Pak. J. Agri. Sci., Vol. 53(1), 151-158; 2016
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI: 10.21162/PAKJAS/16.5018
http://www.pakjas.com.pk

CRYOPRESERVATION OF SHOOT-TIPS FROM DIFFERENT SUGARCANE VARIETIES USING D CRYO-PLATE TECHNIQUE

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Cryopreservation is an effective approach to conserve sugarcane germplasm for long term. This study was conducted to develop D cryo-plate technique to conserve sugarcane's shoot tips. This new and effective cryopreservation technique will serve the purpose to conserve sugarcane germplasm efficiently on the large scale at genebanks. Sugarcane variety Ni-1 were used to optimize the protocol and after optimization 11 other varieties were tested with optimized protocol. It was observed that shoot tips of Ni-1 with a length of 1.5 to 2.0 mm, precultured on semi-solid 1/2 MS medium for 1 day and semi-solid MS medium with 0.3 M sucrose for 1 day, treated with loading solution containing 2.0 M glycerol + 1.2 M sucrose for 30 min and air dehydrated in an air laminar flow's air current for 45 min, removed from alginate gel after cryopreservation and kept in dark for seven days produced maximum regrowth (97.7%). Range of 20.0% to 100% with an average of 52.1 % was observed among sugarcane varieties for regrowth after cryopreservation with optimized D cryoplate protocol.
Keywords: Air dehydration, droplet vitrification, encapsulation dehydration, *Sacharum officinarum*, V cryo-plate

INTRODUCTION

The Food and Agriculture Organization of the United Nations estimates that sugarcane is the world's largest crop by production quantity. Approximately, it is grown in 90 countries and 1.83 billion tons is total production across the world. Sugarcane is not only important for its sugar content, which accounts for 80% of global sugar production, but also for many bi-products used in different industries (Anonymous, 2015). Sugarcane is a cross pollinated crop but is a very shy seed producer and it is not practical to store sugarcane seeds, even though they are orthodox seeds (Roberts, 1973). Sugarcane is mainly conserved in field collections where propagation uses cuttings and accessions need replanting every 1 to 5 years. In Japan, 1975 accessions including 592 wild accessions are maintained in field collections at different locations of the country. There is always the risk of adverse weather, pests or diseases destroying accessions in the field. *In vitro* conservation is an alternative to field collections. Sugarcane germplasm conserved by *in vitro* slow growth at CIRAD-CA Montpellier presently includes 650 sugarcane varieties (Paulet *et al*., 1991). Maintaining a huge *in vitro* collection is laborious because after a certain time re-culturing is required to maintain the germplasm (Withers, 1987) and there is also the risk of contamination. The main concerns during germplasm conservation is the genetic stability, for *in vitro* conservation there is always a chance of somaclonal variation when sugarcane germplasm has been conserved by successive tissue culture.

Cryopreservation (-196°C) of sugarcane is safe and practical for long term storage of sugarcane without fear of genetic changes because at this temperature biological activities completely stopped (Engelmann, 2004). Other benefits are the safety of germplasm from biotic and abiotic hazards, limited maintenance, reduced space and low maintenance costs. In the case of sugarcane, cryopreservation protocols have been developed for various materials: apices of *in vitro* plantlets using the encapsulation-dehydration technique (Gonzalez-Arnao *et al*., 1993; Paulet *et al*., 1993); cell suspensions (Finkle and Ulrich, 1979) and embryogenic callus using classical freezing protocols (Eksomtramage *et al*., 1992; Gnanapragasam and Vasil, 1992; Jian *et al*., 1987) and simplified cryopreservation protocols (Martinez-Montero *et al*., 1998). Moreover, the cryopreservation procedure based on vitrification techniques for somatic embryos is published (Martinez-Montero *et al*., 2008).

