Cryopreservation of subgroup Monthan (ABB) of Indian cooking banana (*Musa* spp.) germplasm

India is recognized as one of the major centres of origin and diversity of *Musa* sp. (bananas and plantains) and is endowed with an extremely rich diversity of this crop. Amongst the Indian cooking bananas, Monthan (ABB) is the most common subgroup grown in all the important banana-growing states, and India is the centre of diversity for this group[1]. In India, nine field genebanks exist for *Musa* sp., that have about 1000 accessions, including some duplicates. Presently, the *In Vitro* Genebank (IVG) at the National Bureau of Plant Genetic Resources (NBPGR), New Delhi, has 405 accessions (of which 30% belongs to the ABB group) of *Musa* spp. in the form of *in vitro* cultures. Currently, cryopreservation is the only effective and economical method in vogue for safe, long-term conservation of vegetatively propagated crop germplasm. Cryopreservation in banana has been reported using seeds[2], zygotic embryos[3], embryonic cell suspensions[4] and *in vitro*-derived meristems[5–9]. Amongst the methods of cryopreservation reported so far, cryopreservation of highly proliferating banana meristems by vitrification method using plant vitrification solution 2 (PVS2) containing dimethylsulphoxide (DMSO), ethylene glycol (EG), glycerol and sucrose was found to be user-friendly, with reasonably high rates of post-thaw regeneration of plantlets[8,9]. This technique requires modification for different species and genera. For successful cryopreservation, optimization of duration of PVS2 treatment to the explants and sucrose in preculture medium are crucial, due to which correct balance between toxicity and adequate dehydration is effected[7]. The present work was carried out to develop an effective and practical cryopreservation protocol for Indian cooking bananas (ABB subgroup) by determining the optimal durations of treatment of sucrose (in preculture medium) and PVS2 to the explants for maximum post-thaw recovery of functional plants.

Three representative cultivars of Indian cooking banana (ABB subgroup), namely 'Kallu Monthan' (IC251010, collected from Kerala), 'Pidi Monthan' (IC250568, collected from Karnataka) and 'Sommarani Monthan' (IC250538, collected from Bihar) were chosen from the *in vitro*-conserved germplasm at IVG. Initially, these cultivars were established *in vitro* by culturing shoot tips following the method described by Banerjee and de Langhe[10]. To multiply shoot cultures, Murashige and Skoog (MS) medium[11], supplemented with 0.09 M sucrose, 10 μM benzyladenine (BA), 1 μM indole-3-acetic acid (IAA), 10 mg/l ascorbic acid and gelled with 0.25% gelrite (M1 medium) was used.

Proliferating cauliflower-like meristem clumps were developed for cryopreservation from the *in vitro*-raised shoots transferred to MS medium (0.09 M sucrose) + 100 μM BA + 1 μM IAA + 10 mg/l ascorbic acid + 0.25% gelrite (M2 medium)[8]. Small clumps of cauliflower-like meristems were selected and subcultured for 4–6 cycles at 4–6 weeks interval, to obtain sufficient explants for cryopreservation experiments. The pH of the medium was adjusted to 5.8 prior to autoclaving at 1.1 kg per sq. cm (121°C) for 20 min. All cultures were raised in culture tubes (25 × 125 mm; Borosil, India) containing approximately 20 ml medium.

Cultivar 'Kallu Monthan' was used for optimization of sucrose preculture duration. The white meristematic clumps (obtained from cauliflower-like meristem cultures) of about 0.8–1.0 cm diameter and containing 12–20 meristematic domes were excised and transferred onto a preculture medium that contained all constituents of the M1 medium, but with 0.4 M sucrose concentration instead of 0.09 M sucrose. These cultures were incubated for 1, 2, 3 and 4 weeks under conditions for normal shoot growth, as described earlier. At the end of this preculture phase, surviving meristems (2–3 mm diameter) containing at least four meristematic domes were excised and used for cryopreservation by the vitrification method.

