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The Aluminum Cryo-plate Increases Efficiency of Cryopreservation Protocols for Potato Shoot Tips

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Abstract The possibility of streamlining general cryoprotection procedures is investigated using aluminium cryoplates with wells, facilitating fluid handling and drying treatment. Precultured shoot tips of potato cultivar 'Sayaka' were embedded in calcium alginate gel in wells of the aluminium cryo-plates. In the V cryo-plate protocol, dehydration was performed for 30 min at 25 °C in plant vitrification solution after osmoprotection. In the D cryo-plate protocol, dehydration was performed by placing the cryo-plates for 2.0 h under an air current in a laminar flow cabinet after osmoprotection. In both protocols, cooling was performed by placing the cryo-plates in uncapped cryotubes, which were immersed in liquid nitrogen. For rewarming, the cryo-plates were immersed in liquid MS medium supplemented with 1.0 M sucrose and diluted for 15 min at room temperature. Under these conditions, regrowth rates of cryopreserved shoot tips in V cryo-plate and D cryo-plate were 96.7 and 93.3 %, respectively. Both protocols will facilitate efficient strategies for preservation, storage, and maintenance of genetic stability of important potato germplasm.

Resumen Se investigá la posibilidad de realizer procedimientos generales de crioprotección mediante el uso de crioplacas de aluminio con cavidades, facilitando el manejo fluido y tratamiento de secado. Se colocaron ápices de tallo de papa pre-cultivados, de la variedad "Sayaca" en crio placas de aluminio y se embebieron en de alginato de calcio. En el protocolo de crio placa -V se hizo la deshidratación por 30 min a 25 °C en una solución de vitrificación de planta después de osmoprotección. En el protocolo de crio placa -D, la deshidratación se hizo colocándolas por 2 h bajo una corriente de aire en una cámara de flujo laminar después de la osmoprotección. En ambos protocolos, el enfriamiento se realizó mediante la colocación de las crio placas en crio tubos sin tapa, que fueron inmersos en nitrógeno líquido. Para la secuperacid se sumergieron las crio placas en un medio líquido MS suplementado con 1.0 M de sacarosa en dilución por 15 min a temperatura ambiente. Bajo estas condiciones, los niveles de secuperacion de los ápices de tallo criopreservados en crio placas -V y -D fueron de 96.7 y 93.3 %, respectivamente. Ambos protocolos facilitarán las estrategias eficientes para preservación, almacenamiento, mantenimiento y estabilidad genética de germoplasma de importacia de accesiones de papa.

Keywords Air dehydration · Aluminum plate · D cryo-plate method · PVS2 · V cryo-plate method · Vitrification

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Introduction

Several protocols have been developed for cryopreservation of potato including the DMSO droplet method (Schäfer-Menuhr et al. 1997), vitrification method (Golmirzaie and Panta 2000), encapsulation vitrification method (Hirai and Sakai 1999), droplet vitrification method (Kim et al. 2006; Panta et al. 2014) and gelled droplet vitrification (Hirai 2011). These methods have been used for cryo-storage of

potato genetic resources in genebanks around the world. However the implementation of cryo-storage as a routine preservation method is still limited because it requires skillful manipulation and involves cumbersome steps such as osmoprotective and dehydration treatments and transfer of samples (Yamamoto et al. 2011b). Consequently, standard cryopreservation protocols are labor intensive and fraught with problems associated with mistiming solution exposure or overmanipulating propagules in solution. These disadvantages may impede the wider utilization of cryostorage for important plant genetic resources.

The cryopreservation method using aluminum cryo-plates has been reported recently for genetic resources of several crops including strawberry, Dalmatian chrysanthemum, mint, mulberry, carnation and mat rush shoot tips/buds (Yamamoto et al. 2011a, b, 2012a, b; Sekizawa et al. 2011; Niino et al. 2013). Aluminum cryo-plates could be used for dehydration of shoot tips either by the vitrification protocol using various vitrification solutions (termed as the V cryo-plate method) or simply air-dehydration (termed as the D cryo-plate method). Both methods are "user-friendly" procedures that can ensure very high cooling and warming rates over 4000 °C min^{-1} of treated explants (Yamamoto et al. 2011b; Niino et al. 2013). As a result, very high regrowth with various plant species has been obtained after cryopreservation (Niino et al. 2013).

