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RESEARCH ARTICLE

Comparison of Three Cryotechniques for Conservation of Banana (*Musa* AAA, Cavendish Subgroup) Genotypes

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The aim of this study was to compare three cryotechniques, namely, droplet vitrification (DV), vitrification-cryo-plate (VCP) and dehydration-cryo-plate (DCP) on proliferating meristems of two *Musa* (AAA, Cavendish Subgroup) genotypes. The experiment was set up as factorial completely randomized design for the two *Musa* accessions (IC 250462 cv 'Borjahaji' and EC 493718 cv 'Williams') in five different dehydration duration (30 -150 min). Regrowth after cryopreservation in all treatments was predominantly of two types - shoot regeneration and callusing. In both the cultivars, shoot regrowth of non-frozen and LN treated meristems were not impacted significantly by duration of dehydration exposure, however, callus growth was significantly different with increasing time of exposure. The mean shoot regrowth for all dehydration treatments in Borjahaji was not significantly different. However, the VCP method in Williams, with mean shoot regrowth of 75.5 ± 2.8% was found to be significantly ($P \leq 0.05$) better than that obtained from DV (63.1 ± 2.5%) and DCP (64.8 ± 1.9%). Quantitatively, highest shoot regrowth was obtained after 120 min PVS2 exposure in both cultivars, but using VCP method in Williams (86.7 ± 7.7%) and DV method in Borjahaji (73.1 ± 2.7%). Taking into consideration practical aspects for each technique, any of the three cryopreservation approaches can be used for cryobanking of proliferation meristems of *Musa*, as DV offers cost-effectiveness, while VCP and DCP have ease of explant handling, and DCP avoids chemical toxicity of PVS2.

Key Words: D-cryo-plate (DCP), Dehydration duration, Droplet vitrification (DV), *Musa* germplasm, Plant vitrification solution 2 (PVS2), Proliferating meristems, V-cryo-plate (VCP)

Introduction

The genus *Musa* (Family Musaceae, Order Zingiberales) native to South-East Asia (Simmonds, 1962), is globally known for the commercially important crops of bananas and plantains. These provide starchy staple fruit/food across some of the poorest parts of the world, including Africa and Asia. Both bananas and plantains serve as an important source of carbohydrates, dietary fibre, protein, fat and essential vitamins such as A, B_6 and C and also contain moderate amount of potassium, magnesium, phosphorus, calcium and iron (Pareek, 2016). They are cultivated in more than 135 countries and the annual world production accounts for about 158 million tons i.e. 116.8 million tons for banana and 41.6 million tons for plantain (FAOSTAT, 2019). Out of recognized 50 subgroups of *Musa*, about 51% of the global production come from Cavendish subgroup of banana (ProMusa, 2016).

India is the leading country in banana production, with current annual production of nearly 30 million t. A large diversity of genus *Musa* comprising seeded wild species to seedless cultivars with a variety of ploidy (2x, 3x, 4x) and different genomic composition (AA, AAA, AAB, AB, ABB, BB, ABBB) are widely distributed especially in the North-eastern India (Uma *et al*., 2019, 2020). Banana production is threatened by several significant pests and diseases such as black Sigatoka, *Fusarium* wilt, bacteria wilt, banana bunchy top virus, banana streak virus, nematodes (Jones, 2018; Ploetz, 2021). Climate change is likely to increase frequency, intensity, and duration of biotic stresses as well as abiotic stresses whether from water, salinity, wind or temperature and can lead to the instability and fluctuation in yield, production and price. The alternative solution is breeding of new cultivars combining disease resistance, abiotic stress tolerance and value-added nutritional and agronomic qualities of cultivated clones.

Plant genetic resources are valuable genepools for many desirable traits including yields, nutrition quality, resistance to biological and environmental effects for

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crop improvement. Safeguarding genetic resources for ensuring food security and for the development of varieties that are more productive and resistant to biotic and abiotic stresses is an urgent need. Complementary conservation of *Musa* genetic resources will enhance the availability and maintenance of plant genetic resources as a safeguard for the changing world (Van den Houwe *et al*., 2020) Conservation of *Musa* genetic resources, including cultivated varieties and wild species, has been carried out in the past in field genebanks and in *in vitro* genebanks and more recently in cryogenebanks (Agrawal *et al*., 2004, 2008, 2019; Panis *et al*., 2020). Numerous germplasm accessions of *Musa* have been collected and conserved around the world, including those in Indonesia, Malaysia, Thailand, the Philippines, India, Honduras, Jamaica, Brazil, Cameroon, and Nigeria, with the largest collections in *in vitro* being at International Transit Centre (ITC) in Leuven, Belgium with a total of 1,617 accessions (Van den houwe *et al*., 2020) and 444 accessions in ICAR-NBPGR, India.

For the long-term and biosecure storage of key germplasm collections, cryo-storage or cryopreservation is the best option as a backup of *in vitro* collection. Cryopreservation in which plant materials are stored at ultra-low temperatures usually in liquid nitrogen (-196˚C) and/or in the vapour phase of LN (-135°C to -180 ºC) is the most practical method for long-term conservation since all physical and chemical reactions and time-related changes are almost arrested at these ultra-low temperatures allowing biological samples to be preserved for unlimited periods (Agrawal and Tyagi, 2014; Panis *et al*., 2020). Currently, it is feasible to use this technique for conservation of different variety of plant germplasm and also different explants including pollen, seeds, shoot tips, dormant buds, cell suspensions, embryonic cultures, somatic and zygotic embryos and callus tissue (Agrawal *et al*., 2019, Bettoni *et al*., 2021).

