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Note

Cryopreservation of mat rush lateral buds by air dehydration using aluminum cryo-plate

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Abstract A cryopreservation protocol based on air dehydration of explants placed on aluminium cryo-plates, termed D cryo-plate, was successfully developed for *in vitro* mat rush (*Juncus decipiens* Nakai) lateral buds. The buds of line 'Hiroshima 4gou(1)' with basal stems were dissected from multiple shoots and precultured overnight at 25°C on solid MS medium containing 0.3 M sucrose. Precultured buds were placed on aluminium cryo-plates, each one with 10 elliptical wells (2.5 mm long, 1.5 mm wide and 0.75 mm deep) and embedded in calcium alginate gel with 0.4 M sucrose and 1 M glycerol. Osmoprotection was performed by immersing the cryo-plates with buds for 30 min at 25°C in loading solution (2 M glycerol + 1.0 M sucrose). Buds were dehydrated to 26% moisture content (fresh weight) by placing the cryo-plates for 2.5 h either in the air current of a laminar flow cabinet or in Petri dishes containing silica gel. Cooling was performed by placing the cryo-plates in uncapped cryotubes, which were immersed in liquid nitrogen. For rewarming, cryo-plates were immersed in liquid medium with 1.0 M sucrose for 15 min at room temperature. Under these conditions, regrowth of cryopreserved buds of line, 'Hiroshima 4gou (1)' was 93% after four weeks culture. The average regrowth of 20 mat rush lines was 88%. The D cryo-plate procedure will facilitate cryostorage of mat rush germplasm.

Key words: Air dehydration, aluminium plate, cryo-plate, D cryo-plate method, mat rush.

Several vitrification protocols using the aluminium cryo-plate method (V cryo-plate method) have been reported recently for strawberry, Dalmatian chrysanthemum, mint, mulberry, carnation and mat rush shoot tips/buds (Yamamoto et al. 2011a, 2011b, 2012a, 2012b; Sekizawa et al. 2011; Niino et al. 2013). This method has two main advantages: 1) it is a user-friendly procedure; and 2) it guarantee higher cooling and warming rates of treated explants. As a result, superior regrowth rate was obtained after cryopreservation with the plant species tested. However, some mat rush lines displayed low regrowth due to their sensitivity to treatment with the plant vitrification solution 2 (PVS2, Sakai et al. 1990) (Niino et al. 2013). Encapsulation-dehydration is a very efficient cryopreservation technique, which is simple and user-friendly to implement and allows overcoming problems associated with sensitivity of plant material to plant vitrification solution (Engelmann et al. 2008). We have recently proposed an air-dehydration method using aluminium cryo-plates (D cryo-plate method) which combines encapsulation-dehydration and V cryo-plate (Niino et al. 2013).

In the D cryo-plate method, shoot tips/buds attached to the cryo-plates are dehydrated under the laminar air flow cabinet after loading treatment with 2 M glycerol and adequate sucrose solution for inducing tolerance to dehydration (osmoprotection). However, some improvements are necessary to standardize this procedure. The points to be addressed include using larger size wells in cryo-plates to accommodate bigger explants, employing silica gel to achieve reproducible dehydration of explants in any environmental conditions, and avoiding that some explants detach themselves from the cryo-plates during manipulations. In this work, we studied these points of the D cryo-plate method using *in vitro* lateral buds of three mat rush lines as experimental materials. We then tested the efficiency of the improved protocol on 17 additional mat rush lines.

The mat rush plants used in this study were obtained from the National Institute of Agrobiological Sciences