The V cryo-plate and D cryo-plate protocols were developed with the aim of simplifying the procedures for cryopreservation and developing a systematic procedure that can be easily performed even by semi-skilled workers (Yamamoto et al. 2012b). In the V cryo-plate and the D cryo-plate protocols, the shoot tips/buds attached to the cryo-plates are dehydrated after osmoprotection in plant vitrification solution 2 (PVS2, Sakai 1990) and under the air current of a laminar flow cabinet, respectively. This paper investigates the possibility of streamlining general cryoprotection procedures using aluminium cryoplates with wells that facilitate fluid handling and drying treatment. For this aim, both methods were applied to in vitro grown shoot tips of potato following the procedure described by Yamamoto et al. (2012b). The protocols developed were tested the effect of the improved protocol on additional 16 potato varieties/lines, resulting in efficient preservation, storage, and maintenance of potato genetic resources in genebanks.

Materials and Methods

Plant Materials

The potato varieties used in this study were obtained from the Genebank of the National Institute of Agrobiological Sciences, Japan. Experiments to apply the V and D cryo-plate protocols to potato shoot tips were performed using variety 'Sayaka'. The optimized procedures were tested with 12 additional varieties and 4 lines of four *Solanum* species related to potato. Culture plants of potato were subcultured every 3 months on solid Murashige and Skoog (1962) medium (MS) with 3.0 % (*w*/*v*) sucrose, 0.03 % (*w*/*v*) calcium chloride and 0.3 % (*w*/*v*) gellan gum. Supplement of calcium chloride reduced the callus formation on the leaves of subcultures from Hirai, personal communication. Cultures were incubated at 20±1 °C with a 16 h light / 8 h dark photoperiod under a light intensity of 104 μmol m^{-2} s^{-1} provided by white fluorescent tubes (standard condition). Nodal segments with lateral bud (about 5 mm) from the shoots were cut, plated on 20 ml solid MS medium in Petri dishes (90×20 mm), and cultured for about 2 weeks under standard conditions (Fig. 1a and b). Shoot tips were dissected from the shoots and precultured overnight on solid MS medium containing 0.3 M sucrose at 25 °C (Fig. 1c). For the optimization of the V cryo-plate and D cryo-plate protocols, about 1.5 mm long explants consisting of the apical meristem with 2 young leaves was used. The optimal size of shoot tips for cryopreservation was evaluated using 0.5, 1.0, 1.5 and 2.0–2.5 mm long explants for the V cryo-plate method and 1.0, 1.5 and 2.0–2.5 mm long explants for D cryo-plate method.

V cryo-plate Procedure

The custom-made cryo-plates (Taiyo Nippon Sanso Corp., Japan) with 10 wells and a size 37 mm×7 mm×0.5 mm were used in all experiments. For this procedure wells are circular in shape with a diameter of 1.5 mm and a depth of 0.75 mm (Yamamoto et al. 2011b). The successive steps of the V cryo-plate procedure are as follows:

1. Pour sodium alginate solution (about 2.0 μl) in the wells of the aluminum cryo-plates using a micropipette at room temperature. The alginate solution contains 2 % (*w*/*v*) sodium alginate (viscosity 300–400 cp, Wako Pure Chem. Ind.) in calcium-free MS basal medium. Place the precultured shoot tips one by one in the wells with the tip of a scalpel blade and lightly press the shoot tips so they fit in the wells (Fig. 1d).
2. Pour calcium chloride solution drop-wise (about 0.3 ml/plate) on the section of the aluminum plates where the shoot tips are located until they are covered and wait for 15 min to achieve complete polymerization. The calcium solution contains 0.1 M calcium chloride in MS basal medium.
3. Remove the calcium chloride solution from the cryo-plates by gently tapping the cryo-plates on filter paper. Then, place the cryo-plates with the attached specimens in a 25 ml pipetting reservoir (NSG Precision Co. Mie,

