Plant Biotechnology 28, 401–405 (2011)
DOI: 10.5511/plantbiotechnology.11.0531a

Technical Note

Cryopreservation of *in vitro*-grown shoot tips of carnation (*Dianthus caryophyllus* L.) by vitrification method using aluminium cryo-plates

Kentaro Sekizawa[1], Shin-ichi Yamamoto[2], Tariq Rafique[3], Kuniaki Fukui[2], Takao Niino[2,*]

[1] National Center for Seeds and Seedlings, Tsukuba, Ibaraki 305-0852, Japan; [2] Genebank, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan; [3] Plant Genetic Resource Programme (IABGR), National Agricultural Research Center, Islamabad, Pakistan
* E-mail: niinot@affrc.go.jp Tel: +81-29-838-8127 Fax: +81-29-838-8465

Received May 13, 2011; accepted May 31, 2011 (Edited by M. Nakano)

Abstract Cryopreservation using an aluminum cryo-plate was successfully applied to *in vitro*-grown carnation (*Dianthus caryophyllus* L.) shoot tips. The shoot tips (1–1.5 mm×1 mm) were dissected from the shoot and precultured at 25°C for 2 days on MS medium containing 0.3 M sucrose. The precultured shoot tips were placed on the aluminum cryo-plate containing ten wells embedded with alginate gel. Osmoprotection was performed by immersing the cryo-plates in loading solution (2 M glycerol and 1.4 M sucrose) for 90 min at 25°C. Then, dehydration was performed by immersing the cryo-plates in PVS2 for 25 min at 25°C. After storage in liquid nitrogen, shoot tips attached to cryoplate, were directly immersed into 2 ml 1 M sucrose solution for regeneration. Using this procedure, the average regrowth level of vitrified shoot tips of 4 carnation cultivars reached 95%. This new method has many advantages and will facilitate the cryostorage of reference cultivars of carnation.

Key words: Aluminum plate, carnation, cryo-plate, encapsulation, vitrification.

Carnation is economically one of the most important flowers cultivated for the market in Japan. Almost all carnation breeders seek new varieties and constantly new registrations are conducted. For a registration, a variety must fully meet the requirements set forth in the Plant Variety Protection system according to Seeds and Seedlings Act in Japan. Examination of the characteristics is performed to examine distinctness, uniformity and stability (DUS testing). The DUS testing is conducted in three forms; growing test, on-site inspection by government officials and documentary examination. Plant Variety Protection Office of Ministry of Agriculture, Forestry and Fisheries (MAFF) decides on how the DUS test should be conducted for each application, mainly depending on the genera or species of the candidate variety and reflecting requirements. The National Center for Seeds and Seedlings (NCSS) has been designated to undertake the growing test. For this test, NCSS has maintained many cultivars of each species as reference cultivars. In the case of carnation (*Dianthus caryophyllus* L.), NCSS maintains about 350 cultivars (fiscal year 2008) in pots or in the field as living plants. Such a collection has several problems such as being time consuming and cumbersome to maintain, requiring both space and labor. Moreover, the plant material thus maintained is exposed to pests, pathogens and environmental stresses.

Cryopreservation of plant materials has proven to be an ideal method for not only long-term but short/medium term preservation of plant germplasm because this method requires minimum of space, labor, medium and maintenance. Cryopreservation techniques are now used for plant germplasm storage at several institutes around the world (Niino 2006). Cryopreservation of carnation shoot tips was reported at first by Seibert (1976). But after this, only few papers were published using the conventional slow freezing method (Fukai 1992; Uemura and Sakai 1980) and the encapsulation vitrification method (Adela and Constantin 2007) with moderate survival levels. Recently, the vitrification method using an aluminium cryo-plate (V-Cryo-plate) method was developed by the National Institute of Agrobiological Sciences (NIAS) Genebank using *in vitro*-grown Dalmatian chrysanthemum (*Tanacetum cinerariifolium*) shoot tips. (Yamamoto et al. 2011). The V- Cryo-plate procedure can be treated easily with lording solution

Abbreviations: BA, benzyl adenine; LN, liquid nitrogen; MS, Murashige and Skoog; PVS, plant vitrification solution

This article can be found at http://www.jspcmb.jp/
Published online August 10, 2011

(LS) and PVS2 without floating and/or clinging of shoot tips and also with no or low level of injury to those shoot tips. In this paper, this V-Cryo-plate procedure was applied for the cryopreservation of carnation.

