the vitrification solution (B5) was devoid of toxic substances such as dimethyl sulfoxide (DMSO) and ethylene glycol (EG). During the exposure of explants with the dehydration solution (B5), it determines the extent of cell dehydration. A long duration of vitrification solution exposure was apparently toxic to the cells (Chen *et al.*, 2011). In supportive of our

Table 2. Effect of loading and dehydration solutions and exposure time on the regeneration rate of chrysanthemum shoot tips before (–LN) and after liquid nitrogen exposure (+LN)

| Loading solution and exposure time (min) | Dehydration solution and exposure time (min) | Regeneration rate (%) | | | |
|--|--|-----------------------|------------------------|-----------------------|------------------------|
| | | ‘Borami’ | | ‘Yes morning’ | |
| | | –LN | +LN | –LN | +LN |
| No loading | B5 (30) | 81.2±2.2 ^a | 9.2±1.5 ^{cd} | 78.7±3.4 ^b | 9.8±3.3 ^{cd} |
| | B5 (60) | 77.4±3.2 ^b | 11.5±3.7 ^{cd} | 82.6±2.7 ^b | 10.5±2.3 ^{cd} |
| | B5 (90) | 82.4±4.2 ^a | 12.3±2.3 ^{cd} | 85.2±1.9 ^b | 9.3±1.5 ^{cd} |
| C6 (30) | B5 (30) | 82.8±3.9 ^a | 33.6±1.8 ^b | 83.6±1.6 ^b | 32.7±1.4 ^b |
| | B5 (60) | 85.5±2.4 ^a | 48.1±0.9 ^a | 88.4±2.5 ^a | 42.8±2.7 ^a |
| | B5 (90) | 79.6±4.4 ^b | 32.5±2.9 ^b | 92.2±3.6 ^a | 29.5±1.8 ^b |
| C6 (60) | B5 (30) | 82.3±4.1 ^a | 24.2±2.8 ^c | 88.7±3.9 ^a | 14.2±2.5 ^{cd} |
| | B5 (60) | 80.8±1.7 ^b | 28.3±3.1 ^{bc} | 85.2±4.7 ^b | 18.3±2.2 ^c |
| | B5 (90) | 84.3±5.2 ^a | 18.5±1.6 ^c | 86.2±2.9 ^b | 15.7±3.3 ^{cd} |

Precultured shoot tips (MS + 0.3M Sucrose for 30 h and then treated in MS + 0.5M sucrose for 16 h) were treated with loading solution (LS, C6) containing 40% of PVS3 (20% of Glycerol + 20% of sucrose) for different time durations ranging from 0 to 60 min and exposed to dehydration solution (B5) containing 80% of PVS3 (40% of Glycerol + 40% of sucrose) for time durations ranging from 0 to 90 min at 25°C, prior to direct immersion in LN for 1 h. Cryopreserved, rewarmed shoot tips were post-cultured on a recovery medium containing MS medium supplemented with 0.1 mg/L IAA, 0.2 mg/L zeatin, 30 g/L sucrose and 2.6 g/L phytagel for recovery. Regeneration rate (%) was recorded 30 days after post-culture. The results are presented as means ± SE. Values in the columns followed by the same letter(s) are not significantly different from each other according to the least significant difference (LSD) ($P < 0.05$).

results, previously Panis *et al.* (2005) stated that the optimal length of exposure to vitrification solution varies from species considerably. In addition, Halmagyi *et al.* (2004) showed that both full strength and diluted PVS2 were successfully employed for cryopreserving chrysanthemum shoot tips.

Effects of post-culture media on regeneration rate (%) of cryopreserved shoot tips of *C. morifolium*

In this study, we investigated whether the ammonium ion concentration in post-thaw culture medium (PCM) affects the viability of cryopreserved *Chrysanthemum* shoot tips. The viability of cooled samples, followed by culturing on NH_4NO_3 -free MS medium for first 5 days was increased to two-fold (80.7%) regrowth rate over those cultured on normal MS medium or MS media containing plant growth regulators. There were no significant differences in regrowth rates among five media used for the treated control (–LN) in two cultivars of *C. morifolium* (Table 3). For cryopreserved (+LN) shoot tips, the lowest regrowth rates were 42.1% and 38.8% for the cultivars ‘Borami’ and ‘Yes morning’,

respectively by normal MS medium (PCM-1; Fig. 2g) while the highest regrowth rates were observed as 80.7% and 75.5% for the cultivars ‘Borami’ and ‘Yes morning’, respectively in NH_4NO_3 -free MS medium (PCM-2). The next highest regeneration rate (%) was obtained by the MS medium supplemented with 1.0 mg/L BA + 0.1 mg/L NAA (PCM-3). From our results, Table 3 shown that the best optimal post-culture medium was PCM-3 (NH_4NO_3 -free MS medium) for both the cultivars of *C. morifolium*. The complete plantlets (Fig. 2h) were obtained from regenerated shoot tips were transferred to the potting soil (Fig. 2i) under greenhouse conditions for their acclimatization.