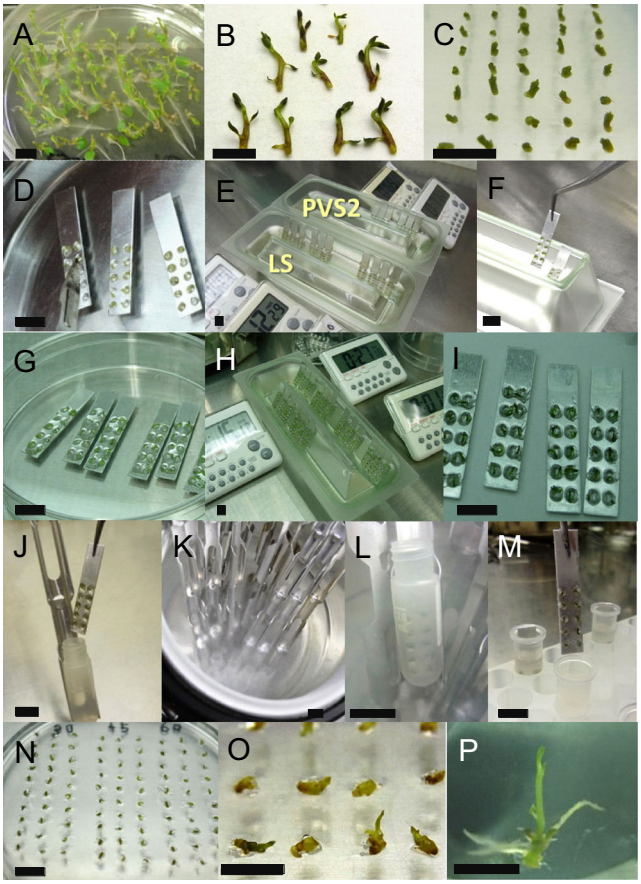

Fig. 1 Overview of the V cryo-plate and D cryo-plate methods for cryopreservation of in vitro grown potato shoot tips. (**a**) Potato plantlets; (**b**) Preparation of potato shoot tips for cryopreservation; (**c**) Preculture of excised shoot tips; (**d**, **e**, **f**) V cryo-plate method; (**d**) Shoot tips mounted on aluminum cryo-plate with Ca alginate gel; (**e**) Osmoprotection in LS (*left*) and dehydration in PVS2 (*right*); (**f**) Transferring the cryo-plate to next step; (**g**, **h**, **i**) D cryo-plate method; (**g**) Shoot tips mounted on aluminum cryo-plate; (**h**) Treatment with LS; (**i**) Desiccation of shoot tips under the air current of a laminar flow cabinet; (**j**) Transferring the cryo-plate with shoot tips into cryotube after dehydration; (**k**) Immersion into liquid nitrogen; (**l**) Vitrified shoot tips in liquid nitrogen; (**m**) Retrieval of cryo-plate from liquid nitrogen and warming in 1 M sucrose solution; (**n**) Plating the cryopreserved shoot tips from the V cryo-plate method; (**o**) Regenerated plants from the V cryo-plate method at 1 week after plating; (**p**) Regenerated plant from the D cryo-plate method at 3 weeks after plating. *Bars* indicate 10 mm

Japan) filled with about 20 ml loading solution (LS, Fig. 1e). The LS solution contains 2 M glycerol + 0.6–1.6 M sucrose in liquid MS basal medium (Nishizawa et al. 1992; Niino et al. 2013). The specimens are osmoprotected at 25 °C for 30 or 60 min. The condition for the standard procedure in this step and succeeding steps is 30 min.