Dictated by the results of optimum sucrose concentration for preculture treatment of cv. 'Kallu Monthan', optimization of PVS2 treatment was done for cultivars 'Kallu Monthan', 'Pidi Monthan' and 'Sommrani Monthan'. The sucrose-precultured meristems were transferred to 2 ml sterile cryovials (Greiner, Germany) containing 1.5 ml loading solution (LS) for 20 min at room temperature. The composition of LS was 2 M glycerol and 0.4 M sucrose dissolved in MS medium (pH 5.8). The LS was removed and 1.5 ml PVS2 (ice-cold)[12] was added into cryovials containing precultured meristems. The PVS2 solution consisted of 30% (3.26 M) glycerol, 15% (2.42 M) EG, 15% (1.9 M) DMSO and 0.4 M sucrose dissolved in the MS medium (pH 5.8). The meristems were exposed to PVS2 for 30, 60, 90, 120 and 150 min at 0°C. Just prior to the end of the incubation period, 1 ml of fresh ice-cold PVS2 was added to replace the old one. After completion of the PVS2 treatment, cryovials containing 7–10 clumps were plunged into liquid nitrogen (LN) and cryostored for at least 1 h.

Subsequent to cryostorage, the frozen explants in cryovials were rapidly thawed by stirring in a water bath at 40°C for 80 s. After thawing, the PVS2 was removed and replaced by a deloading solution [1.2 M sucrose dissolved in MS medium (pH 5.8)] for 15 min at room temperature. Then meristem clumps were placed onto two sterile filter papers (Whatmann No. 1) on top of a semi-solid hormone-free MS medium containing 0.3 M sucrose (20 ml) in a petri dish (9 cm diameter; Greiner, Germany). After 2 days, the meristems were transferred to a liquid regeneration medium (M3 medium), i.e. MS medium (0.09 M sucrose) + 2.22 μM BA [30 ml in 100 ml Erlenmeyer flask (Borosil, India)] on a rotary shaker (LabTec, Korea) at 70 rpm and at 25°C. Control (precultured and PVS2-treated, but non-frozen) meristems were also recultured similarly. After 1 week of incubation in the dark, cultures were transferred to semi-solid regeneration medium (M3), i.e. MS medium (0.09 M sucrose) + 2.22 μM BA + 0.3% gelrite (20 ml in 9 cm diameter petri dish). All the cultures were incubated at 25 ± 2°C at 16 h photoperiod and light intensity of 40 μE per sq. m per s, provided by 40 W cool-white fluorescent tubes (Philips, India).

Regrowth (shoot regeneration and callus formation) rate was calculated as the percentage of meristematic clumps that showed survival and produced shoot buds in 4–6-week-old cultures on semi-solid M3 medium. A total of 30–50 explants for PVS2 treatment (non-frozen

Table 1. Effect of sucrose preculture duration on post-thaw regrowth of proliferating meristems of banana cv. 'Kallu Monthan' on MS + 0.4 M sucrose (culture age: 1 month)

	PVS2-treated control (non-frozen)		LN-treated	
Duration of sucrose preculture (weeks)	Shoot (%)	Callus (%)	Shoot (%)	Callus (%)
1	61.1 ± 38.5^{c}	38.9 ± 38.5	0 ± 0^{c}	23.3 ± 8.8
2	66.7 ± 8.2^{bc}	14.3 ± 0	35.3 ± 7.4^{a}	19.0 ± 15.0
3	95.8 ± 0^{a}	7.2 ± 0	18.6 ± 6.9^{b}	21.7 ± 14.9
4	72.7 ± 0^{b}	18.2 ± 0	14.5 ± 4.8^{b}	22.0 ± 9.8

Values superscripted by different alphabets in one column are significantly different for shoot regeneration according to Duncan's Multiple Range Test at $P = 0.05$.

control) and 70–150 explants for freezing in LN were used for each cultivar; experiments were repeated three times for each treatment. Thus, data on regrowth are based on a total of 90–150 control explants and 210–450 LN-treated explants. For further growth and elongation, shoot buds were transferred to MS medium (0.09 M sucrose) + 1 µM BA + 0.25% gelrite (M4 medium) for 4–6 weeks. To induce rooting, 3–5 cm long shoots were excised and cultured on MS medium (0.09 M sucrose) + 0.5% activated charcoal (M5 medium). In each cultivar, 27–30 shoots regenerated from explants after optimum duration of PVS2 treatment and 36–45 from LN-treatment were subjected to rooting at different occasions. The number of roots/shoot and root length (cm) was measured after 2 weeks on rooting medium. Rooted plantlets were hardened in plastic pots (15 cm diameter) containing a soilrite mixture of horticultural grade 1 pelrite : 1 Irish peat moss : 1 vermiculite (Kelpelrite, Bangalore, India) moistened with ¼ MS major salt solution and incubated at 25 ± 2°C. The plantlets were covered with transparent perforated polythene bags for 1–2 weeks to maintain high level of humidity (75–85%) and subsequently exposed to *ex vitro* conditions. The 3-week-old hardened plantlets were transplanted to soil in earthen pots (30 cm diameter) and allowed to grow in a greenhouse under natural light. They were subsequently transplanted in the field with a spacing of 2 × 2 m (plant to plant) for further evaluation. The plants were irrigated with water as and when required.