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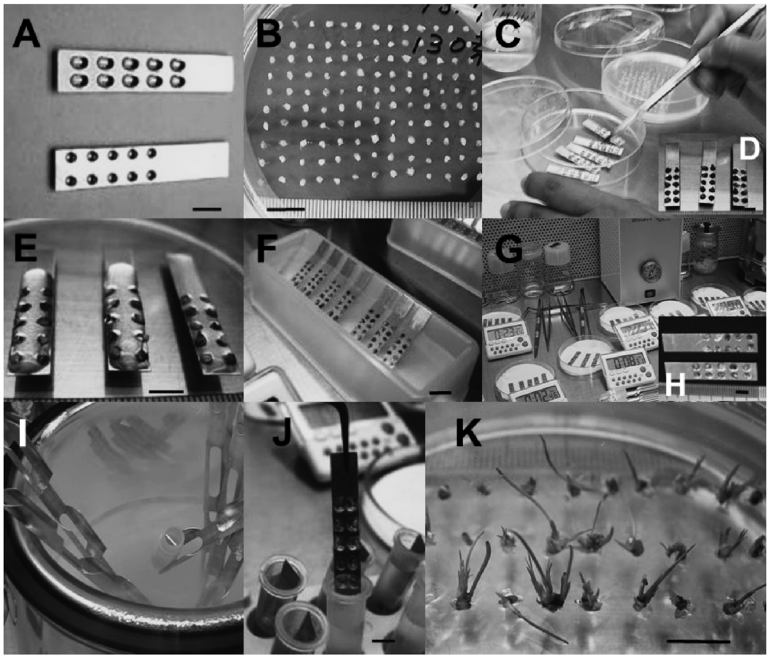

Figure 1. Successive steps of the D cryo-plate procedure. (A) Cryo-plates with different well sizes. Top: No. 3, oval shape wells; bottom: No. 2, circular shape wells; (B) preculture of buds on MS medium with 0.3 M sucrose after excision; (C) transfer of precultured buds one by one in the wells with the tip of a scalpel blade; (D) shoot tips mounted on aluminium cryo-plates; (E) polymerization of calcium alginate gel; (F) treatment with LS; (G) desiccation of buds under the air current of a laminar flow cabinet; (H) cryo-plates with attached buds after desiccation; (I) immersion in LN of cryotubes clipped to cryo-canes; (J) retrieval of cryo-plates from LN and warming in 1 M sucrose solution; (K) regenerated plants 3 weeks after plating, line 'Hiroshima 4gou (1).' Scale bars indicate 10 mm (B, F, K) and 5 mm (A, D, E, H, J).

(NIAS) genebank, Japan. Experiments aimed to improve the D cryo-plate protocol were performed with three lines, 'Hiroshima 4gou (1),' 'Okayama 1gou' and 'Okayama 2gou.' The improved protocol was tested with seventeen additional lines. *In vitro* culture plants of mat rush were subcultured every 2 months on solid Murashige and Skoog (1962) medium (MS) with 0.9 μM benzyladenine (BA), 2.5% (w/v) sucrose, and 0.8% (w/v) agar. Cultures were incubated at 25±1°C with a 16 h light/8 h dark photoperiod under a light intensity of 52 μmol m^{-2} s^{-1} provided by white fluorescent tubes (standard conditions). Multiple shoots were induced using the roller culture protocol developed by Niino et al. (2007). Buds excised from shoots were inoculated in liquid MS medium with 8.9 μM BA and 2.5% (w/v) sucrose and cultured on the roller drum of a roller culture apparatus for 60 days. Established multiple shoots were cut into small explants (each containing four-five shoots), plated on solid MS medium with 0.9 μM BA, 2.5% (w/v) sucrose in Petri dishes (90 mm×20 mm), and cultured for 1 week in the standard conditions. The shoots grown in the Petri dishes were then cold-hardened at 5°C with an 8 h light/16 h dark photoperiod under a light intensity of 26 μmol m^{-2} s^{-1} provided by white fluorescent tubes for 1 to 2 months. Buds with a basal stem were dissected from the cold-hardened shoots and precultured on the solid MS medium containing 0.3 M sucrose at 25°C for 16 h. In plant cryopreservation, the size of shoot tips/buds to be cryopreserved is 1–1.5 mm long usually, resulting taking a lot time to excise and damage to meristem dome during manipulation. In experiments aimed to optimize the D cryo-plate protocol, about 1.5–2.0 mm long and about 1.0–1.5 mm wide explants consisting of buds covered with base sheaths and basal stems were usually used (Figure 1B). For two lines, 'Kumamoto 7gou' and 'Chikugozairai,' larger specimens were used (about 2.0–2.5 mm long and about 1.5–2.0 mm wide), because of their large shoots.