4. Remove the cryo-plates from LS and transfer to a 25 ml pipetting reservoir filled with PVS2 (about 20 ml). The shoot tips are dehydrated at 25 °C for 0, 15, 30, 45, 60, 75 or 90 min (Fig. 1e and f).
5. After dehydration, transfer the cryo-plates to 2 ml uncapped cryotubes, which are held on cryo-canes, and plunge them directly in LN where they are kept for at least 30 min (Fig. 1j, k and l).
6. For rewarming, the cryotubes are retrieved from LN. Then the cryo-plates are immersed in 2 ml cryotubes containing 2 ml MS basal medium with 1 M sucrose (Fig. 1m), and are incubated for 15 min at room temperature. Shoot tips are plated on solid MS medium and cultured under standard condition (Fig. 1n).

D cryo-plate Procedure

The wells on cryo-plates for this procedure had an oval shape and a length of 2.5 mm, a width of 1.5 mm and a depth of 0.75 mm (Niino et al. 2013). The successive steps for the D cryo-plate procedure are basically same as those described for the V cryo-plate protocol, but with modifications in steps 1, 2 and 4 and the modification for these three steps are as follows:

1. Pour sodium alginate solution (about 4.0 μl) in the wells of the aluminum cryo-plates using a micropipette at room temperature. The alginate solution contains 2 % (*w*/*v*) sodium alginate in calcium-free MS basal medium with 0.4 M sucrose. After placing one shoot tip in each well, pour sodium alginate solution (about 1.0 μl) on the shoot tips using a micropipette so that the shoot tips adhere firmly to the cryo-plate.
2. Pour calcium chloride solution drop-wise (about 0.3 ml/plate) on the section of the aluminum plates where the shoot tips are located (Fig. 1g). The calcium solution contains 0.1 M calcium chloride in MS basal medium with 0.4 M sucrose.
4. Remove the cryo-plates from LS (Fig. 1h) and transfer to a petri dish with filter paper to remove the excess LS solution. The shoot tips attached to the cryo-plates are desiccated under the air current of a laminar flow cabinet (HC-1600FS, Oriental Co. Japan) for 1.0, 1.5, 2.0, 2.5 or 3.0 h at 25 °C, with 40–50 % RH (Fig. 1i). The condition for the standard procedure in this step is 2.5 h.

For large scale cryostorage, the optimized V and D cryo-plate procedures were assessed on 16 additional potato varieties/lines. We also assessed the utility of both protocols as a routine procedure for cryopreservation by comparing the results obtained of four operators with no previous experience in cryopreservation.

Survival Assessment and Statistical Analyses

Post LN regrowth was evaluated after 2 weeks (V cryo-plate) or 3 weeks (D cryo-plate) of culture under standard conditions

by counting the number of explants which developed into normal shoots. Three replicates for each of the 10 samples were tested in each experimental treatment. Statistical analysis was performed with the Tukey's test (http://www.gen-info.osaka-u.ac.jp/MEPHAS/tukey.html) to compare the means and determine significant differences ($P<0.05$).

Results and Discussion

Optimization of the V cryo-plate Procedure

The key points in developing an efficient protocol for cryopreservation include the size of shoot tips, sucrose concentration of LS for osmoprotection and exposure time by PVS2 for dehydration. In the V cryo-plate protocol, the exposure time to PVS2 is crucial in order to obtain a high post LN regrowth. A higher regrowth of cryopreserved shoot tips of potato was observed between 15 and 60 min exposure to the PVS2 and almost all samples tested grew with 96.7–100 % regrowth without any significant statistical difference (Table 1). Such a wide range of exposure time to the PVS2 giving very high regrowth is one of the characteristics of V cryo-plate method and droplet vitrification method (Niino et al. 2013; Tanaka et al. 2011). Based on this result, 30 min is considered the optimal dehydration time. In addition, high regrowth of cryopreserved shoot tips was observed (43.3 %) even without PVS2 treatment (0 min in Table 1). This was due to partial dehydration during osmoprotection brought about by high sucrose concentration in LS. It has been suggested that even if the materials were insufficiently dehydrated, rapid cooling and warming to avoid the lethal ice formation in cells could be achieved by using cryo-plates made of aluminum which have a very high thermal conductivity (Yamamoto et al. 2011b; Niino et al. 2013).