The carnation plants used in this study were obtained from the Nishinihon Station, NCSS, sub-bank of NIAS Genebank Project, in Japan in April 2010. Four cultivars of carnation, 'Melissa', 'Grana', 'Terra Cotta' and 'Yell', were tested. Apical shoots, excised from *in vivo* explants, were surface-sterilized successively with 70% ethanol for 1 min, and 0.5% sodium hypochlorite for 10 min, and then rinsed twice with sterilized distilled water. After surface sterilization, shoot tips with basal plates (1 mm long×1 mm wide) were excised from the shoots and cultured on 70 ml of 0.4% (w/v) gellan gum-solidified MS medium (Murashige, Skoog 1962) in a culture flask (80 mm×100 mm) containing 1 mg l^{-1} thidiazuron, 1 mg l^{-1} naphthalene acetic acid and 3% (w/v) sucrose (Jose et al. 2010). After 50 days of culture, shoots were transferred onto gellan gum-solidified MS medium devoid of plant growth regulators. Then stock plants were subcultured every two months on MS medium. Cultures were incubated at 25°C with a 16 h photoperiod under white fluorescent light (52 μmol m^{-2} s^{-1}) in the culture flask (standard condition). For cryopreservation of *in vitro* shoot tips of carnation, the V-Cryo-plate procedure was applied. The size of the aluminum cryo-plate used was 7 mm×37 mm×0.5 mm with ten wells (diameter 1.5 mm, depth 0.75 mm) (Figure 1B). These plates fit in 2 ml cryotube. The different steps of the V-Cryo-plate procedure are as follows;

1) Cut shoots (5 mm) with a lateral bud and plate on solidified MS medium and culture for 2 weeks at 25°C in standard conditions (Figure 1A). Then, dissect shoot tips with basal plate (1–1.5 mm long×1 mm wide) from the shoots and preculture for 2 days at 25°C on the MS medium with 0.3 M sucrose.
2) Place an aluminum cryo-plate in Petri-dish and pour 2.0–2.5 μl 2% (w/v) Na-alginate solution with 0.4 M sucrose in calcium-free MS basal medium on a well.
3) Place the precultured shoot tips in the well, one by one, with the tip of scalpel blade and slightly press the shoot tips to make them fit into the plate's well (Figure 1B).
4) Pour a calcium solution drop (about 0.3 ml in total) on the section of the aluminum plate where the shoot tips are located until they are covered and leave for 15 min to achieve complete polymerization (Figure