Currently, effective V and D cryo-plate methods have been developed for many plant species (Vujovic *et al*., 2015). These protocols make the handling of shoot tips easy during the procedure and also have increased cooling and warming rate of shoot tips hence are effective and convenient for cryopreservation of various crops. In our former work, the V cryo-plate method has been successfully applied to 11 sugarcane varieties with an average regrowth rate of 70.3%

(Rafique *et al*., 2015). The D cryo-plate method was proposed by Niino *et al*. (2013) using *in vitro* mat rush. This method could be used with large explants, which are very sensitive to physical damage and cryoprotectant toxicity (Niino *et al*., 2014).

The objectives of this study are to determine the applicability and optimization of the D cryo-plate method for use with sugarcane germplasm.

MATERIALS AND METHODS

***Plant materials and tissue culture protocols*:** Sugarcane germplasm was acquired from sub-genebanks of Japan. Sugarcane variety Ni-1 was used to optimize the conditions of the successive steps of the D cryo-plate method and also used in the droplet vitrification and encapsulation dehydration procedures. After optimization of the protocol, 11 additional varieties were also tested using the cryopreservation protocol developed with variety Ni-1. Lateral buds from fully grown sugarcanes were excised, washed with "Muse" (Proctor and Gamble, Japan) medicated LN soap and surface-sterilized with 70% ethanol for 1 min and sodium hypochlorite solution with 2% active chlorine for 15 min. After that buds were washed thrice with sterile water and from these sterilized buds, 2–4 mm shoot tips were excised using a stereomicroscope. Excised shoot tips were placed on 1/2 MS semi-solid medium (Murashige and Skoog, 1962) containing 3% sucrose, 0.01 mg/l naphthalene acetic acid (NAA) and 0.1 mg/l benzyladenine (BA) in test tubes (Gonzalez-Arnao and Engelmann, 2006). Initially all the cultures were kept in the dark for 7 days and after that incubated in light with a photoperiod of 16 h light (52 µmol/m^2s, standard condition). *In vitro* shootlets were then transferred to 1/2 MS semi-solid medium containing 3% sucrose and 1 ml/l Plant Preservative Mixture (PPM) (manufactured by Plant Cell Technology, Inc. US) in jars with the size of 80×102 mm (Fig. 1A).

***Preconditioning and preculture*:** After removal of dead leaves, *in vitro* grown shoots of sugarcane containing shoot tips were cut to a size of 5–7 mm (Fig. 1B) and plated on semi-solid 1/2 MS medium having 3% sucrose in Petri dishes for 1 week to obtain uniform material for shoot tips. The shoot tips used were 1.5–2.0 mm long with a basal part (Fig. 1C). The excised shoot tips were cultured on semi-solid 1/2 MS medium containing 3% sucrose for 1 day and precultured on semi-solid MS medium containing 0.3M sucrose for 1 day at room temperature (RT). For the experiment studying the