Results are presented as mean ± standard error. Data were subjected to analysis of variance and Duncan's Multiple Range Test (using SPSS-10 statistical package) to test significant differences.

After 4–6 weeks of cryopreservation, meristematic clumps exhibited four types of responses: (i) remained pale or white and did not show any further change, (ii) turned partially or completely black, (iii) formed non-morphogenic callus, and (iv) regenerated shoot buds/shoots. The latter two were considered as responding explants.

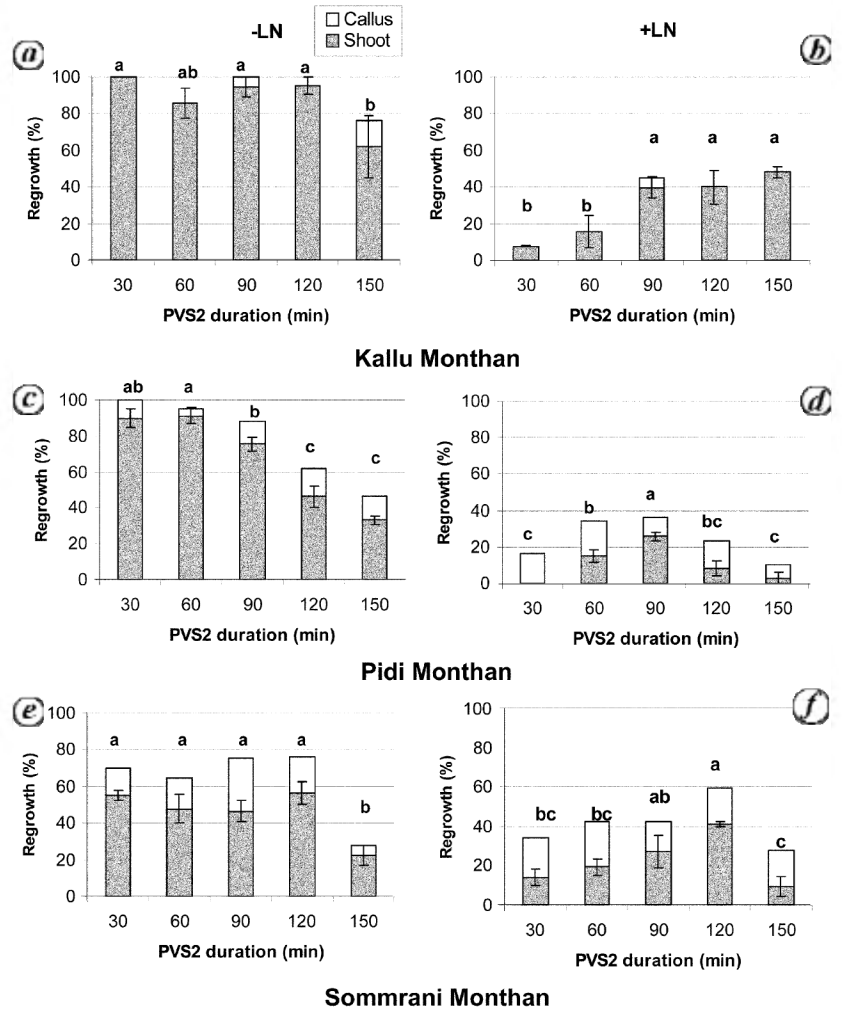

Figure 1. Regrowth (shoot regeneration and callus formation) in PVS2-treated non-frozen control (–LN) and LN-treated (+LN) meristematic clumps of banana cultivars 'Kallu Monthan', 'Pidi Monthan' and 'Sommrani Monthan'. Bars marked with different letters are significantly different for shoot regeneration using DMRT at $P = 0.05$. *Y*-bars represent standard error for shoot regeneration.