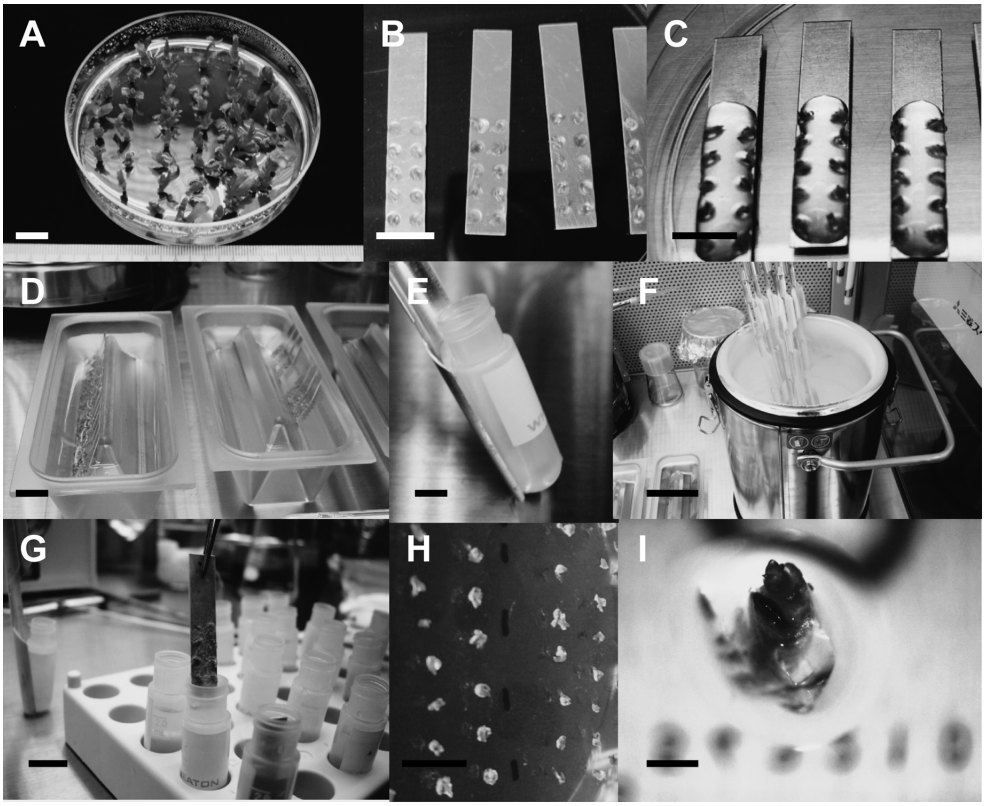

Figure 1. The V-Cryo-plate procedure and appearance of *in vitro*-grown carnation after cryopreservation. (A) Preconditioning of shoot for making uniform material. (B) Aluminium plates and mounting excised shoot tips on the aluminium plates. (C) Hardening of the alginate gel by $CaCl_2$ solution. (D) Treatment by LS and PVSs for osmo-protection and dehydration using the plates. (E) Uncapped cryotube held on a cryo-cane for immersion in LN. (F) Placing the plates into the cryotubes and directly immersed into LN. (G) Removal the plates from LN and warming them into 2 ml 1 M sucrose solution in a cryotube. (H) Plating the vitrified shoot tips with gel on the medium. (I) Regenerated plantlets 3 days after plating. Bars in photos indicate 1 mm (I), 10 mm (A), 5 mm (B, C, D, E, G, H) and 50 mm (F), respectively.

1C). The calcium solution contains 0.1 M calcium chloride in MS basal medium with 0.4 M sucrose.

5) Remove the calcium solution from the cryo-plate by sucking it gently up with a micropipette. Shoot tips adhere to the cryo-plate by the alginate gel.
6) Place the cryo-plate with shoot tips in a 25 ml pipetting reservoir filled with about 20 ml LS (Nisizawa et al. 1992), which contains 2 M glycerol + 1.4 M sucrose in liquid MS basal medium. Shoot tips are thus osmoprotected at 25°C for 30–90 min (Figure 1D).
7) Remove the cryo-plate from LS and place it in a 25 ml pipetting reservoir filled with about 20 ml PVS2 (Sakai et al. 1990). Shoot tips are dehydrated at 25°C for 15–35 min (Figure 1D).
8) After dehydration, transfer the cryo-plate to an uncapped 2 ml plastic cryotube, which is held on a cryo-cane (Figure 1E), and directly plunge into LN where it is kept for at least 30 min (Figure 1F). For long-term storage, the cryotube containing the cryo-plate and LN is capped and stored in LN tank.
9) For regeneration, retrieve the cryotube from LN, take the cryo-plate with shoot tips out of the cryotube and immerse it in 2 ml 1 M sucrose solution in a 2 ml cryotube (Figure 1G). Shoot tips are incubated in this solution for 15 min at room temperature and then transferred onto gellan gum-solidified MS medium (Figure 1H). Post-thaw regrowth (regrowth level) was evaluated after 4 weeks of culture at 25°C under standard conditions.