The dimensions of the aluminium cryo-plates used were a length of 37 mm, a width of 7 mm and a thickness of 0.5 mm with 10 wells. Cryo-plates with two different well sizes (No. 2 and No. 3) were compared for accommodating explants with different sizes. On cryo-plates No. 2, the wells had a diameter of 1.5 mm and a depth of 0.75 mm (Figure 1A bottom). On cryo-plates No. 3, the wells had an oval shape and a length of 2.5 mm, a width of 1.5 mm and a depth of 0.75 mm (Figure 1A top). The volume of No. 3 wells was twice that of No. 2 wells. The cryo-plates were custom-made by Taiyo Nippon Sanso Corp Tokyo, Japan. The aluminium grade of cryo-plates is A1050, which means that aluminium purity is over 99.5%. These plates were specially designed to fit in 2 ml cryotubes (Yamamoto et al. 2011b).

The successive steps of the D cryo-plate procedure were as follows. In steps 1, 3 and 5 below, the * indicates the condition used in the standard procedure.

1. Pour sodium alginate solution (cryo-plate No. 2; about 2.0 μl, No. 3*; about 4.0 μl) in the wells of the aluminium cryo-plates using a micropipette at room temperature. The alginate solution contains 2% (w/v) sodium alginate (viscosity 300–400 cps, Wako Pure Chem. Ind.) in calcium-free MS basal medium with 0.4 M sucrose and 0.0*, 1.0 or 2.0 M glycerol.

2. Place the precultured specimens (length 1.5–2.0 mm, width 1.0–1.5 mm) one by one in the wells (one bud/well) with the tip of a scalpel blade and slightly press the buds to make them fit in the wells (Figure 1C); drip sodium alginate solution (about 1.0 μl) again on the buds using a micropipette (Figure 1D).

3. Pour calcium chloride solution drop-wise (about 0.1 ml/plate) on the section of the aluminium plates where the buds are located until they are covered and wait for 15 min to achieve complete polymerization of calcium alginate (Figure 1E). The calcium solution contains 0.1 M calcium chloride in MS basal medium with 0.4 M sucrose and 0.0, 1.0* or 2.0 M glycerol.

4. Remove the calcium chloride solution from the cryo-plates by gently tapping the cryo-plates on filter paper.

5. Place the cryo-plates with the encapsulated specimens in a 25 ml pipetting reservoir (NSG Precision Co. Mie, Japan) filled with about 20 ml loading solution (LS, Nishizawa et al. 1992, Figure 1F). The LS solution contains 2 M glycerol+1.0 M sucrose in liquid MS basal medium (Niino et al. 2013). The specimens are treated with LS at 25°C for 15, 30* or 60 min to induce dehydration tolerance (osmoprotection).

6. Remove the cryo-plates from LS and place them in a Petri dish on filter paper after removing the excess LS solution. The specimens on the cryo-plates are desiccated under the air current of a laminar flow cabinet (HC-1600FS, Oriental Co. Japan) for 1.5, 2.0, 2.5* or 3 h at 25°C, with 40–50% RH (Figures 1G, H), or exposed on filter paper in a Petri dish (9 cm diameter) with 35 g silica gel.

7. After dehydration, transfer the cryo-plates in 2 ml cryotubes, which are held on cryo-canes, and plunge them directly in LN where they are kept for at least 30 min (Figure 1I).

8. For rewarming, the cryotubes are retrieved from LN. The cryo-plates are immersed in cryotubes containing 2 ml MS liquid medium with 1 M sucrose (Figure 1J), in which they are incubated for 15 min at room temperature. Buds are transferred to solid MS medium under standard conditions.

Moisture content (MC) of encapsulated buds was determined by weighting them after each dehydration duration. Dry weight was determined after drying for 48 h at 102°C (Niino et al. 2013). Six individual plates with 10 buds were tested for each experiment. Post-LN regrowth was evaluated after 4 weeks of culture under standard conditions by counting the number of explants, which had developed normal stems. Three replicates of 10 specimens were tested in each experimental treatment. Statistical analyses were performed using Excel statistics, and then the Scheffe non parametric multiple comparison test was used to compare the means. Significant differences were set at $p \leq 0.05$.