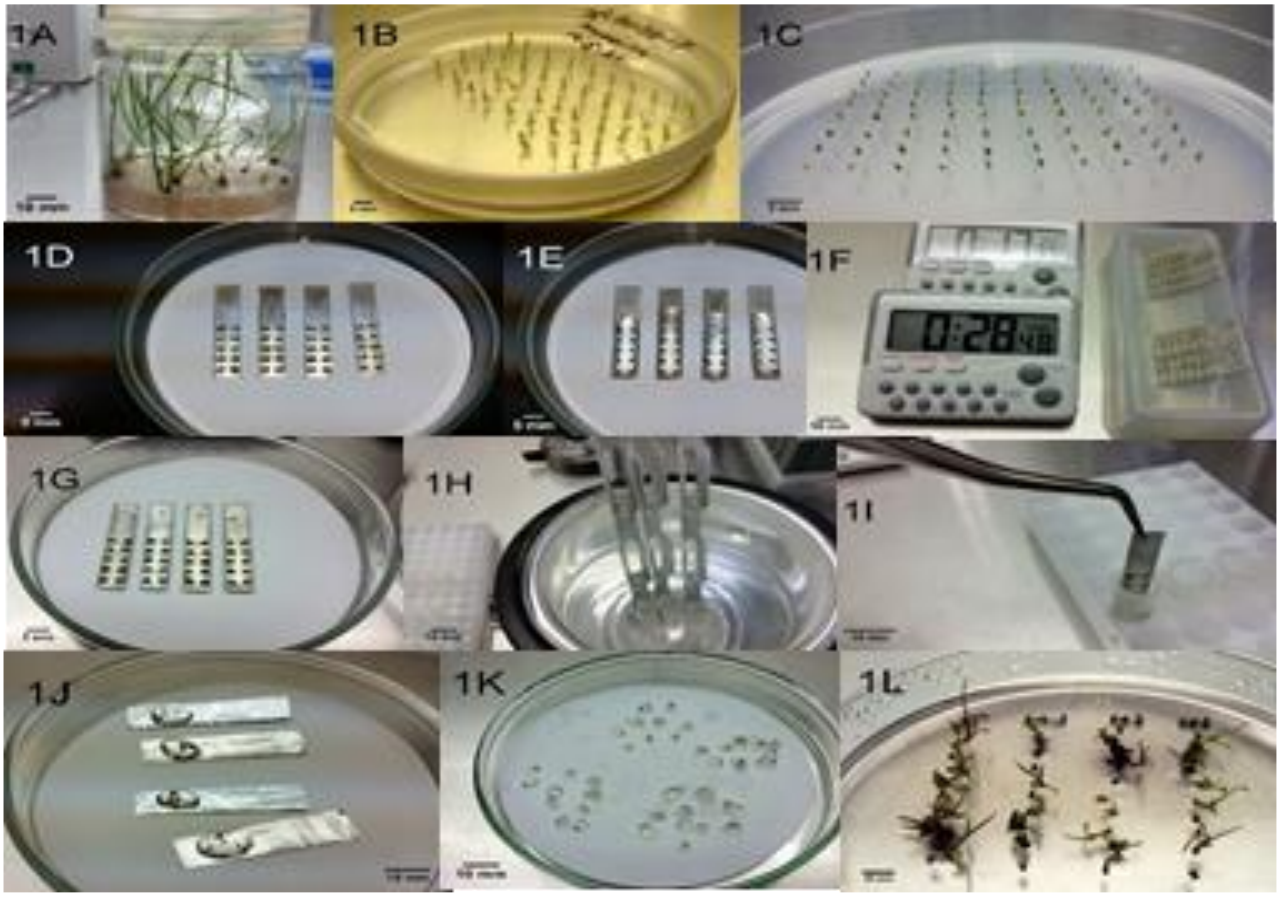

Figure 1. **D cryo-plate procedure for sugarcane and regrowth after cryopreservation.**

effect of shoot tip size, three different sizes were used; 0.5–1.0, 1.0–1.5 and 1.5–2.0 mm. For the preculture experiment, semi-solid MS medium containing 0.3 M or 0.5 M sucrose were used for 1 day preculture at RT.

***D cryo-plate procedure*:** The steps of D cryo-plate procedure followed as. The * indicates the condition used in the standard procedure.