Three replicates of 10 shoot apices each were tested in each experimental treatment. Results of the replicates are shown as averages ± SEM. Statistical analysis was performed using the Tukey test and significant differences ($P<0.05$) were indicated by different letters.

In the V-Cryo-plate procedure, shoot tips are encapsulated with a thin layer of alginate gel (2.0–2.5 μl). The first step was to determine the optimal exposure time to the LS and PVS2 solutions. The optimal osmoprotection was obtained by 90 min exposure to the LS solution (containing 2.0 M glycerol + 1.4 M sucrose) and resulted in high regrowth rate (80%) (Table 1). No significant differences were obtained when PVS2 dehydration took place in the range of 15–35 min (Table 2).

Using the optimal procedure, we tested the regrowth levels of shoots excised from 4 cultivars of carnation. Regrowth was very high for all cultivars, ranging from 93 to 97%, with an average of 95% for the 4 cultivars (Table 3). PVS3 (containing 50% (w/v) glycerol and 50% (w/v) sucrose; Nisizawa et al 1993), which is another vitrification solution without dimethyl sulfoxide (DMSO) and ethylene glycol (EG), was also tested with the same protocol except dehydration duration. Regrowth after dehydration treatment with PVS3 (50 min at 25°C) resulted in the same level of post-thaw regeneration compared to PVS2 treatment (Table 3). The shoot tips treated with both PVS treatments resumed growth within three days of plating and developed normal shoots without any intermediary callus formation (Figure 1I).

In conventional vitrification procedures, small shoot tips are usually treated in a cryotube floating or suspending in a solution. Treatments, like pipetting, result sometimes in the damage and/or loss of shoot tips. Also, vitrification procedures generally show a very narrow spectrum of optimal treatment time (Niino et al. 2007). In the droplet vitrification procedure, osmoprotected and dehydrated shoot tips have to be

Table 1. Effect of different exposure times to LS (2 M glycerol and 1.4 M sucrose) on the regrowth of cryopreserved shoot tips using the aluminum cryo-plate

Exposure time in LS solution	Regrowth rate (%)
30 min	40 ± 10^{a}
60 min	60 ± 10^{ab}
90 min	80 ± 10^{b}

Shoot tips were treated as follows: Carnation shoot-tips of the cv 'Grana' were precultured for 2 days at 25°C on MS with 0.5 M sucrose, loaded in 2.0 M glycerol and 1.4 M sucrose solution for 30–90 min at 25°C, exposed to PVS2 for 15 min at 25°C. Ten shoot tips were tested for each of the three replicates.

[ab] Responses with the same letter are not significantly different at $P=0.05$ by Tukey test.

Table 2. Effect of different exposure time to PVS2 on regrowth of treatment control (TC) and cryopreserved (LN) shoot tips using the aluminum cryo-plate

Exposure time in PVS2 solution	Regrowth rate (%)	
	TC	LN
15 min	97 ± 6^{a}	87 ± 6^{a}
25 min	93 ± 6^{a}	87 ± 6^{a}
35 min	93 ± 6^{a}	83 ± 6^{a}

Shoot tips were treated as follows: Carnation shoot-tips of the cv 'Grana' were precultured for 2 days at 25°C on MS with 0.3 M sucrose, loaded in 2.0 M glycerol and 1.4 M sucrose solution for 90 min at 25°C and exposed to PVS2 for 15–35 min at 25°C. Ten shoot tips were tested for each of the three replicates.

[a] Responses with the same letter are not significantly different at $P=0.05$ by Tukey test.