Experiments performed by Niino et al. (2013) when establishing the original D cryo-plate procedure showed that improvements were needed, including the production of cryo-plates with larger wells to accommodate larger explants and the use of silica gel to ensure more reproducible dehydration. Using larger size wells offers the possibility to use larger shoots/buds, to increase the volume of alginate gel encapsulating the explants and to reduce the frequency of buds dropping from the new cryo-plates during LS and PVS2 treatment. Cryo-plates with wells of two sizes, cryo-plates No. 2 (1.5 mm diameter and 0.75 mm deep wells) and cryo-plates No. 3 (oval shape, 2.5 mm long, 1.5 mm wide and 0.75 mm deep wells) were tested with three mat rush lines using the original D cryo-plate procedure. Even though differences in recovery of buds of the three lines cryopreserved using No. 2 and No. 3 cryo-plates were not significant, recovery rates measured with larger wells (No. 3) was higher compared to smaller wells (No. 2) (Table 1). The number of buds dropping from the cryo-plate during LS treatment was 0.4 buds/cryo-plate and 1.0 buds/cryo-plate for larger and smaller

Table 1. Effect of well size of cryo-plates and of glycerol concentration in 2 g l^{-1} Na-alginate solution with 0.4 M sucrose on regrowth (%) and on number of buds dropping from the cryo-plates of three mat rush lines cryopreserved using the D cryo-plate procedure.

Well size of cryoplate	Conc. of glycerol in Na-alginate solution with 0.4 M sucrose	Regrowth (%±SEM)			Number of drop buds (buds/plate)
		Line			
		Hiroshima 4gou (1)	Okayama 1gou	Okayama 2gou	
Small (No. 2)	0 M*	86.7±3.3^{a}	90.0±0.0^{a}	76.7±3.3^{ab}	1.0
Large (No. 3)	0 M*	90.0±0.0^{a}	93.3±3.3^{a}	83.3±3.3^{ab}	0.4
Large (No. 3)	1.0 M**	93.3±3.3^{a}	96.7±3.3^{a}	90.0±0.0^{a}	0.2
Large (No. 3)	2.0 M***	70.0±0.0^{b}	76.7±3.3^{a}	66.7±3.3^{b}	0.2

Different letters in columns indicate significant differences between treatments ($p \leq 0.05$). The Ca solutions used were * with 0.4 M sucrose, ** with 0.4 M sucrose+1.0 M glycerol and *** with 0.4 M sucrose+2.0 M glycerol, respectively. Different letters indicate significant differences between treatments ($p \leq 0.05$). Ten shoot tips were tested for each of the three replicates.

wells, respectively. The well of No. 2 may be too small for mat rush buds to adhere steadily on the plates. It is important that shoot tips adhere steadily to the cryo-plates throughout the whole procedure for efficient performance. Adding 1 M glycerol to the sodium alginate solution employed for encapsulating buds was effective in avoiding buds dropping from the cryo-plates and had no detrimental effect on regrowth of cryopreserved buds (Table 1). However, encapsulating buds in sodium alginate solution containing 2 M glycerol reduced recovery after LN exposure and regrowing shoots turned brown or red. The number of buds dropping from cryo-plates during the LS treatment was 0.4 buds/cryo-plate (0 M glycerol) and 0.2 buds/cryo-plate (1 M and 2 M glycerol). The addition of glycerol to the alginate gel may render stickier. Also, dropping decreased to 0.01 buds/cryo-plate when we employed cryo-plates which had already been used once (data not shown). This may be due to the lower surface tension on these cryo-plates, which had been washed and autoclaved after their first use. Only No. 3 cryo-plate (large wells) were used in further experiments.