Cryopreservation protocols in *Musa* have been reported in embryogenic cell suspension cultures using slow-freezing method (Panis *et al*., 1990), simple freezing of sucrose precultured proliferating meristematic clumps (Panis *et al*., 1996), vitrification of banana apical meristems (Thinh 1999), droplet vitrification (DV) of *Musa* proliferating meristems (Agrawal *et al*., 2004), droplet vitrification of *Musa* shoot tips (Panis *et al*., 2005) and air-desiccation method for seeds/zygotic embryos of diploid *Musa* spp.(Singh *et al*., 2021). In recent years, to facilitate easier handling of samples during cryopreservation stages, new-age protocols called vitrification cryo-plate (VCP), dehydration cryo-plate (DCP) and cryo-mesh have been successfully developed for several species (Yamamoto *et al*., 2011; Niino *et al*., 2013; Funnekotter *et al*., 2017). The cryo-plates techniques are adaptation to the encapsulation-vitrification and encapsulation-dehydration protocols, aimed at providing greater stability, safety and tolerance of the explants to sudden changes in temperature, using aluminum microplates containing 10-12 oval wells. The VCP is based on PVS2 dehydration, while the DCP is based on air dehydration in the laminar flow chamber for a controlled time. However, there is constraint that the use of specific protocol is genotype specific i.e. some genotypes are recalcitrant to some protocols. Although about 100 accessions of *Musa* have been cryobanked in our laboratory at ICAR-NBPGR, results show high genotypic variability, with some accessions remaining recalcitrant to the DV protocol using proliferating meristems. Thus, the aim of this study was to compare three cryotechniques (DV, VCP and DCP) on proliferating meristems of two *Musa* (AAA, Cavendish Subgroup) genotypes, as a pilot study to further optimize cryobanking of diverse *Musa* genetic resources.

Material and Methods

Plant Material

In vitro shoot cultures of *Musa* belonging to genomic group AAA and subgroup Cavendish were sourced from the *in vitro* Gene Bank (IVGB) of ICAR-National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India. Two genotypes namely 'Borjahaji' (IC 250462) and 'Williams (Bell South Johnstone)' (EC 493718, henceforth referred as 'Williams') were used for this study. Shoot cultures were multiplied on a standard P5 multiplication medium comprising MS salts supplemented with 10 µM 6-benzylaminopurine (BAP), 1 µM Indole-3-acetic acid (IAA), 1 µM ascorbic acid, 3% sucrose (0.09 M) and gelled with 0.25% Phytagel™ (Panis, 2009). All chemicals used were of Sigma Aldrich, USA, except sucrose which was from HiMedia Lab, Mumbai, India. Cultures were maintained at 25 ± 2°C with 40 µE $m^{-2}s^{-1}$ photon flux density at 16 h light/8 h dark photoperiod.

Generation of Proliferating Meristems

The basic steps for cryopreservation were followed as per previous protocol of Agrawal et al. (2014a). Shoot cultures of the two accessions were subcultured by splitting the shoots vertically into two halves, to trigger proliferation of axillary meristems at leaf bases. These were raised on P5 medium for two culture cycles, after which they were subcultured on P4 medium (P5 medium with 10x higher BAP concentration i.e 100 µM), and incubated in the dark (Panis, 2009). Subsequently, the smallest shoots with clusters of meristems at their leaf bases were selected and subcultured on p4 medium, until groups of proliferating 'cauliflower-like meristems' (about 0.5 cm in diameter) are produced by subculturing about one-month intervals for 6-12 subculture cycles, depending on the cultivar. White proliferating meristematic clumps with 6-10 mm in diameter were excised and precultured on C0 medium containing all P5 medium constituents but with 0.4 M sucrose for pre-growth desiccation of 2 weeks and incubated at 25 ± 2°C in the dark.

Cryopreservation by Three Techniques

Meristematic explants (2-3 mm dia) containing at least 3-5 apical domes were excised from pregrown clusters and placed in a plastic container containing filter-sterilized loading solution (LS). The LS comprised 2 M glycerol + 0.4 M sucrose in MS medium, at pH 5.8 (Sakai *et al*., 1990). Explants were treated with LS at room temperature (RT, 25 ± 1°C) for 20 min, after the last meristematic clump was excised. After this step, explants were subjected to three different techniques for the subsequent steps of desiccation and freezing.

Droplet Vitrification Technique (DV)

After LS treatment, the meristematic clumps were transferred into a Petri dish (5 cm dia) containing ice-cold plant vitrification solution 2 (PVS2) (Sakai *et al*., 1990). The filter-sterilized PVS2 solution consisted of 30% glycerol (3.26 M) + 15% ethylene glycol (2.42 M) + 15% dimethyl sulfoxide (DMSO) (1.9 M) + 0.4 M sucrose in MS medium, at pH 5.8. The explants were subjected to PVS2 treatment at 0°C for 30, 60, 90, 120, 150 min. Five minutes before the completion of each incubation period, 10 meristematic clumps were transferred to a drop of fresh, chilled PVS2 solution placed on a strip of pre-autoclaved aluminum foil (20×5 mm). Then, the aluminum foil strips with the meristems were plunged directly into liquid nitrogen (LN) and carefully placed into sterile cryogenic tubes (2 ml). The cryotubes were held in a polycarbonate cryobox, kept immersed in LN in a storage dewar. Explants were held in the LN for at least 30 min.