Table 2. Hardening and survival of PVS2-treated (control, at optimum PVS2 treatment) and LN-treated (cryopreserved) plants of three cultivars of Indian cooking banana (ABB)

Cultivar	Treatment (duration)	No. of plantlets hardened	Survival of plants in potted soil and field (% mean ± SE)
'Kallu Monthan'	PVS2 (150 min)	30	93.6 ± 3.2
	LN (1 h)	36	86.1 ± 2.8
'Pidi Monthan'	PVS2 (90 min)	33	90.9 ± 0.49
	LN (1 h)	30	88.1 ± 2.4
'Sommrani Monthan'	PVS2 (120 min)	27	96.3 ± 3.7
	LN (1 h)	45	90.6 ± 5.8

Results of optimum duration of sucrose preculture treatment in cv. 'Kallu Monthan' are shown in Table 1. For LN-treated explants, it was found that 2-week preculture on M1 medium (0.4 M sucrose) was optimum and gave significantly higher post-thaw shoot regeneration (35.3%). Preculture of the cauliflower-like meristems of banana on sucrose has been shown to be essential for the acquisition of tolerance towards the severe dehydration associated with cryopreservation[5,6]. Sucrose imparts tolerance to desiccation and freezing in plant tissues, by preserving the membrane integrity and protein structure[7]. The concentration of sucrose at 0.4–0.5 M in the preculture medium for 2–4 weeks has been reported to be effective for high rate of post-thaw survival (42.5%) in cv. 'Bluggoe' (ABB subgroup)[5]. Our results in terms of post-thaw survival and shoot regeneration (35.3%) in cv. 'Kallu Monthan' are comparable after preculture treatment of 0.4 M sucrose for 2 weeks. Hence, in all the subsequent experiments, meristematic clumps of cultivars 'Pidi Monthan' and 'Sommarani Monthan' were precultured on M1 medium with 0.4 M sucrose for 2 weeks.

For optimization of duration of PVS2 treatment, post-thaw survival and shoot regeneration for cultivars 'Kallu Monthan', 'Pidi Monthan' and 'Sommrani Monthan' are shown in Figure 1. In 'Kallu Monthan', shoot recovery in PVS2-treated non-frozen (–LN) explants ranged from 85.7% to 100%, when PVS2 treatment was given for 30–120 min (Figure 1 *a*). In the LN-treated (+LN) explants, 7.7% and 15.4% shoot regrowth was obtained in explants exposed to PVS2 for 30 and 60 min respectively. With increased duration of PVS2 treatment (90–150 min), shoot regeneration was significantly increased to 39.7% and 48.1% (Figure 1 *b*). It is important to note that, in general, callus formation was absent in all the treatments. In 'Pidi Monthan', –LN explants exhibited shoot regeneration in 75.4–91.1% cultures with the treatment of PVS2 for 30–90 min (Figure 1 *c*). In the +LN explants, at 60 min duration of PVS2 treatment, 15.3% shoot regeneration was achieved, which significantly increased to 25.7% at 90 min treatment of PVS2 (Figure 1 *d*). In 'Sommrani Monthan', shoot regeneration in –LN explants ranged from 46.4% to 56.5%, when PVS2 treatment was given for 30–120 min (Figure 1 *e*). In the +LN explants, highest shoot regeneration (41%) was obtained in cultures treated with PVS2 for 120 min. Unlike in cv. 'Kallu Monthan', increased duration of PVS2 treatment (150 min) was not beneficial.