We studied the effect of duration of loading with a LS containing 2 M glycerol and 1.0 M sucrose on regrowth of cryopreserved buds. Regrowth after 15, 30 and 60 min LS treatment was 86.7%, 93.3% and 73.3% in 'Hiroshima 4gou (1),' and 86.7%, 86.7% and 76.7% in 'Okayama 2gou,' respectively (Figure 2). A 30 min LS treatment, which led to the highest regrowth of cryopreserved buds

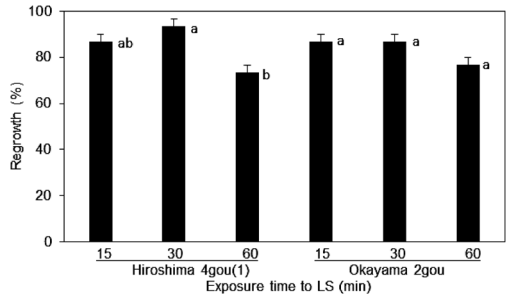

Figure 2. Effect of exposure time to LS on regrowth (%) of cryopreserved buds of mat rush lines 'Hiroshima 4gou (1)' and 'Okayama 2gou' using the D cryo-plate procedure. Different letters in variety indicate significant differences between treatments ($p \leq 0.05$). Ten shoot tips were tested for each of the three replicates.

was enough effect for osmoprotection and employed in further experiments.

In the original D cryo-plate protocol, the dehydration rate can vary between laboratories due to differences in room temperature, relative humidity of the air and laminar air flow speed. In order to standardize the dehydration step, the use of silica gel was compared with air flow dehydration. The MC of air-dehydrated and silica gel-dehydrated mat rush buds reached 31% after 1.5 h and 25% after 3 h, and 30% after 1.5 h and 25% after 3 h, respectively (Table 2). Silica gel dehydration was faster as compared to air dehydration. The highest regrowth of cryopreserved buds was obtained after 2.5 h dehydration with both methods, reaching 93.3% after air-dehydration and 90.0% after silica gel desiccation. Based on the results obtained, the revised details of D cryo-plate steps using No. 3 cryo-plate for mat rush buds are as follows (numbers are corresponded to the steps indicated above):

2. Pour sodium alginate solution (about 4.0 μl) in the wells of aluminium cryo-plates No. 3 using a micropipette. The alginate solution contains 2 g l^{-1} sodium alginate in calcium-free MS basal medium with 0.4 M sucrose and 1.0 M glycerol.

5. The LS contains 2 M glycerol+1.0 M sucrose in liquid MS basal medium. The explants are osmoprotected at 25°C for 30 min.

6. Place the cryo-plates on a filter paper in a closed Petri dish (9 cm diameter) with 35 g silica gel for 2.5 h at 25°C or place them in open Petri dishes in the air current of a laminar flow cabinet for 2.5 h at 25°C, with 40–50% RH.

This optimized procedure was applied to buds of 20 mat rush lines (Table 3). Regrowth was very high for all lines, ranging from 70.0 to 96.7%, with an average of 87.7%. Buds resumed growth within 3–5 days after plating and developed normal stems (Figure 1K). Also, the buds of 'Hiroshima 4gou (1)' stored in LN for 6 months by this procedure was monitored. The regrowth rate was 93%, which was the same regrowth level of initial storage, indicating maintain the regrowth ability in LN (data not shown).

There are two steps which can be affected by operator skills: one is the excision of shoot tips/buds and the other is the implementation of the cryopreservation

Table 2. Effect of dehydration method and of dehydration duration on moisture content (MC, % FW) and on regrowth (%) of cryopreserved buds of mat rush line 'Hiroshima 4gou (1)' using the D cryo-plate procedure.

Duration of dehydration (h)	Laminar air flow		Silica gel	
	Buds MC (% FW)	Regrowth (%±SEM)	Buds MC (% FW)	Regrowth (%±SEM)
1.5	31	76.7±3.3[a]	30	80.0±5.8[a]
2.0	28	90.0±0.0[a]	27	86.7±3.3[a]
2.5	26	93.3±3.3[a]	26	90.0±0.0[a]
3.0	25	73.3±3.3[a]	25	70.0±5.8[a]

Different letters in columns indicate significant differences between treatments ($p \leq 0.05$). Ten shoot tips were tested for each of the three replicates.