Vitrification Cryo-plate Technique (VCP)

This technique was followed according to Yamamoto *et al.* (2011) and Niino *et al*. (2014), using aluminum cryo-plates No. 3 (37 mm length, 7 mm breadth, 0.5 mm thickness), with 10 oval-shaped wells of 1.5 mm dia, 2.5 mm long and 0.75 mm depth. Sterilized cryo-plates were placed in a Petri dish (9 cm) and 2% (w/v) sodium alginate solution (~2.0–2.5 µl) in a calcium-free MS basal medium with 0.4 M sucrose were poured in each well of the cryo-plate. The osmoprotected explants (in LS) were individually placed in the depth of cryo-plate and slightly pressed to allow the explants to position firmly in the well. Thereafter, calcium solution (0.1 M calcium chloride in basal MS medium and 0.4 M sucrose) was poured drop-wise on the section of the aluminum plates where the explants were located until it covered all the samples and left for 15 min to achieve complete polymerization of alginate gel. Excess calcium solution was removed by tapping the cryo-plates on autoclaved filter papers. The cryo-plate with encapsulated explants was transferred into Petri dish containing 20 ml ice-cold PVS2 solution. Exposure time to PVS2 were 30, 60, 90, 120, 150 min at 0°C. After appropriate time, the cryo-plate with the explants was transferred into a 2 ml sterile cryogenic tube immersed in LN and was kept for at least 30 minutes.

Dehydration Cryo-plate Technique (DCP)

The D-plate technique of Niino *et al*. (2013) was adapted, in which the explant embedding in alginate was same as in V-plate. Thereafter, the cryo-plates were desiccated by air-drying in a laminar flow cabinet for 30, 60, 90, 120 and 150 min to reduce the moisture content of the explants. Subsequently, cryo-plates with explants were transferred to a 2 ml cryotube immersed in LN with exposure time of at least 30 min.

Recovery, Regeneration and Data Analysis

After exposure to LN, the aluminum foil strips in DV technique and cryo-plates in V-plate and D-plate techniques were taken out and rapidly rinsed in 10 ml of recovery/reloading solution (RS) held in Petri dish (5 cm dia) at RT for 20 min. The RS comprised MS medium supplemented with 1.2 M sucrose, at pH 5.8.

Control in each technique consisted of explants along with aluminum foils/cryo-plates subjected to desiccation but not freezing; the explants were directly washed with RS solution after PVS2/air dehydration treatment without plunging in LN.

For regrowth, explants were placed on two sterile filter papers (Whatman No. 1) on top of semi-solid hormone-free MS medium (recovery medium) containing 0.3 M sucrose in Petri dish, and incubated in the dark for 48 h. Then meristems were transferred to the semi-solid regeneration (C3) medium (MS medium + 2.22 µM BA + 0.09 M sucrose + 0.25% phytagel) without filter papers and continuously keeping in the dark for 14 d. Thereafter meristems were transferred to p6 regeneration medium (MS medium + 0.09 M sucrose +1 µM BAP + 0.25% phytagel) and kept in 16 h light/8 h dark (40 μMm^{-2} s^{-1}) at 25±2°C.

Post-thaw recovery data was recorded after 4-6 weeks. Shoot regeneration and/or non-morphogenic callus formation of surviving explants were observed under a binocular microscope at weekly intervals up to 6 weeks; meristematic clumps which remained pale or white and did not show any further change and which turned partially or completely black were considered non-responsive. For each experiment, 5–20 explants were used for control treatment (non-LN-exposed) and 8–44 explants were used for LN treatment. As the experiments were performed three times for each treatment, a total of 75-92 control explants and 172-209 LN-treated explants were used. The experiment was set up as factorial completely randomized design (CRD). The percentage responses of the cryopreserved meristems that produced shoot and/or callus in 6-week-old cultures were calculated. Results are presented as means (%) ± standard error (SE). Data were subjected to analysis of variance (ANOVA) to test significant differences using SPSS ver. 22.0 package.

Results

Proliferating 'cauliflower like' meristems were generated after 8-12 subculture cycles in P4 medium in *Musa* cv Borjahaji while in Williams it took only 6-7 subcultures. The different steps of three cryopreservation techniques (DV, VCP and DCP) and post-regeneration growth are represented in Fig. 1. Regrowth after cryopreservation in all treatments was predominantly of two types – shoot regeneration and callusing. Some 5-10% infection