1. Pour sodium alginate solution in the wells of the aluminium cryo-plates using a micropipette at RT. The alginate solution contains 2% (w/v) sodium alginate (viscosity 300–400 cps, Wako Pure Chem. Ind.) in calcium-free MS basal medium.
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; drip sodium alginate solution (about 1.0 µl) again on the buds using a micropipette (Fig. 1D).
3. Pour calcium chloride solution drop-wise (about 0.1 ml/plate) on the section of the aluminum plates where the buds are located until they are covered and wait for 15 min to achieve complete polymerization of sodium alginate (Fig. 1E). The calcium solution contains 0.1 M calcium chloride in MS basal medium.
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 filled with about 20 ml loading solution (LS) (Fig. 1F). The LS solution contains 2 M glycerol + 0.8, 1.2* and 1.6 M sucrose in liquid MS basal medium. The specimens are treated with LS at 25°C for 30 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 0, 15, 30, 45*, 60, 75, 90, 105 and 120 min at 25°C, with 40–50% relative humidity (RH) (Fig. 1G).
7. After dehydration, transfer the cryo-plates in 2 ml cryotubes, which are held on cryo-canes, and plunge them directly in liquid nitrogen (LN) where they are kept for at least 30 min (Figure 1H).
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 (Fig. 1I), in which they are incubated for 15 min at RT.
9. Shoot tips were transferred to semi solid 1/2 MS medium without growth regulators or with 0.01 mg/l NAA + 0.1 mg/l BA* or with 0.01 mg/l NAA + 0.1 mg/l BA + 0.01 mg/l PVP under standard conditions.
10. After transfer to regrowth medium, initially all the cultures were kept in the dark for 7 days and then transferred into light under standard conditions. In one experiment aiming at observing the effect of light exposure, the shoot tips were placed under light.

***Measurement of shoot tips moisture content*:** Decrease in moisture content of sugarcane's shoot tips by air dehydration were calculated by weighing them after 15 min intervals of dehydration from 0 to 120 min. Dry weight was determined after drying the shoot tips for 48 hours at 102°C (Niino *et al*., 2014).

***Droplet vitrification*:** Steps for droplet vitrification followed as.

1. Sugarcane shoot tips were placed in LS containing 1.2M sucrose for 30 min.
2. Vitrification were carried out by plant vitrification solution number 2 (PVS2) which is composed of 0.4 M sucrose, 150 g/l glycol, 150 g/l dimethyl sulfoxide and 300 g/l glycerol, for 30 min at RT.
3. After that shoot tips were transferred to aluminium strips having 10 µl drop of PVS2 (10 shoot tips per drop) (Fig. 1J) and directly plunged in to LN for 30 min.
4. For rewarming of shoot tips, aluminum strips were transferred to 1.0 M sucrose solution for 15 min at RT.
5. Shoot tips were then transferred to semi solid 1/2 MS medium containing 0.01 mg/l NAA + 0.1 mg/l BA
6. For first 7 days after cryopreservation the cultures were held in the dark and then transferred to the light afterwards.

***Encapsulation dehydration*:** 2% sodium alginate were used to encapsulate shoot tips with a diameter of 4-5 mm for beads (Fig. 1K). Alginate beads containing shoot tips were kept in LS, for 30 min, comprising of 1.2M sucrose. Afterwards alginate beads containing shoot tips were dehydrated in laminar flow's air current for 30 min and then submerged into LN for 30 min. Subsequently the shoot tips were rewarmed in 1.0M sucrose solution at RT for 15 min and place on recovery medium as used for the D cryo-plate method. The cultures were retained in dark for 7 days and transferred to the light afterwards.

***Data analysis*:** Regrowth (shoot elongation with or without root formation) was evaluated 4 weeks after rewarming of cryopreserved shoot tips. Each experimental treatment was replicated thrice with each replication containing 10 shoot tips. Statistical analysis was done by means of ANOVA and after that Tukey's honest significant difference (HSD) was used for means comparison. Significant differences were set at $P \leq 0.05$. Means of replicates with standard error are presented in the Tables and Figures.

RESULTS

In the D cryo-plate procedure, preconditioning, preculture, osmoprotection with LS, dehydration with air and silica gel and post-rewarming handling of shoot tips are the key parameters for successful cryopreservation.

***Preconditioning and preculture*:** Shoot tip size significantly affects regrowth after cryopreservation, with increased shoot tip size success in regrowth improves (Table 1). Shoot tips having 1.5–2.0 mm size, displayed maximum (96.7%) regrowth after cryopreservation. In order to prepare the shoot tips for cryopreservation and to make them suitable to obtain higher regrowth after cryo-storage, their preculture on medium having sucrose is an important step and when precultured on medium having 0.3 M sucrose for 1 day at RT, regrowth was maximum (96.7%) after cryopreservation, whereas regrowth was lower (86.7%) in high sucrose concentration (0.5M) preculture medium (Table 2).