Table 3. Regrowth (%) of buds of 20 mat rush lines cryopreserved using the optimized D cryo-plate procedure.

Line	Regrowth (%±SEM)	Line	Regrowth (%±SEM)
Hiroshima 4gou (1)	93.3±3.3	Tokiwazairai (3)	80.0±5.8
Okayama 1gou	96.7±3.3	Chikugozairai	76.6±3.3
Yonagizairai (1)	90.0±0.0	Okayama 2gou	90.0±0.0
Izumo 9gou	83.3±3.3	Nishizairai (2)	93.3±3.3
Hirokei 309	93.3±3.3	Ooi 3gou (3)	90.0±5.8
Kijokazairai B	90.0±0.0	Kumamoto 9gou	86.7±3.3
Kumagawa	96.7±3.3	Kumamoto 7gou	70.0±5.8
P-114	83.3±3.3	NW258	90.0±5.8
Koukei 274	86.7±3.3	Tokiwazairai	90.0±0.0
Okayama Mkei	86.7±3.3	Seto 5gou	86.7±3.3

Ten buds were tested for each of the three replicates.

Table 4. Effect of operator performing excision of buds and the cryopreservation experiment on regrowth of buds cryopreserved using the D cryo-plate method.

Operator	Regrowth (%±SEM)	
	Excision of buds step	Cryopreservation procedure step
1	90.0±0.0[a]	90.0±0.0[a]
2	93.3±3.3[a]	93.3±3.3[a]
3	83.3±3.3[a]	76.7±6.7[a]
4	93.3±3.3[a]	86.7±3.3[a]
5	76.7±3.3[a]	90.0±0.0[a]

Different letters in columns indicate significant differences between treatments ($p \leq 0.05$). Ten shoot tips were tested for each of the three replicates.

procedure. Excision of small size samples requires skills, precision and delicacy. In the D cryo-plate method, we employed large size specimens (about 1.5–2.0 mm long and about 1.0–1.5 mm wide) consisting of buds covered with base sheaths and basal stems. Five operators, who had little experience of this D cryo-plate procedure but had experience in tissue culture techniques, performed these two steps. Excision of 130 buds of this type took about 2.5 h (based on an average of five persons, ranging between 1.5 and 3.5 h). Regrowth of cryopreserved buds did not reveal any significant differences between operators, who all achieved high regrowth levels, indicating that semi-skilled staff were able to easily excise large buds and to efficiently implement the D cryo-plate procedure (Table 4).

Keller et al. (2008) reported that variations in cryopreservation response might arise from differences in staff skills and competence as well as in differences in equipment and minor technical details. Similarly, Reed et al. (2004) reported that differences in cryopreservation results between laboratories arise because of differences in culture systems, technical procedures and operator skills. Yamamoto et al. (2012a, b) suggested that in order to allow the establishment of cryopreserved collections, it is necessary to develop robust and user-friendly cryopreservation techniques, which can be performed by semi-skilled staff with limited expertise. The cryo-plate method was developed with the aim of limiting the problems related to operator skills and of simplifying the procedure. It avoids damaging and losing shoot tips during lengthy manipulations (Niino et al. 2013). The entire process including excision of buds and D cryo-plate cryopreservation, was performed without any difficulty by five operators. This could be achieved due to the optimization of the size of buds, of the size of wells, and to the addition of glycerol to the alginate gel. Selecting large buds/shoot tips for cryopreservation allowed minimizing damage during excision, facilitated their transfer on the cryo-plates and ensured rapid regrowth after rewarming compared to small ones (around 1 mm). Using large wells for large buds/shoot tips facilitated their transference on cryo-plates and resulted in reduced dropping of buds/shoot tips from the cryo-plates during LS treatment and retrieval from LN. The addition of 1 M glycerol and 0.4 M sucrose to the sodium alginate gel was also instrumental in avoiding that buds dropped from the cryo-plates. However, it will be important to check with other species, whether the addition of 1 M glycerol in the alginate gel has, or not, a negative effect on regrowth after rewarming. In order to standardize the dehydration step, the use of silica gel was considered because dehydration rate can fluctuate between laboratories due to differences in room temperature, air RH and laminar air flow speed. Dehydration could be performed either using the laminar flow or silica gel. If an air conditioner is installed in the transfer room, using the laminar air flow for desiccation may be easier. If this is not the case, then silica gel-dehydration should be used.