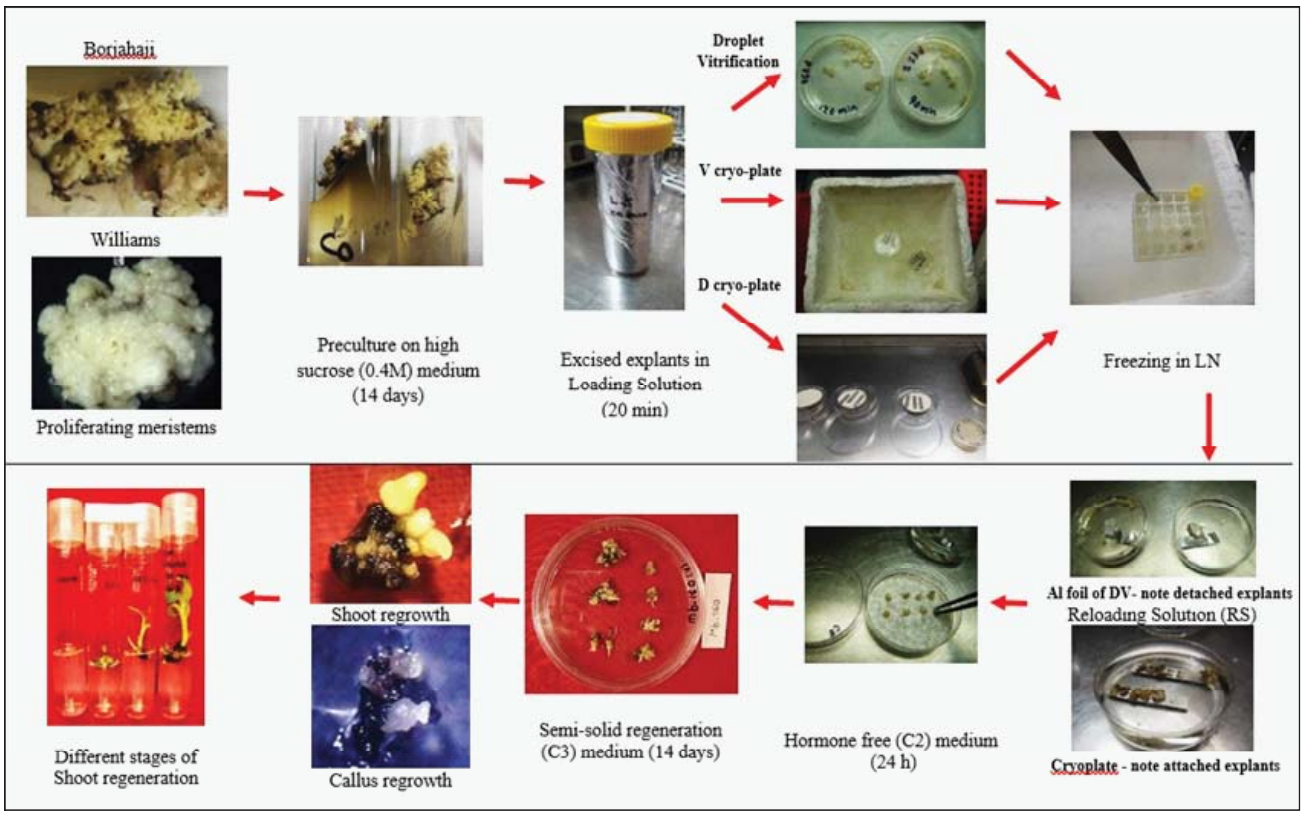

Fig. 1. Different stages of the three cryopreservation techniques – Droplet Vitrification (DV), V Cryo-plate (VCP) and D Cryo-plate (DCP), in *Musa* cv Borjahaji

(bacterial) was obtained in a few samples initially, and these were discarded and not used for computation of results.

Effect of dehydration duration

To determine the optimal duration of dehydration treatment, meristem explants in both the cultivars Borjahaji and Williams were treated with 30, 60, 90, 120 and 150 min. Post-thaw regeneration of shoot and callus in different duration of dehydration treatments for three cryopreservation techniques is shown in Table 1. In both the cultivars, shoot regrowth of non-frozen meristems was >82-85%, and not impacted significantly by duration of dehydration exposure (Table 2). In contrast, callus formation increased significantly ($P \leq 0.01$) in the non-frozen explants with increase in dehydration duration (120 and 150 min) in both the genotypes (Table 1, 2). Similar trend was recorded overall with the LN treated samples, where shoot regrowth was not meaningfully impacted with increase in dehydration duration but callus growth increased very significantly ($P \leq 0.01$) with increasing time of exposure of PVS2. The callus developed in all explants failed to convert into shoots at any stage, and eventually turned black and necrotic. In general, longer dehydration duration increases not only shoot regeneration, but also callus growth (Table 1).

Effect of technique and genotype

The mean regrowth of control and LN treated meristematic explants in the three techniques (DV, VCP, DCP) for Borjahaji and Williams is represented in Fig. 2. In Borjahaji, irrespective of the technique applied, it was found that 60-150 min duration of dehydration exposure gave statistically similar shoot regrowth after LN, within a technique (Table 1, 2). The mean shoot regrowth for all dehydration treatments in Borjahaji were 67.5 ± 2.8% in DV, 66.5 ± 2.4% in VCP and 58.4 ± 3.7% in DCP (Fig. 2A). In case of Williams, dehydration duration had no significant effect within a technique with respect to shoot regrowth after LN treatment (Table 1, 2). However, unlike Borjahaji, the VCP method in Williams, with mean shoot regrowth of 75.5 ± 2.8% was found to be significantly ($P \leq 0.01$) better than that obtained from DV (63.1 ± 2.5%) and DCP (64.8 ± 1.9%) (Fig 2B). In absolute terms, highest shoot regrowth was obtained after 120 min dehydration

Table 1. Shoot and callus regeneration (%) after different PVS2 treatment/dehydration durations, in *Musa* cvs Borjahaji and Williams, in control and LN treated meristematic explants.