Previously, Decruse and Seeni (2002) reported the importance of reducing ammonium nitrate in the culture medium to get maximum recovery of cryopreserved shoot tips of *Holostemma annulare*. Under the long preconditioning treatment, MS medium free of NH_4NO_3 allowed maximum regeneration rate in LN exposed shoot tips after 45 days of post-freeze culture. Similar results were also observed in the present study with respect to the absence of NH_4NO_3 in MS medium (Table 3). On the other hand,

Table 3. Effects of post-culture media on regeneration rate (%) of the treated control (–LN) and cryopreserved (+LN) shoot tips of the two cultivars of *C. morifolium* by droplet-vitrification

| Post-culture medium (PCM) ^x | Regeneration rate (%) | | | |
|--|-----------------------|-----------------------|-----------------------|-----------------------|
| | ‘Borami’ | | ‘Yes morning’ | |
| | –LN | +LN | –LN | +LN |
| PCM-1 | 85.3±5.2 ^b | 42.1±3.1 ^b | 90.3±1.4 ^b | 38.8±1.5 ^c |
| PCM-2 | 88.3±2.3 ^b | 80.7±1.8 ^a | 93.4±2.4 ^a | 75.5±2.2 ^a |
| PCM-3 | 90.3±1.9 ^a | 77.3±3.2 ^a | 90.8±2.8 ^b | 73.7±2.2 ^a |
| PCM-4 | 92.3±3.1 ^a | 41.8±2.5 ^b | 88.4±3.1 ^b | 39.1±1.2 ^c |
| PCM-5 | 90.2±1.4 ^a | 75.2±2.2 ^a | 95.3±2.7 ^a | 61.3±3.5 ^b |

Shoot tips (2.0 mm) excised from terminal buds of 4 weeks old stock cultures were precultured with MS+0.3M Sucrose for 30 h and then treated in MS+0.5M sucrose for 16 h. Precultured and loaded shoot tips were exposed to loading solution (LS, C6) for 30 min and exposed to dehydration solution (B5) for 60 min prior to a direct immersion in LN. Rewarmed shoot tips were post-cultured on various media for shoot recovery. Two week later, all the plantlets were transferred to normal MS media. The regeneration rate (%) of the cryopreserved shoot tips was recorded 30 days after inoculation. The results are presented as means ± SE. The values in the columns followed by the same letter(s) are not significantly different from each other according to the least significant difference (LSD) ($P < 0.05$). ^x PCM-1: Normal MS medium; PCM-2: NH₄NO₃-free MS medium; PCM-3: MS + 1.0 mg/L BA + 0.1 mg/L NAA; PCM-4: MS + 1.0 mg/L BA + 0.2mg/L NAA; PCM-5: MS + 0.1 mg/L IAA + 0.2 mg/L zeatin + 0.05 mg/L GA₃.

Kuriyama *et al.* (1989) found that NH₄⁺ was beneficial for the growth of new rice cells, but it was toxic to cells that had been exposed to LN. Our results from post-culture treatment following cryopreserved shoot tips are most similar to other results developed recently by Wang *et al.* (2014) in *C. morifolium*, where thawed shoot tips were post-cultured on MS medium supplemented with 0.05 mg/L GA₃ in the dark for 3 days, followed by incubating under standard culture conditions. In contrast, studies with *C. morifolium* by encapsulation-dehydration were not satisfactory, resulting in a low survival and regrowth through callus (Halmagyi *et al.*, 2004), while there was not callus formation from the shoot tips in our report. This result shows droplet-vitrification would be a promising method for cryopreserving *Chrysanthemum* germplasm. In a conclusion, this study presents a protocol for cryopreservation of *Chrysanthemum* germplasm resources by droplet-vitrification, and thus it appears that shoot tips of various cultivars of *C. morifolium* germplasm can be safely conserved by droplet-vitrification method. Further study is necessary to confirm the genetic stability of regenerated plantlets by cytological, biochemical and molecular analyses.

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