Table 1. Effect of explant size on the regrowth of cryopreserved shoot tips using the D cryo-plate method.

Explant size (mm)	Regrowth (%±SD)
0.5 – 1	20.0 ± 26.5 b
1 – 1.5	20.0 ± 17.3 b
1.5 – 2	96.7 ± 05.7 a

Mean values followed by different letters differ significantly at the 0.05 probability level.

***D cryo-plate procedure (osmoprotection and dehydration)*:** After preculture of shoot tips on semi-solid MS medium with 0.3M sucrose, the effect of osmoprotection with LS containing

Table 2. Effect of sucrose concentration in preculture medium on cryopreserved shoot tips using the D cryo-plate method.

Sucrose concentration	Regrowth (%±SD)
0.3 M sucrose, 1 day	96.7 ± 5.7 a
0.5 M sucrose, 1 day	86.7 ± 5.7 b

Mean values followed by different letters differ significantly at the 0.05 probability level.

Different sucrose concentrations and duration of dehydration with air drying was evaluated (Table 3). The results suggest that maximum regrowth (96.7%) after cryo-storage was obtain with LS having 2.0M glycerol + 1.2M sucrose and dehydrated under air laminar flow's air current for 45 min. These condition were selected as the optimal for other sugarcane varieties.

The relation of moisture content of shoot tips with alginate gel and the regrowth after cryo-storage at 15 min intervals of drying from 0 to 120 min was estimated after LS treatment with 2 M glycerol + 1.2M sucrose. The regrowth percentage compared with the moisture content in shoot tips (Fig. 2 and 3) indicated that 28 to 35% moisture content in shoot tips is optimum prior to cryo-storage.

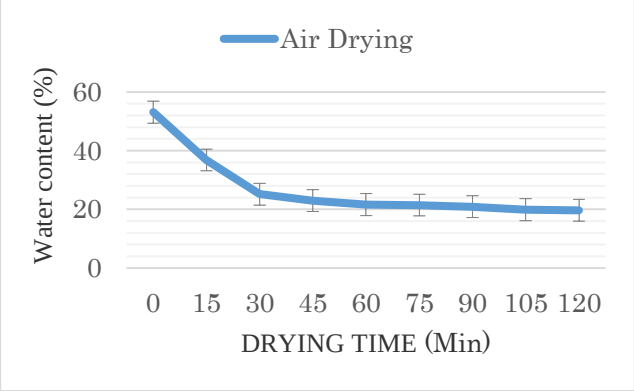

Figure 2. Decrease of moisture content of shoot tips with exposure to air drying.

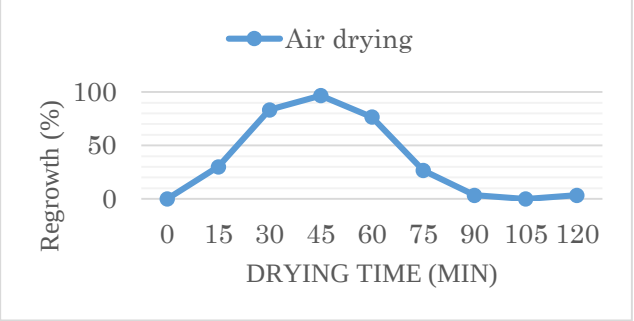

Figure 3. Regrowth curve of cryopreserved shoot tips with respect to air drying time.

Table 3. Effect of sucrose concentration in LS and drying time by air drying on the regrowth of cryopreserved shoot tips using the D cryo-plate method.

Sucrose concentration in LS	Drying Time	Regrowth rate (% +SD)
LS 0.8	30 min	83.3 ± 15.3 a
	45 min	83.3 ± 5.8 a
	60 min	70 ± 10 abc
LS 1.2	30 min	86.7 ± 5.8 a
	45 min	96.7 ± 5.8 a
	60 min	76.7 ± 11.5 ab
LS 1.6	30 min	66.7 ± 5.8 abcd
	45 min	66.7 ± 11.5 abcd
	60 min	33.3 ± 11.5 def

Mean values followed by different letters differ significantly at the 0.05 probability level.