Technique	Dehydration duration (min)	Borjahaji				Williams			
		Regrowth response (-LN)		Regrowth response (+LN)		Regrowth response (-LN)		Regrowth response (+LN)	
		Shoot (%)	Callus (%)	Shoot (%)	Callus (%)	Shoot (%)	Callus (%)	Shoot (%)	Callus (%)
DV	30	95.8 ± 1.9 ^{ab}	7.5 ± 0.0 ^{cde}	67.5 ± 4.3 ^{a}	27.5 ± 4.3 ^{cd}	90.0 ± 4.5 ^{a}	8.8 ± 0.1 ^{de}	56.7 ± 6.6 ^{cd}	18.4 ± 2.2 ^{e}
	60	95.0 ± 2.2 ^{ab}	6.3 ± 0.3 ^{de}	66.6 ± 2.0 ^{a}	25.7 ± 0.4 ^{cde}	93.3 ± 3.0 ^{a}	12.4 ± 0.1 ^{cd}	60.9 ± 9.5 ^{bcd}	19.1 ± 2.1 ^{e}
	90	82.2 ± 4.3 ^{b}	12.0 ± 0.8 ^{abc}	72.4 ± 4.4 ^{a}	27.9 ± 7.0 ^{cd}	93.3 ± 3.0 ^{a}	19.5 ± 1.2 ^{ab}	64.9 ± 5.8 ^{bcd}	28.1 ± 1.6 ^{cd}
	120	95.0 ± 2.2 ^{ab}	15.5 ± 0.2 ^{ab}	73.1 ± 2.7 ^{a}	35.7 ± 5.1 ^{bc}	85.0 ± 2.6 ^{a}	23.3 ± 0.2 ^{a}	64.3 ± 5.2 ^{bcd}	41.9 ± 5.8 ^{a}
	150	87.5 ± 3.2 ^{ab}	15.0 ± 0.0 ^{ab}	58.1 ± 9.8 ^{ab}	45.6 ± 2.5 ^{ab}	89.7 ± 3.4 ^{a}	18.0 ± 0.8 ^{b}	68.9 ± 8.7 ^{bcd}	40.0 ± 2.9 ^{a}
VCP	30	100.0 ± 0.0 ^{a}	11.0 ± 1.2 ^{bcd}	62.1 ± 2.9 ^{ab}	11.3 ± 0.8 ^{f}	100.0 ± 0.0 ^{a}	9.8 ± 0.6 ^{cde}	70.8 ± 5.3 ^{abcd}	24.0 ± 0.6 ^{de}
	60	100.0 ± 0.0 ^{a}	11.0 ± 1.2 ^{bcd}	71.1 ± 3.1 ^{a}	16.1 ± 2.0 ^{def}	100.0 ± 0.0 ^{a}	17.5 ± 0.3 ^{b}	72.5 ± 1.4 ^{abc}	32.5 ± 4.3 ^{bc}
	90	95.0 ± 2.2 ^{ab}	11.4 ± 1.8 ^{abcd}	67.4 ± 9.0 ^{a}	24.3 ± 2.3 ^{cde}	88.9 ± 5.0 ^{a}	14.6 ± 1.4 ^{bc}	75.2 ± 2.2 ^{ab}	42.5 ± 0.2 ^{a}
	120	95.0 ± 2.2 ^{ab}	11.5 ± 0.8 ^{abcd}	61.0 ± 4.0 ^{ab}	24.5 ± 2.9 ^{cde}	95.0 ± 2.2 ^{a}	17.8 ± 1.6 ^{b}	86.7 ± 7.7 ^{a}	36.7 ± 1.9 ^{ab}
	150	91.7 ± 3.7 ^{ab}	16.5 ± 1.6 ^{a}	70.9 ± 6.9 ^{a}	49.7 ± 4.7 ^{a}	92.9 ± 3.2 ^{a}	19.0 ± 0.6 ^{ab}	72.5 ± 7.2 ^{abc}	32.5 ± 1.4 ^{bc}
DCP	30	94.4 ± 2.5 ^{ab}	5.5 ± 0.1 ^{e}	45.5 ± 7.9 ^{b}	5.1 ± 0.1 ^{f}	100.0 ± 0.0 ^{a}	5.0 ± 0.0 ^{e}	63.9 ± 5.2 ^{bcd}	18.8 ± 2.0 ^{e}
	60	94.4 ± 2.5 ^{ab}	11.0 ± 0.3 ^{bcd}	61.1 ± 7.3 ^{ab}	7.9 ± 1.2 ^{f}	100.0 ± 0.0 ^{a}	5.5 ± 0.1 ^{e}	69.4 ± 1.6 ^{bcd}	27.8 ± 3.2 ^{cd}
	90	94.4 ± 2.5 ^{ab}	12.5 ± 0.6 ^{abc}	62.9 ± 7.4 ^{ab}	6.3 ± 0.2 ^{f}	100.0 ± 0.0 ^{a}	6.8 ± 0.5 ^{e}	68.1 ± 1.2 ^{bcd}	25.3 ± 2.4 ^{cde}
	120	100.0 ± 0.0 ^{a}	12.5 ± 0.1 ^{abc}	62.6 ± 9.6 ^{ab}	14.2 ± 1.4 ^{ef}	100.0 ± 0.0 ^{a}	9.4 ± 0.9 ^{de}	67.5 ± 4.3 ^{bcd}	23.0 ± 1.5 ^{de}
	150	100.0 ± 0.0 ^{a}	12.0 ± 0.0 ^{abc}	59.7 ± 10.1 ^{ab}	14.2 ± 1.4 ^{ef}	96.7 ± 1.5 ^{a}	12.5 ± 0.6 ^{cd}	55.0 ± 2.9 ^{d}	20.2 ± 0.2 ^{e}

Data represents mean ±SE of three replicates, recorded 8 weeks after culture; Values followed by the same letter are not significantly different ($P < 0.05$).