Table 1 Effect of exposure time to PVS2 solution on regrowth of cryopreserved shoot tips of potato variety 'Sayaka' by the V cryo-plate method

Exposure time to PVS2	Regrowth (%±SEM)
0 min	43.3b±5.8
15 min	96.7a±5.8
30 min	100.0a±0.0
45 min	96.7a±5.8
60 min	100.0a±0.0
75 min	86.7a±15.3
90 min	70.0ab±26.5

Shoot tips were excised to 1.5 mm in length, precultured for 16 h at 25 °C on MS with 0.3 M sucrose, loaded in 2.0 M glycerol and 0.8 M sucrose solution for 30 min at 25 °C and exposed to PVS2 for 0–90 min at 25 °C. Different letters indicate significant differences between treatments ($P\leq 0.05$). Ten shoot tips were tested for each of the three replicates

The effect of treatment time and sucrose concentration in the LS containing 2 M glycerol on regrowth of cryopreserved shoot tips was studied. Regrowth of cryopreserved shoot tips treated with LS containing 1.2 M sucrose for 30 min or 60 min was the same. Moreover regrowth of cryopreserved shoot tips in LS treatment with 0.8, 1.2 and 1.6 M sucrose for 30 min was 100, 96.7 and 100 %, respectively (Table 2).

Optimization of the D cryo-plate Procedure

The dehydration time by laminar air flow desiccation is crucial to obtain high regrowth in the D cryo-plate protocol especially for specimens of different sizes (Niino et al. 2013; Engelmann et al. 2008). The highest regrowth of cryopreserved shoot tips of potato was obtained after 2.0 h air-dehydration reaching 93.3 % (Table 3). A high regrowth was also obtained after 1.5 to 2.5 h air-dehydration without a significant statistical difference. Even after only 1.0 h air-dehydration, the regrowth of cryopreserved shoot tips reached 73.3 %. Regrowth of cryopreserved shoot tips in LS containing 2 M glycerol with the 0.6, 0.8 and 1.0 M sucrose LS treatment was 90.0, 90.0 and 96.7 %, respectively (Table 4).

Size of Shoot Tips

Also, we performed an experiment to study the effect of size of specimens on regrowth after LN exposure. Specimens of four different size ranges [large (2.0–2.5 mm length), medium (about 1.5 mm), small (about 1.0 mm) and very small (about 0.5 mm)] were used in the two cryopreservation techniques (V cryo-plate and D cryo-plate) employed. Even though differences were in most cases not significant, regrowth of medium and larger specimens was higher compared to smaller specimens following cryopreservation using the D cryo-plate protocol (Table 5). Regrowth was higher and equivalent with the smaller and medium size of specimens using the V cryo-plate

Table 2 Effect of exposure time to LS and concentration of sucrose in LS solution on regrowth of cryopreserved shoot tips of potato variety 'Sayaka' using the V cryo-plate method

Sucrose concentration in LS	Exposure time	Regrowth (%±SEM)
0.8 M	30 min	100.0a±0.0
1.2 M	30 min	96.7a±5.8
1.2 M	60 min	96.7a±5.8
1.6 M	30 min	100.0a±0.0

Shoot tips were excised to 1.5 mm in length, precultured for 16 h at 25 °C on MS with 0.3 M sucrose, loaded in 2.0 M glycerol and 0.8, 1.2 and 1.6 M sucrose solution for 30 or 60 min at 25 °C and exposed to PVS2 for 30 min at 25 °C. Different letters indicate significant differences between treatments ($P\leq 0.05$). Ten shoot tips were tested for each of the three replicates

Table 3 Effect of dehydration period on regrowth of control (-LN) and cryopreserved (+LN) shoot tips of potato variety 'Sayaka' using the D cryo-plate method

Dehydration period	Regrowth (%±SEM)	
	-LN	+LN
1.0 h	100.0a ±0.0	73.3b±8.8
1.5 h	100.0a±0.0	86.7a±3.3
2.0 h	100.0a±0.0	93.3a±3.3
2.5 h	96.7a±3.3	90.0a±0.0
3.0 h	93.3a±3.3	76.7b±3.3