***Post-warming regrowth*:** Post-warming manipulations are important to obtain high regrowth after cryopreservation. Removing alginate gel from shoot tips significantly increased post-LN regrowth compared to shoot tips covered with alginate gel (Table 4). The initial light conditions for cryopreserved shoot tips after rewarming affected regrowth significantly as if placed in standard light conditions, their regrowth was significantly lower compared to shoot tips which were held in dark for 7 days (Table 5). The hormonal composition in the culture media also affected regrowth after cryopreservation. The addition of 0.01 mg/l NAA and 0.1 mg/l BA in the culture medium produced maximum regrowth (100%), whereas regrowth was only 80% on medium having 0.01 mg/l NAA + 0.1 mg/l BA + 0.01 mg/l PVP and regrowth of 40% was observed on medium devoid of growth regulators (Table 6).

Table 4. Effect of alginate beads presence or absence on cryopreserved shoot tips using the D cryo-plate method.

Ag beads	Regrowth (% ± SD)
Present	40.0 ± 10 b
Removed	96.7 ± 5.7 a

Mean values followed by different letters differ significantly at the 0.05 probability level.

Table 5. Effect of light on the regrowth of cryopreserved shoot tips using the D cryo-plate method.

Regrowth condition	Regrowth (% ± SD)
In dark for 7 days	96.7 ± 5.7 a
Without dark	36.7 ± 5.7 b

Mean values followed by different letters differ significantly at the 0.05 probability level

***Optimal D cryo-plate procedure*:** The optimal conditions established for Ni-1 were as follows: 1.5 to 2.0 mm long shoot tips were placed on semi-solid 1/2 MS medium having 3% sucrose for 1 day, 1 day preculture on semi-solid MS medium containing 0.3 M sucrose, 30 min Osmoprotection by LS having 2.0 M glycerol + 1.2 M sucrose at 25°C and air dehydrated in air flow laminar hood for 45 min at RT before direct dipping in LN. For regeneration, cryopreserved shoot tips were rewarmed in 1.0M sucrose solution for 15 min at RT and plated on semi-solid 1/2 MS medium containing 3% sucrose, 0.01 mg/l NAA and 0.1 mg/l BA after removal of the alginate gel. Cultures were retained in the dark for 7 days and shifted in standard light afterwards.

These optimal conditions were applied to an additional 11 varieties of sugarcane. Regrowth ranged from 20 to 100%, with an average of 52.1% for the twelve varieties tested (Table 7).

***Encapsulation dehydration and droplet vitrification*:** The regrowth percentage of sugarcane shoot tips after cryopreservation using the encapsulation dehydration and droplet vitrification methods were 13.3 and 30%, respectively and significantly lower than the D cryo-plate method (Table 8).

A) In vitro plantlet of sugarcane in jar; B) Preconditioning of shoots on 1/2 MS medium to get uniform shoot tips;
C) Preculture on semi-solid MS medium with 0.3 M sucrose; D) Shoot tips mounted on cryo-plate with sodium alginate solution; E) Hardening of gel with calcium solution; F) Shoot tip treatment with LS; G) Air drying in laminar flow; H) Immersion of cryotubes containing cryo-plates in LN; I) Warming in 1.0 M sucrose solution after LN exposure; J) Shoot tips on aluminum foil strips in 10 m PVS2 soln. for droplet vitrification; K) Encapsulation of shoot tips in alginate beads for encapsulation dehydration; L) Regenerated shoot tips after LN exposure (4 weeks after rewarming).

Table 8. Comparison of the regrowth percentage of sugarcane varieties after cryopreservation with optimum D cryo-plate method, encapsulation dehydration and droplet vitrification.