Table 3. Regrowth of cryopreserved shoot tips of 4 carnation cultivars using two PVSs

Cultivar	Regrowth rate (%)	
	PVS2	PVS3
Melissa	93 ± 6	97 ± 6
Grana	97 ± 6	93 ± 12
Terra Cotta	93 ± 6	97 ± 6
Yell	97 ± 6	90 ± 0

Shoot tips were treated as follows: shoot-tips were precultured for 2 days at 25°C on MS with 0.3 M sucrose, loaded in 2.0 M glycerol and 1.4 M sucrose solution for 90 min at 25°C, exposed to PVS2 for 25 min or PVS3 for 50 min at 25°C. Ten shoot tips were tested for each of the three replicates.

transferred onto aluminum strips with a droplet of PVS2 or PVS3 just before immersion into LN, which is cumbersome (Kim et al. 2009). The V-Cryo-plate method can overcome these disadvantages since all treatments can be carried out only by moving and transferring the cryo-plate (with attached shoot tips) from one solution to another. Also, this procedure can be performed by semi skilled staff with a little expertise of mounting shoot tips (Yamamoto et al. 2011).

For successful cryopreservation, key factors are preconditioning, hardening, preculture, osmoprotection by LS, dehydration by PVS treatments, and post-thaw handling (Sakai et al. 2008). In any of the cryogenic protocols, the cells and tissues to be cryopreserved must be in a physiologically optimal status for the acquisition of dehydration tolerance and to produce vigorous recovery of growth (Dereuddre et al. 1988; Withers 1979). Preconditioning of shoot tips is necessary to obtain uniform and vital shoot tips to start with. For this, carnation shoots grown for 2-weeks from lateral buds were used for shoot tips to be cryopreserved. As such, there is no need of cold acclimation in carnation. Secondly, preculture on MS medium with high sucrose concentrations and osmoprotection was effective for the induction of osmotolerance towards PVS2 (Niino et al. 2003; Niino et al. 2007). In potato cryopreservation by encapsulation vitrification, the LS solution (a mixture of 2 M glycerol plus 0.6 M sucrose) was effective in increasing osmotolerance towards PVS2 (Hirai and Sakai 1999). Also, Hirai and Sakai (2003) showed that in sweet potato cryopreservation, both a higher concentration of sucrose (1.6 M) in the LS and a longer period of osmoprotection (3 h at 25°C) were necessary to increase the osmotolerance. Kim et al. (2009) indicated for developing a new LS solution for the droplet vitrification procedure that the loading treatment may act as an osmotic stress neutralizer and/or induce a physiological adaptation of tissues and cells prior to both dehydration and vitrification. Also, they pointed out that appropriate LS should be selected for plant species which are highly sensitive to the cryotoxicity of the PVS solutions. In the case of carnation, 90 min osmoprotection by the LS solution containing 1.4 M sucrose was most effective. The last key parameter for successful cryopreservation by vitrification is the carefully controlled procedures for dehydration and prevention of injury by chemical toxicity or excessive osmotic stresses during treatment with PVS solutions. With the V-Cryo-plate it is possible to control these procedures more easily. Using this procedure, in the case of carnation, optimal exposure time to PVS2 has showed wide spectrum of efficiency (from 15 to 35 min). This was also the case when applying the droplet method to shoot tips of black chokeberry (Tanaka et al. 2011). This proves that cooling and warming rates of the V-Cryo-plate method is probably at the same level as that of the droplet method (Yamamoto et al. 2011). Also, the regrowth level after PVS2 dehydration was comparable to PVS3. This means that carnation is not sensitive to PVS2, especially to its toxic compounds DMSO and EG. This V-Cryo-plate method is thus a very practical cryopreservation method for carnation germplasm and also appears to be promising for the cryopreservation of other plants.

Acknowledgements

The authors thank Dr. Ir. Bart Panis (Lab. of Tropical Crop Improvement, Katholieke Universiteit Leuven) for the reviewing this manuscript and suggestions. We are also grateful to Ms. A. Nishiuchi, Ms. N. Nohara and Ms. N. Ishikura for their excellent technical assistance.