Table 2. Analysis of variance (ANOVA) for shoot and callus regeneration in the two *Musa* cvs Borjahaji and Williams

Regrowth	Source		Borjahaji				Williams			
		DF	SS	MS	F	P	SS	**MS**	**F**	**P**
Shoot (Control)	Techniques	2	291.2	145.6	1.7	0.198	618.4	309.2	3.2	0.057
	Dehydration duration	4	280.5	70.1	0.8	0.520	160.5	40.1	0.4	0.800
	Techniques*Dehydration duration	8	421.1	52.6	0.6	0.755	281	35.1	0.4	0.934
	Error	30	2551.9	85.1			2938.6	98.0		
Callus (Control)	Techniques	2	19.5	9.7	0.9	0.413	678.8	339.4	38.7	0.000**
	Dehydration duration	4	258.2	64.5	6.1	0.001**	493.9	123.5	14.1	0.000**
	Techniques*Dehydration duration	8	132.7	16.6	1.6	0.181	186.9	23.4	2.7	0.024*
	Error	30	320.2	10.7			263.0	8.8		
Shoot (LN)	Techniques	2	753.9	377.0	2.8	0.077	1356.6	678.3	7.2	0.003**
	Dehydration duration	4	479.1	119.8	0.9	0.483	443.3	110.8	1.2	0.343
	Techniques*Dehydration duration	8	869.0	108.6	0.8	0.603	716.9	89.6	1.0	0.494
	Error	30	4044.9	134.8			2838.5	94.6		
Callus (LN)	Techniques	2	4110.8	2055.4	40.8	0.000**	860.1	430.1	21.3	0.000**
	Dehydration duration	4	2765.8	691.4	13.7	0.000**	1041.1	260.3	12.9	0.000**
	Techniques*Dehydration duration	8	919.2	114.9	2.3	0.049*	1157.7	144.7	7.2	0.000**
	Error	30	1511.9	50.4			604.8	20.2		

* Significant ** Highly significant

exposure in both cultivars, but using VCP method in Williams (86.7 ± 7.7%) and DV method in Borjahaji (73.1 ± 2.7%).

Combined effect of genotype, technique and dehydration duration

Table 3 presents the overall ANOVA of combined effect of the two genotypes, three techniques and five dehydration duration tested in the present work, with respect to shoot and callus regrowth in non-frozen controls and LN treated meristematic tissues of banana. Shoot regeneration rate in dehydration treatment (control) was slightly influenced ($P \leq 0.014$) by cryotechnique applied, but interaction of genotypes and dehydration duration was not significant. Callus formation was highly influenced by genotypes, techniques and dehydration duration ($P \leq 0.01$), in both non-frozen and frozen meristems. In terms of shoot regrowth after LN treatment, which is the most important parameter, it is seen that genotype *per se* has no significant effect, but technique influences shoot recovery at highly significant level ($P \leq 0.005$) and genotype × technique has significant (P

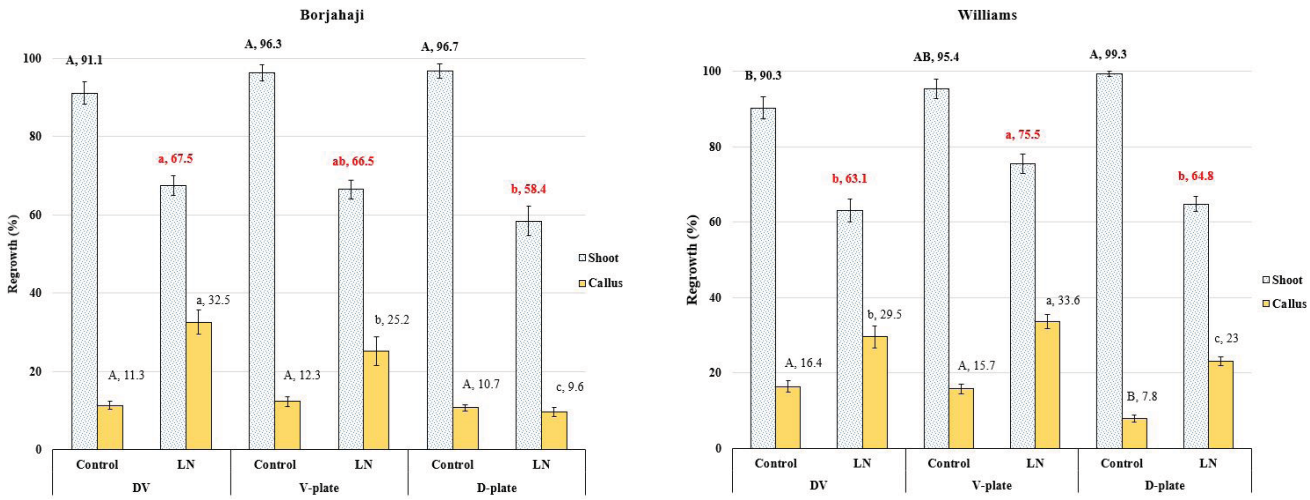

Fig. 2. Shoot and callus regeneration in control and LN treated meristem explants of the two cultivars of *Musa* (A-Borjahaji, B-Williams) using the three cryopreservation techniques. Same alphabets amongst C and LN treated samples for shoot and callus denote non-significant differences (P≤0.05)

Table 3. Analysis of variance (ANOVA) for shoot and callus regeneration (control and LN) for both genotypes, different techniques and various dehydration duration in cryopreserved *Musa* meristems