Shoot tips were excised to 2.0 mm in length, precultured for 16 h at 25 °C on MS with 0.3 M sucrose, loaded in 2.0 M glycerol and 0.8 M sucrose solution for 30 min at 25 °C and dehydrated by the air current of a laminar flow cabinet at 25 °C for 1.0–3.0 h. Different letters indicate significant differences between treatments ($P \leq 0.05$). Ten shoot tips were tested for each of the three replicates

procedure (Table 5). In mat rush smaller specimens (the meristematic dome exposed) are more rapidly and uniformly dehydrated by PVS2, in comparison with larger specimens (Niino et al. 2013). This may be the same in potato. By contrast, physical dehydration in the D-cryo-plate might be more uniform in larger specimens, thus explaining the higher regrowth obtained (Niino et al. 2013). Among medium and smaller type specimens, the most vigorous growth was observed after cryostorage from 1.5 mm shoot tips in the V cryo-plate procedure.

Optimized Procedure for Cryopreservation

Based on the above results, we developed an optimized procedure for efficient application of the V cryo-plate and D cryo-plate protocols in cryopreservation of potato shoot tips as follows:

Table 4 Effect of concentration of sucrose in LS on regrowth of control (-LN) and cryopreserved (+LN) shoot tips of potato variety 'Sayaka' using the D cryo-plate method

Sucrose concentration in LS	Regrowth (%±SEM)	
	– LN	+ LN
0.6 M	93.3a±3.3	90.0a±5.8
0.8 M	96.7a±3.3	90.0a±5.8
1.0 M	100.0a±0.0	96.7a±3.3
1.2 M	80.0b±5.8	66.7b±3.3

Shoot tips were excised to 2.0 mm in length, precultured for 16 h at 25 °C on MS with 0.3 M sucrose, loaded in 2.0 M glycerol and 0.6–1.2 M sucrose solution for 30 min at 25 °C and dehydrated by the air current of a laminar flow cabinet at 25 °C for 2.5 h. Different letters indicate significant differences between treatments ($P \leq 0.05$). Ten shoot tips were tested for each of the three replicates

Table 5 Effect of size of shoot tips of potato variety 'Sayaka' to be cryopreserved on regrowth (%) by the V-cryo-plate and the D-cryo-plate method

Size of shoot tips	Regrowth (%±SEM)	
	V cryo-plate	D cryo-plate
0.5 mm	100.0a±0.0	—
1.0 mm	100.0a±0.0	76.7a±6.6
1.5 mm	96.7a±3.3	93.3a±3.3
2.0–2.5 mm	56.7b±3.3	90.0a±5.8

Shoot tips were excised, precultured for 16 h at 25 °C on MS with 0.3 M sucrose, loaded in 2 M glycerol and 0.8 M sucrose solution for 30 min at 25 °C. In the V cryo-plate, shoot tips were dehydrated by PVS2 for 30 min at 25 °C and in the D cryo-plate, dehydrated by the air current of a laminar flow cabinet for 2.5 h at 25 °C. Different letters indicate significant differences between treatments ($P \leq 0.05$). Ten shoot tips were tested for each of the three replicates

1. Cut the shoots and culture on MS medium for 2 weeks at 20 °C. Shoot tips covered by young leaves with length about 1.5 mm (V cryo-plate) and about 2.0 mm (D cryo-plate) are dissected from shoots and precultured overnight at 25 °C on MS medium containing 0.3 M sucrose.
2. Pour sodium alginate solution in the wells of each aluminum cryo-plates using a micropipette. Place the precultured explants one by one in the wells with the tip of a scalpel blade and slightly press the shoot tips into the wells. In the D cryo-plate method, pour additional sodium alginate solution (about 1.0 μl) on the shoot tips using a micropipette.
3. Pour about 0.3 ml calcium chloride solution drop-wise on the section of aluminum plates where the shoot tips are located and wait for 15 min to achieve complete polymerization.
4. Remove the calcium chloride solution from the cryo-plates by gently tapping the cryo-plates on filter paper and transfer in a 25 ml pipetting reservoir filled with about 20 ml LS. The LS solution contains 2 M glycerol + 0.8 M (V cryo-plate) or 1.0 M (D cryo-plate) sucrose in liquid MS basal medium. The explants are osmoprotected at 25 °C for 30 min.