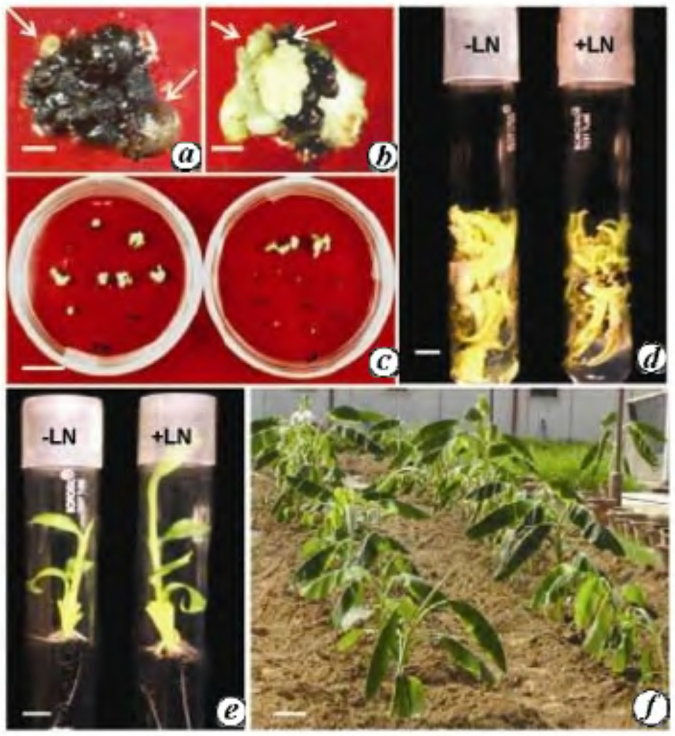

Figure 2. Cryopreservation of meristematic clumps of banana cv. 'Sommrani Monthan'. ***a***, Four-week-old explant culture showing post-thaw callus regeneration on M3 medium as indicated by arrows (bar = 1 mm). ***b***, Four-week-old explant culture showing post-thaw shoot bud regeneration on M3 medium as indicated by arrows (bar = 1 mm). ***c***, Ten-week-old cultures showing shoot regeneration from –LN and +LN explants after 90 min of PVS2 treatment (bar = 20 mm). ***d***, Fourteen-week-old cultures showing shoot development in –LN and +LN explants on M4 medium (bar = 10 mm). ***e***, Rooting in individual shoots (after 2 weeks) on M5 medium (bar = 10 mm). ***f***, Partial view of plants (6-month-old) in field (bar = 30 cm).

The effects of duration of PVS2 exposure on banana have also been investigated[7], but the explants used were single meristem (not meristem clumps) excised from rooted *in vitro* plantlets and the method for cryopreservation was droplet vitrification (not vitrification). The study[7] reported that 30 min PVS2 treatment was in general good for banana, but also found that genotype-specific responses and longer PVS2 duration treatments (50–60 min) were better for

accessions containing one or two B genomes (ABB, AAB and AAB plantains), as some relation is established with the B genome which tends to be more drought-tolerant than those with only the A genome[13]. It indicates that PVS2 exposure time is critical and not only genotype-specific but also explant- and method-specific (employed for cryopreservation). Also, droplet vitrification method using single meristem (isolation of uninjured single meristem from monocot is tedious and time-consuming) is not found to be user-friendly for cryopreservation of bulk banana germplasm[8].

Figure 2 *a–d* shows the different sequential stages of conversion of cryopreserved meristematic clumps into shoots. Shoots regenerated from +LN explants attained 2–3 cm height after 2–3 weeks compared to 3–5 cm of those regenerated from –LN explants. Irrespective of the cultivars and treatments, when 3–5 cm long shoots were transferred on M5 medium, 100% cultures regenerated 3–5 roots/shoot with a length 3–4 cm within 2 weeks on the rooting medium (Figure 2 *e*). No morphological difference was observed between the plantlets obtained from either –LN and +LN explants. Considering all cultivars and treatments together, 86–96% (non-significantly different) rooted plantlets survived hardening treatment and all the hardened plants survived in potted soil and field (Table 2) and grew normally (Figure 2 *f*).

The above described cryopreservation method was effective, practical and user-friendly for banana cultivars of subgroup Monthan. With the advantages of easy operation, less expenditure on laboratory equipment and a short processing time, this method will be useful for long-term conservation of banana germplasm. Using this method banana germplasm is being cryopreserved and till date, ten cultivars of banana (AB, AAB and ABB) have been cryopreserved at NBPGR. The cryopreserved plants have also been transplanted in the field for further evaluation.

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Effect of different number of coatings on gloss and in controlling moisture entry into mango wood

Very often, even with properly seasoned wood, dimensional instability is encountered due to possible moisture exchange between the product and the surroundings, wherein the usual suspect is the coating finish that is applied. Each coating material differs in its capacity to block the movement of moisture in and out of the product[1]. This is compounded by the lack of understanding regarding the amount of coating or the number of coats that needs to be applied. Usually, moisture excluding effectiveness (MEE) or water uptake coefficient (WUC) is determined to assess the ability of a coating to inhibit moisture penetration or escape to/from the wood. 'Seepage' or 'breathing' of moisture through the protective film and osmotic pressure of moisture in wood against the film cause adverse effects like reduction in gloss, cracking, blushing, haziness, blistering or peeling of the surface coating and the movement in wood, resulting in swelling and shrinkage[2]. This transport of moisture through the coating is determined either by steady-state methods, wherein the amount of moisture transported through a defined area in a specified time is measured or by sorption/desorption methods, where the change in weight of a test sample when the surrounding conditions change is measured. A mathematical treatment of these methods is discussed by Crank[3]. Feist *et al.*[4] have also discussed in detail about the determination of MEE, which has limitations due to the domination of wood characteristics rather than the finishing coat in the calculations. The water uptake method, on the other hand, estimates the actual amount of moisture intake by a defined weight of wood material in a given time.

The appearance of a wooden product is of prime importance as far as marketing is