	Source	DF	SS	MS	F	P
Shoot (Control)	Genotypes	1	1.8	1.8	0.0	0.889
	Techniques	2	845.6	422.8	4.6	0.014*
	Dehydration duration	4	329.4	82.3	0.9	0.470
	Genotypes*Techniques	2	64.1	32.0	0.4	0.706
	Genotypes* Dehydration duration	4	111.5	27.9	0.3	0.874
	Techniques* Dehydration duration	8	268.1	33.5	0.4	0.934
	Genotypes*Techniques*Dehydration duration	8	434.0	54.3	0.6	0.780
	Error	60	5490.4	91.5		
Callus (Control)	Genotypes	1	81.8	81.8	8.4	0.005**
	Techniques	2	432.1	216.1	22.2	0.000**
	Dehydration duration	4	718.1	179.5	18.5	0.000**
	Genotypes*Techniques	2	266.1	133.0	13.7	0.000**
	Genotypes* Dehydration duration	4	34.0	8.5	0.9	0.485
	Techniques* Dehydration duration	8	196.3	24.5	2.5	0.019*
	Genotypes*Techniques*Dehydration duration	8	123.3	15.4	1.6	0.149
	Error	60	583.2	9.7		
Shoot (LN)	Genotypes	1	306.7	306.7	2.7	0.107
	Techniques	2	1351.0	675.5	5.9	0.005**
	Dehydration duration	4	806.6	201.7	1.8	0.149
	Genotypes*Techniques	2	759.5	379.7	3.3	0.043*
	Genotypes* Dehydration duration	4	115.8	29.0	0.3	0.907
	Techniques* Dehydration duration	8	242.5	30.3	0.3	0.975
	Genotypes*Techniques*Dehydration duration	8	1343.4	167.9	1.5	0.190
	Error	60	6883.3	114.7		
Callus (LN)	Genotypes	1	900.3	900.3	25.5	0.000**
	Techniques	2	3908.0	1954.0	55.4	0.000**
	Dehydration duration	4	2906.7	726.7	20.6	0.000**
	Genotypes*Techniques	2	1062.9	531.5	15.1	0.000**
	Genotypes* Dehydration duration	4	900.1	225.0	6.4	0.000**
	Techniques* Dehydration duration	8	1253.8	156.7	4.4	0.000**
	Genotypes*Techniques*Dehydration duration	8	823.1	102.9	2.9	0.008**
	Error	60	2116.6	35.3		

* Significant ** Highly significant

$\leq$ 0.043) impact. In our study, duration of dehydration alone, or in combination with genotype and technique was not important factor to change the shoot recovery results (Table 3).

Discussion

The droplet vitrifcation (DV) and cryo-plate methods (VCP and DCP) are contemporary cryopreservation techniques that are being increasingly used to successfully cryopreserve shoot tips of many tropical and temperate plant species (Wang *et al*., 2021). In case of *Musa,* vitrification and DV of isolated shoot tips or proliferating meristems have been practically applied for cryobanking of germplasm at the *Musa* International Transit Centre (ITC), Leuven, Belgium (Panis *et al*., 2020) as well as at

ICAR-NBPGR, India (Agrawal et al., 2019) with varying rates of post-thaw recovery (20-90%). However, the VCP and DCP protocols have not been applied so far. Hence, the present study was undertaken to test the comparative efficacy DV, VCP and DCP in cryopreservation of *in vitro* raised proliferating meristems in two Cavendish (AAA) cultivars of *Musa*. We focused on effect of dehydration periods (30, 60, 90, 120 and 150 min) before immersion in LN in all the three techniques and two genotypes (Borjahaji and Williams).

Finding the best duration of treatment with a cryoprotection solution is important to determine the correct balance between toxicity and acceptable dehydration of explants, by reducing the chance of lethal ice crystal formation in the regenerating tissue while avoiding overproduction of reactive oxygen species which cause serious oxidative damage to proteins, nucleic acids and polyunsaturated lipids (Turner *et al*., 2000; Panis *et al*., 2005). The optimal dehydration treatment of different plant species (with variable water content and membrane permeability) varies considerably (Faltus *et al*., 2021). In the present work, osmotic dehydration using varied duration (30 to 150 min) of PVS2 in both DV and VCP methods did not prove harmful for the tissues in non-frozen controls and LN treated samples. Shoot conversion rates were high in Borjahaji (58.1 – 73.1% in DV; 61.0-71.1% in VCP) as well as Williams (56.7 – 68.9% in DV; 70.8-86.7% in VCP) explants subjected to LN treatment. The high regrowth rate is attributed to the optimal cryoprotection by PVS2. The conversion of cytoplasmic and extracellular solutions to a glass by vitrification following cooling avoids the damaging stresses associated with the crystalline ice formation and cryodehydration, which increases viability following the recovery from cryopreservation (Agrawal *et al*., 2004). Our results are in agreement with the findings of Faltus *et al*. (2021) who have demonstrated that PVS2 in full concentration give optimal cooling and warming rates and its toxicity is significantly reduced when used at 0°C, as done in the present study.

Formation of non-morphogenic callus in the two genotypes was positively correlated with increase in PVS2 duration. This is in contrast to the results obtained in the Monthan subgroup (ABB) by Agrawal *et al*. (2008), where callus formation (0-30%) was genotype-dependent and not impacted greatly with PVS2 duration (30-120 min). Similarly, Agrawal *et al*. (2014b) investigating on five wild *Musa* species for different duration of PVS2 treatment reported that four species (*M. acuminata, M. basjoo, M. jackeyi* and *M. textilis*) could tolerate PVS2 dehydration up to 120 min, whereas in *M. balbisiana* a 90 min PVS2 incubation time was optimal in DV of proliferating meristems. The optimal length of PVS2 treatment (30-50 min) at 0°C was observed by Panis *et al.* (2005) in isolated shoot tips (not proliferating meristems) where DV cryopreservation on eight different groups of *Musa* spp. including AAA genomic group, gave 68-74% regeneration for Williams (AAA) and 74-83% for Grand Naine (AAA) genotypes.

Panis *et al*. (2005) reported that in banana cryopreservation, callus formation occurs in meristematic samples by mechanical injury of tissue due to excision and processing. Hence, it was expected that in VCP and DCP techniques, due to use of alginate gels to secure samples to aluminum cryo-plates, may reduce some mechanical injuries during handling and thus the callus formation. As seen in Fig. 2 and Table 3, all three factors, namely technique, genotype and desiccation duration and their interaction were highly significant variables that determined degree of callus development. Hence, not only mechanical injury but the genotype and method applied are critical in case of callus formation in *Musa* cryopreservation.