Methods	Regrowth (% ± SD)
D cryoplate	96.7 ± 5.7 a
Encapsulation dehydration	13.3+ 11.5 b
Droplet vitrification	30.0+ 10.0 b

Table 6. Effect of recovery medium on the regrowth of cryopreserved shoot tips using the D cryo-plate method.

Medium	Regrowth (% ± SD)
½ MS + 30 g/l sucrose	40. 0 ± 20 b
½ MS + 30 g/l sucrose + NAA+ BA	96.7 ± 5.7 a
½ MS + 30 g/l sucrose + NAA + BA + PVP	80.0 ± 10 a

Mean values followed by different letters differ significantly at the 0.05 probability level

Table 7. Regrowth percentage of sugarcane varieties after cryopreservation with optimum D cryo-plate conditions.

S. No	1	2	3	4	5	6	7	8	9	10	11	12
Varieties	Chunnee	NiF4	NiF3	Ni27	Badilla	Ni12	KNOO-114	Kouchi Zairai	Wakayama	NiF8	Ni-1	NiN2
Regrowth	50.0	100.0	47.0	77.0	60.0	50.0	20.0	27.0	20.0	57.0	96.7	20.0
± SD	±17.3	±0.0	±11.5	±5.7	±10.0	±10.0	±10.0	±11.5	±0.0	±5.7	±5.7	±10.0

Mean values followed by different letters differ significantly at the 0.05 probability level.

Mean values followed by different letters differ significantly at the 0.05 probability level.

DISCUSSION

There are very few reports on sugarcane cryopreservation using *in vitro* grown shoot tips. Successful cryopreservation was accomplished by the encapsulation dehydration procedure (Gonzalez-Arnao, 1993; Paulet *et al*., 1993). Recently, successful cryopreservation *in vitro* shoot tips of two sugarcane varieties were carried out by encapsulation-dehydration and droplet vitrification with recovery 53–60% and 20–37%, respectively (Barraco *et al*., 2011). Using 11 sugarcane varieties regrowth rates of 56.7 to 100%, with an average of 70.3%, was achieved using an optimally developed V cryo-plate procedure (Rafique *et al*., 2015).