5–1 For the V cryo-plate method, remove the cryo-plates from LS and place them in a 25 ml pipetting reservoir filled with PVS2 (about 20 ml). The shoot tips are dehydrated at 25 °C for 30 min.

5–2 For the D cryo-plate method, remove the cryo-plates from LS and place them in a Petri dish on filter paper to remove excess LS solution. Place the cryo-plates in open petri dishes in the air current of a laminar flow cabinet for 2.0 h at 25 °C, with 40–50 % RH.

6. After dehydration, transfer the cryo-plates to 2 ml cryotubes, which are held on cryo-canes, and plunge them directly in LN where they are kept for at least 30 min.

This optimized procedure was applied to shoot tips of 12 potato varieties and 4 wild *Solanum* species (Table 6). Regrowth using the V-cryo-plate method was very high for all lines, ranging from 93.3 to 100.0 %, with an average of 98.6 %. Regrowth using the D-cryo-plate method was very high for all lines, ranging from 80.0 to 100.0 %, with an average of 91.6 %. Shoot tips resumed growth within 3 days after plating and developed normal shoots without initial callus formation (Fig. 1o) and they grew into complete normal plantlets with roots (Fig. 1p).

Key Point to Optimize Cryo-plate Procedures

Yamamoto et al. (2012b) developed a flowchart of the general procedure for the V cryo-plate protocol and a guide to optimize the protocol for cryopreservation of new species. In this flowchart, osmoprotection by LS and dehydration by plant vitrification solution (PVS) are crucial steps. An effective osmoprotective treatment appears to be essential for improving post-cryopreservation regrowth. Treating the explants with LS containing adequate sucrose concentration is very effective to induce osmotolerance to PVS (Yamamoto et al. 2012b). The precise control of dehydration with PVS2 is also a key factor for successful cryopreservation in order to prevent injury by chemical toxicity or excessive osmotic stress. The V cryo-plate method makes it possible to control this step more easily than other methods (Yamamoto et al. 2012b). Damage to the specimens to be cryopreserved can be reduced by avoiding the use of PVS2, using materials with comparatively higher moisture content, and minimal excision of young leaves and/or sheaths. The D cryo-plate method can overcome the problems associated with insufficient or excessive dehydration, damage to and loss of material during excision and manipulations (Niino et al. 2013). Currently the DMSO droplet method is being improved by the application of alternating preculture temperature for cold acclimatization of shoots under 8-h photoperiod at 21/8 °C day/night temperature for 7 days (Kaczmarczyk et al. 2008). Cold hardening of potato germplasm had a positive effect on the regrowth after cryopreservation using the droplet vitrification procedure (Folgado et al. 2014; Panta et al. 2014). Although cold acclimatization treatment was not tested in this study, it is possible to combine the treatment with cryo-plate procedures for stable regeneration after LN storage.

Table 6 Regrowth (%) of shoot tips of 16 potato varieties/lines cryopreserved using optimized the V cryo-plate and D cryo-plate procedures

Variety/Line	V cryo-plate Regrowth (%±SEM)	D cryo-plate Regrowth (%±SEM)
Solanum tuberosum		
Whitefryer	100.0±0.0	100.0±0.0
North Chip	100.0±0.0	100.0±0.0
Kitahime	96.7±5.8	96.7±3.3
Tokachi Kogane	100.0±0.0	96.7±3.3
Ohoutsuku Chip	93.3±5.8	96.7±3.3
Snow March	100.0±0.0	96.7±3.3
May Queen	96.7±5.8	90.0±0.0
Natsufubuki	100.0±0.0	90.0±0.0
Danshakuimo	100.0±0.0	90.0±0.0
Snowden	100.0±0.0	86.7±3.3
Inca-no-Mezame	100.0±0.0	83.3±3.3
Waseshiro	100.0±0.0	80.0±0.0
S. chacoence 34	96.7±5.8	96.7±3.3
S. phureja 114	100.0±0.0	93.3±3.3
S. bulbocastanum	96.7±5.8	90.0±0.0
S. pinnatisectum 109	96.7±5.8	83.3±3.3