Cryopreservation protocols for more than 25 species (e.g. *Allium*, *Mentha*, *Saccharum*, *Solanum* etc.) by the VCP method and at least 15 species (e.g. *Solanum*, *Dendranthema*, *Juncus*, *Saccharum* etc.) by DCP are successfully reported (Niino *et al*., 2019). Yamamoto et al. (2011) reported the shoot regrowth rate of 65–90% (with an average of 77%) for seven genotypes of Dalmatian chrysanthemum (*Tanacetum cinerariifolium*) by VCP technique. Comparable regeneration rates have been reported using both VCP and DCP techniques in *Prunus* spp. (Vujović *et al*., 2015) and *Solanum* spp. (Yamamoto *et al*., 2015), while higher regeneration rates using DCP than VCP in *Ullucus tuberosus* (Arizaga *et al*., 2017). Higher regeneration rate for VCP than DCP is published in *Clinopodium odorum* (Sylvestre and Engelmann, 2015). Niino and coworkers, who successfully developed the DCP technique, reported cryo-storage of mat rush germplasm obtaining regeneration rates of 73–97% with a mean shoot regrowth of 86.3% across the 20 tested genotypes (Niino *et al*., 2013). The DCP technique is recommended for species which are sensitive to cryoprotective chemicals like PVS2 (Niino *et al*., 2019).

Table 4. Comparative advantages and drawbacks for droplet vitrification versus cryo-plate techniques of cryopreservation

Process/ steps during cryopreservation	DV technique	Cryoplate (VCP and DCP) technique
Support for mounting explants during freezing	aluminum foils are cost-effective and universally available.	The cryoplates are so far only available in Japan and costlier than aluminum foils.
Steps for handling during preparation for freezing	Steps are fewer and overall time taken is less. However, treating with LS and PVS2 often results in damage or loss of meristematic clumps by repeated pipetting or handling with forceps.	There are more handling steps (mounting explants on cryoplate, alginate drop on cryoplate, explant mounting, polymerization with calcium solution followed by desiccation) which needs extra time and well-trained technician.
Freezing tissue in LN	Placing the aluminum foil into cryovial requires skill and practice, as sometimes the foil folds during placing in cryovials (especially in larger explants like *Musa* proliferating meristems)	Overall handling of material and placing the samples in cryovials for freezing is easier, as explants are securely adhered to cryoplates with alginate matrix.
Thawing of frozen tissue	When frozen samples are thawed and placed in recovery solution, the tissue easily detach from the aluminum foil and readily float in solution, undergoing rapid thawing.	Frozen samples often remain attached to the cryo-plates in recovery solution (in DCP technique) due to the alginate matrix. Sometimes, it requires physical detachment with help of forcep/needle, and this can cause mechanical injuries to the explants.

So far, there is no publication related to cryopreservation of proliferation meristems of *Musa* using VCP and DCP techniques. The present work has shown that in *Musa* (AAA) cv Williams, VCP can be the preferred technique as compared to DV and DCP while for cv Borjahaji, all the three methods are equally effective (Table 1, Fig. 2). It is suggested that in general, VCP and DCP lead to higher and uniform survival after cryopreservation and have a wider spectrum for desiccation duration, which is especially useful for samples which are sensitive to exposure to toxic chemicals (Niino *et al*., 2013, 2014; Tanaka *et al*., 2018). There are findings in *Prunus* spp. (Vujović *et al*., 2015), *Solanum* spp. (Yamamoto *et al*., 2015) and mat rush (Niino *et al*., 2013) in which longer durations of dehydration treatment is required for DCP compared to VCP techniques. We hypothesize that longer air desiccation periods in DCP may increase higher regeneration rate in *Musa* proliferating meristems after cryopreservation. Also, due to absence of use cryoprotecting agents like PVS2 in DCP, it may be considered as an alternative choice for *Musa* germplasm cryobanking, as it would eliminate the risks of chemical stress and possible genetic alternations (Benson, 2008; Harding, 2004; Wang *et al*., 2014; 2018).

In terms of practical cryobanking of germplasm of *Musa*, use of proliferating meristems has the advantage in ease of explants isolation, although some genotypes are recalcitrant to proliferate (Agrawal *et al*., 2014a; Panis *et al*., 1990). Based on the findings of the present work and published literature (Yamamoto *et al*., 2011; Matsumoto 2017; Niino *et al*., 2019), Table 4 provides a comparative summary of advantages and drawbacks for DV, VCP and DCP techniques.

In India, a large diversity of genus *Musa* with a variety of ploidy and different genomic composition (AA, AAA, AAB, AB, ABB, BB, ABBB, etc.) are widely distributed. So far, some 100 accessions of *Musa* have been cryobanked at ICAR-NBPGR, New Delhi, out of which nearly 65 accessions are conserved in the form of proliferating meristems, using either simple vitrification or DV technique (Agrawal *et al*., 2014 a,b, 2019). The present study has demonstrated that besides DV, the VCP and DCP technique can also be successfully applied for cryobanking of *Musa* germplasm, provided cryo-plates are easily available to the genebank curators and managers. The DCP technique has the additional advantage of absence of use of PVS2, a toxic reagent. Both DCP and VCP may prove more useful in isolated meristems, which are more delicate to handle. However, more studies across all genotypes of *Musa* are required before practical cryobanking using DCP or VCP techniques. Taking into consideration practical aspects for each technique, any of the three cryopreservation approaches can be used for cryobanking of proliferation meristems of *Musa*, as DV offers cost-effectiveness, while VCP and DCP have ease of explant handling, and DCP avoids chemical toxicity of PVS2.

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