It is important to have different protocols for cryo-preservation, because there are many types of plant propagules and plant species/varieties to be cryopreserved (Niino and Arizaga, 2015). Also, the protocols might efficiently complement one another (Niino *et al*., 2014). Despite research conducted to date, some genotypes are considered 'recalcitrant materials'. Kaczmarczyk *et al*. (2011) emphasized that regrowth after cryopreservation in different genotypes of potato is varied greatly because of the regeneration capacity of genotypes. As some genotypes of potato does not have regeneration ability *in vitro* and it is essential to store them as tuber (Keller *et al*., 2011). Several cryopreservation protocols developed are useful to overcome the different responses of genotype. These genotypic response differences include sensitivity to biochemical and osmotic toxicity of plant vitrification solution, weakness to dehydration or sub-zero temperatures, damage to plant material during dissection and post regrowth. In this paper, we assessed the feasibility of sugarcane shoot tip cryopreservation using the D cryo-plate method after optimizing different conditions. Average regrowth rate of 12 sugarcane varieties ranged from 20 to 100%, with an average of 52.1%. This average regrowth rate is higher than most previous studies but lower than that reported for the V cryo-plate procedure (Rafique *et al*., 2015). Comparing the regrowth rates of nine varieties by both procedures in detail, 4 varieties (Ni-1, Ni27, Badilla and Ni12) were similar, 4 varieties (Chunnee, NiF3, KN00-114 and NiN2) had higher regrowth rates with the V cryo-plate method, and 1 variety (NiF4) had a higher regrowth rates with the D cryo-plate method (Rafique *et al*., 2015). These results show that some but not all varieties of sugarcane respond better to the V cryo-plate method. In a mat rush cryopreservation experiment, the regrowth using D cryo-plate method was much more successful than the V cryo-plate method and regrowth rate ranged from 73.3 to 96.7%, with an average of 86.3%, compared to 52.5% for the V cryo-plate method. The 11 lines which displayed regrowth less than 50% with the V cryo-plate method, showed regrowth between 73.3-93.3% with the D cryo-plate method (Niino *et al*., 2013). In an experiment with potato germplasm, average regrowth rates of cryopreserved shoot tips in V cryo-plate and D cryo-plate methods were 98.6% and 91.6, respectively (Yamamoto *et al*., 2014). In cherry plum and plum cultivars, regrowth rates shoot tips cryopreservation by V cryo-plate and D cryo-plate methods were 41.7% and 77.5%, 44.6% and 47.5%, respectively (Vujovic *et al*., 2015). Recently, the D cryo-plate method has been adapted to several plant species, data palm (Salma *et al*., 2014), *Chenopodium odorum* (Engelmann-Sylvestre and Engelmann, 2015), persimmon (Matsumoto *et al*., 2015), blueberry (Dhungana *et al*., 2015) and taro (Fukui *et al*., 2015). These results indicate that the D cryo-plate procedure helps to overcome problems associated with sensitivity to vitrification solutions, mistiming dehydration and damage to plant material during dissection (Vujovic *et al*., 2015). In the D cryo-plate protocol, the duration of physical dehydration is crucial for obtaining high post-LN recovery. The dehydration of shoot tips are influenced by the size/volume of shoot tips, the structure of shoot tips, preculture and LS conditions, and desiccation method. Usually, simple and small shoot tips may be appropriate for the V cryo-plate method and complicated and large shoot tips may be suitable for the D cryo-plate method. To explain these results it can be hypothesized that smaller specimens with the buds (the meristematic dome) exposed are more rapidly and uniformly dehydrated by PVS2, compared to larger shoots with the buds (the meristematic dome) covered by leaf sheaths. By contrast, physical dehydration in the D cryo-plate might be more uniform, explaining the equivalent regrowth obtained with small and large specimens (Niino *et al*., 2013).

Major advantages of D cryo-plate technique can be summarized as: handling of samples throughout the procedure is simple and quick; the possibility of injuring and losing shoot tips is considerably reduced compared with other methods; larger specimens can be used; and the window of optimal moisture contents for cryopreservation is very broad. Depending on the species and variety both the D cryo-plate and V cryo-plate methods are efficient and practical for cryo-banking. The D cryo-plate protocol described here appears promising for cryopreservation of, not only sugarcane, but also other plants after marginal modifications of the procedure.

Reed (2001) stated that the physiological condition of the stock plants from which explants are taken as well as growth conditions after rewarming have a major impact on cryopreservation results. Investigations of preconditioning, preculture, osmoprotective treatment, an efficient tissue culture procedure and post-rewarming handling system are necessary for achieving high recovery after LN exposure in cryopreservation by V cryo-plate procedure (Rafique *et al*., 2015). In this paper, the following factors affected regrowth using the D cryo-plate procedure: shoot tip size, sucrose

concentration in preculture medium, sucrose concentration in the LS and drying time by air flow, regrowth medium, regrowth light condition and removal of alginate gel from shoot tips. It is necessary to test each plant species at each step of both the V and D cryo-plate procedure to develop the best cryo-banking protocol. After modifications, the procedure can be used for practical cryobanking. To realize comprehensive cryo-storage of plant genetic resources, further development of cryopreservation techniques, diligent attention to protocol, and also best procedures for establishment of *in vitro* shoot culture system are necessary.

***Acknowledgements*:** We are grateful to Shigeki Nakayama for providing germplasm for the experiments and to Ms. Naoko Nohara, Ms. Akiko Nishiuchi, Ms.Noriko Ishikura and Ms. Yumi Sato for their technical assistance. This work was mainly supported by a grant from Ministry of Agriculture, Forestry, and Fisheries of Japan (Genomics-based Technology for Agricultural Improvement, CRS-1001).

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