References

Adela H, Constantin D (2007) Cryopreservation of carnation (*Dianthus caryophyllus* L.) shoot tips by encapsulation-vitrification. *Scientia Hort* 113: 300–306

Casas JL, Olmos E, Piqueras A (2010) In vitro propagation of carnation (*Dianthus caryophyllus* L.). In: Jain SM, Ochatt SJ (eds) *Protocols for In Vitro Propagation of Ornamental Plants*. Humana Press (Springer) LLC, New York, pp 109–116

Dereuddre J, Fabre J, Bassaglia C (1988) Resistance to freezing in liquid nitrogen of carnation (*Dianthus cryophyllus* L. var Eolo) apical and axillary shoot tips excised from different-aged in vitro plantlets. *Plant Cell Rep* 7: 170–173

Fukai S (1992) Studies on the cryopreservation of shoot tips of *Dianthus* and *Chrysanthemum. Memoirs Fac Agr Kagawa Univ* 56: 1–79

Hirai D, Sakai A (1999) Cryopreservation of in vitro grown meristems of potato (*Solanum tuberosum* L.) by encapsulation-vitrification. *Potato Res* 42: 153–160

Hirai D, Sakai A (2003) Simplified cryopreservation of sweet potato (*Ipomoea batatas* (L.) Lam.) by optimizing conditions for osmoprotection. *Plant Cell Rep* 21: 961–966

Kim HH, Yoon JW, Pak SU, Lee SC, Baek HJ, Cho EG, Engelmann F (2009) Development of alternative loading solutions in droplet-vitrification procedures. *CryoLetters* 30: 291–299

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–479

Niino T, Tanaka D, Ichikawa S, Takano J, Ivette S, Shirata K, Uemura M (2003) Cryopreservation of *in vitro*-grown apical shoot tips of strawberry by vitrification. *Plant Biotechnol* 20: 75–80

Niino T (2006) Developments in plant genetic resources cryopreservation technologies. In: Jung-Hoon Kang (ed) *Effective Genebank Management in APEC Member Economies*, NIAB, Korea, pp 197–217

Niino T, Tanaka D, Tantely RR, Fukui K, Shirata K (2007) Cryopreservation of basal stem buds of *in vitro*-grown mat rush (*Juncus* spp.) by vitrification. *CryoLetters* 28: 197–206

Nishizawa S, Sakai A, Amano Y, Matsuzawa T (1992) Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration

by a simple freezing method. *CryoLetters* 13: 379–388

Nishizawa S, Sakai A, Amano Y, Matsuzawa T (1993) Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification. *Plant Sci* 91: 67–73

Sakai A, Kobayashi S, Oiyama I (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Rep* 9: 30–33

Sakai A, Hirai D, Niino T (2008) Development of PVS-based vitrification and encapsulation-vitrification protocols. In: Reed B (ed) *Plant Cryopreservation, A practical guide*. Springer LLC, New York, pp 33–58

Seibert M (1976) Shoot initiation from carnation shoot apices frozen to −196°C. *Science* 191: 1178–1179

Tanaka D, Matsumoto T, Nishiuchi A, Niino T (2011) Cryopreservation of in vitro grown black chokeberry shoot tips by droplet vitrification. *Acta Hort* (in press)

Uemura M, Sakai A (1980) Survival of carnation (*Dianthus caryophyllus* L.) shoot apices frozen to the temperature of liquid nitrogen. *Plant Cell Physiol* 21: 85–94

Withers LA (1979) Freeze preservation of somatic embryos and clonal plantlets of carrot (*Daucus carota* L.). *Plant Physiol* 63: 460–467

Yamamoto S, Rafique T, Priyantha WS, Fukui K, Matsumoto T, Niino T (2011) Development of a cryopreservation procedure using aluminium cryo-plates. *CryoLetters* 32: 256–265