In the V cryo-plate method, shoot tips (1.5 mm long) were excised, precultured for 16 h at 25 °C on MS with 0.3 M sucrose, loaded in 2.0 M glycerol and 0.8 M sucrose solution for 30 min at 25 °C and exposed to PVS2 for 30 min at 25 °C. In the D cryo-plate method, shoot tips (2 mm long) were excised, precultured for 16 h at 25 °C on MS with 0.3 M sucrose, loaded in 2.0 M glycerol and 1.0 M sucrose solution for 30 min at 25 °C and dehydrated by the air current of a laminar flow cabinet at 25 °C for 2.0 h. Ten shoot tips were tested for each of the three replicates

Routine Procedure for Cryopreservation

In order to make cryostorage routine work in genebank, the procedures for cryopreservation should be easely transferred (Keller et al. 2008). We investigated whether the V cryo-plate and D cryo-plate procedures could be easily performed in the laboratory. Four operators with little or no experience with the V and D cryo-plate procedures but with enough experience in tissue culture techniques performed cryopreservation procedures from the excision of shoot tips to the plating cryopreserved shoot tips on medium (Table 7). The result of regrowth level of cryopreserved shoot tips shows no significant

Table 7 Effect of operators performing the cryopreservation experiment on regrowth of cryopreserved shoot tips of potato variety 'Sayaka' by the V and D cryo-plate methods

Operator	V cryo-plate Regrowth (%±SEM)	D cryo-plate Regrowth (%±SEM)
1	100.0a±0.0	90.0a±0.0
2	100.0a±0.0	93.3a±3.3
3	96.7a±5.8	76.7a±6.7
4	93.3a±11.5	90.0a±0.0

The V cryo-plate and D cryo-plate procedures are same as indicated in Table 6

difference with high regrowth level obtained by the four operators. This indicates that the V cryo-plate and D cryo-plate procedures can be easily performed in the laboratory and can be easily adopted as an alternative protocol for preservation of important plant genetic resources. One of the major factors to be considered to ensure success in cryopreservation is to prepare uniform and vigorous plant materials. It was possible to obtain those materials by growing nodal segments with a lateral bud under rather intense light conditions (104 μ-mol m^{-2} s^{-1}) and cool temperature (20 °C) for 2 weeks.

Conclusion

Both the V cryo-plate and D cryo-plate methods using aluminum cryo-plates are efficient for cryopreservation of potato germplasm. With careful selection of explants for cryopreservation, a higher regrowth of the cryopreserved samples was obtained after rewarming because of the rapid cooling and warming steps reduced the damage to explants during the cryopreservation process (Yamamoto et al. 2012b; Niino et al. 2013). Each step in the procedure has been simplified thereby eliminating problems linked to operator skills associated with lengthy manipulations that results in damaging and losing shoot tips. We have shown that operators with little experience on both procedures could easily get used to the routine procedures from the step of excision of shoot tips to the step of plating cryopreserved shoot tips on medium. These results indicate that the procedures we developed can be easily adopted as a routine procedure for cryopreservation.

In this work, we showed that the V cryo-plate and the D cryo-plate protocols are efficient and practical methods for cryopreservation of potato germplasm. Both procedures were applied to a wide variety of potato germplasm including wild *Solanum* species and diploid variety such as 'Inca-no-Mezame' even without changing the dehydration conditions. Results were similarly effective for wild species and cultivated varieties. These protocols were easily optimized after minor modifications of the procedure. Both methods efficiently complement each other and appear highly promising to facilitate large scale cryobanking of potato germplasm in genebanks by providing efficient strategies for preservation, storage, and maintenance of genetic stability of important potato germplasm. Choice of the method may depend on the laboratory situation, cost, sensitivity of explant to PVS and/or staff skills (Reed et al. 2004).

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