Cryo-storage of mat rush buds has been started in NIAS genebank by D cryo-plate method above mentioned. The time schedule of this cryo-storage is as follows. Preparation of mat rush material for cryopreservation took about 6–10 months, including primary culture (2–5 months), multiple stem induction (2 months), preconditioning (1 week) and cold-hardening (1–2 months). The flow of the D cryo-plate procedure for cryostorage of two accessions was the following. One accession was usually cryo-stored using

13 cryo-plates (130 shoot tips/buds), including 10 cryo-plates for long term storage and three cryo-plates for monitoring. One cryo-plate for monitoring was used to check the initial regrowth. Cryo-storage (two mat rush accessions) required 2 days. The first day, buds with a basal stem were dissected from cold-hardened shoots. Excision of 130 buds required about 2.5 h/accession. Subsequently, they were precultured overnight at 25°C on medium with 0.3 M sucrose. On the second day, two sets of Petri dishes with cryo-plates were prepared for the two accessions. One set comprised three Petri dishes with five, four and four cryo-plates, respectively. Embedding the buds of the first set of five cryo-plate took 5–6 min and polymerization of alginate with calcium chloride took 15 min. During this time, the buds were placed on the second and third sets of cryo-plates. Then the cryo-plates were transferred to LS solution for 30 min. During the LS treatment of the first line, buds of the second line were placed on the cryo-plates in a similar manner. After the LS treatment, the cryo-plates were placed on filter paper in Petri dishes and desiccated under the laminar air flow cabinet for 2.5 h. Then the cryo-plates were put into cryotubes and immersed in LN. The cryotubes were capped and transferred to the LN tank for long-term storage. In total, the second day's manipulations took up about 4.5 h for the two accessions treated.

In this work, we showed that the optimized D cryo-plate procedure is a practical and efficient method for cryopreservation of mat rush germplasm. This protocol appears promising for cryopreservation of other plant species after marginal modifications of the procedure. It efficiently complements the V cryo-plate method. Smaller buds with the meristematic dome exposed are more rapidly and uniformly dehydrated with PVS2, compared to larger buds with the meristematic dome covered by leaf sheaths. By contrast, the physical dehydration employed in the D cryo-plate protocol might be more uniform, thus explaining the equivalent regrowth obtained with larger shoot tips cryopreserved with the D cryo-plate and the V cryo-plate (Niino et al. 2013). We recommend using the V cryo-plate method with 1.0–1.5 mm long explants, which can be easily obtained from shoot tips without excision damage and which can tolerate exposure to PVS2 solution. The D cryo-plate method may be used with larger explants, which are very sensitive to physical damage and cryoprotectant toxicity.

Cryopreservation should be considered as a backup to field collections to insure against loss of plant germplasm (Niino et al. 2007). Cryopreservation techniques for shoot tips and embryos are now well developed. However, in minor crops, the development of micro propagation systems is crucial for successful cryopreservation because the cryopreservation procedure is usually based on tissue culture, except when preserving seeds, pollen and dormant buds. The explants must be in an optimal physiological and morphological state to ensure high recovery % and vigorous regrowth after LN exposure (Engelmann et al. 2008; Sakai et al. 2008). Also, obtaining a high level of plant regrowth after cryopreservation is very important. Oxidative processes involved in cryopreservation protocols may be responsible for the reduced viability of explants after LN exposure. Adding antioxidants that counteract these reactions to the cryoprotectant and/or to recovery media may improve recovery (Uchendu et al. 2010a, b). Developing tissue culture systems which allow rapid multiplication and which stimulate regrowth after retrieving samples from LN will be the next challenge in cryopreservation research.

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