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CRYOPRESERVATION OF *IN VITRO* GROWN SHOOTS OF GINGER (*Zingiber officinale* Rosc.)

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Abstract

An efficient cryopreservation technique for *in vitro* grown shoots of ginger (*Zingiber officinale* Rosc) was developed based on encapsulation dehydration, encapsulation vitrification and vitrification procedures. Pregrowth and serial preculture were needed to obtain the best regrowth for all techniques. The vitrification procedure resulted in higher regrowth (80%) when compared to encapsulation vitrification (66%) and encapsulation dehydration (41%). In the vitrification procedure shoots were: precultured in liquid Murashige-Skoog medium containing 0.3 M sucrose for 3 days; cryoprotected with a mixture of 5% DMSO and 5% glycerol for 20 min at room temperature; osmoprotected with a mixture of 2 M glycerol and 0.4 M sucrose for 20 min at 25ºC; before being dehydrated with a highly concentrated vitrification solution (PVS2) for 40 min at 25ºC. The dehydrated shoots were transferred to 2 ml cryotubes, suspended in 1 ml PVS2 and plunged directly into liquid nitrogen. In all the three cryopreservation procedures tested, shoots grew from cryopreserved shoot tips without intermediary callus formation. The genetic stability of cryopreserved ginger shoot buds were confirmed using ISSR and RAPD profiling.

Keywords: conservation, cryopreservation, dehydration, encapsulation, genetic stability, germplasm, ginger, ISSR, RAPD, vitrification.

Abbreviations: BA, 6 benzyl adenine; NAA, naphthalene acetic acid; LS, loading solution; DMSO, dimethyl sulfoxide; LN, liquid nitrogen; MS, Murashige and Skoog medium; PVS2, plant vitrification solution 2; En Vi, encapsulation vitrification; En De, encapsulation dehydration; RAPD, random amplified polymorphic DNA; ISSR, inter simple sequence repeat.

INTRODUCTION

Ginger (*Zingiber officinale* Rosc.) belongs to the family *Zingiberaceae* of the order *Zingiberales* (*Scitamineae*). It is not known to occur in a truly wild state. It is believed to have originated in Southeast Asia, and is distributed in tropical and subtropical Asia and Far East Asia. It has been cultivated from time immemorial in India and China. Ginger is used as an ingredient in many spice mixes. It is also used in the preparation of gingerbread, biscuits, cakes puddings, soups, pickles, ginger beer, wine, and is an important ingredient in the traditional Indian, Chinese and Japanese systems of medicine. Ginger is believed to have carminative and digestive properties and is used to treat anorexia and dyspepsia and to suppress inflammation (32). Over 800 accessions of ginger germplasm are available at the National Conservatory for Ginger (32) at the Indian Institute of Spices Research (IISR). The major constraints involved in the conservation of the germplasm of ginger are two soil-borne

diseases: rhizome rot, caused by *Pythium* spp. (*P. aphanidermatum, P. myriotylum and P. vexans*); and bacterial wilt, caused by *Ralstonia solanacearum* (*Pseudomonas solanacearum*). Added to this, infection by leaf fleck virus also poses serious problems for conservation. These diseases are extremely difficult to control under field conditions. Hence, at IISR, ginger germplasm is conserved in specially made cement tubs under 50 % shade, as a nucleus gene bank to safeguard the material from deadly diseases and to maintain the purity of germplasm. *In vitro* conservation of ginger germplsm is a safe and complementary strategy to protect genetic resources from epidemic diseases and other natural disasters. This is an excellent method to supplement the conventional conservation strategies. Conservation of ginger germplasm under *in vitro* conditions by slow growth is standardized at IISR (13) and over 100 unique accessions are held in medium-term *in vitro* storage (13, 28, 32). The possibility of storage at relatively high ambient temperatures (24-29ºC), by subjecting the ginger and related taxa to stress factors, has been explored (6).

In vitro preservation of tissue and organs can constitute a complementary approach to genotype conservation that overcomes some of the problems associated with field collections. In fact, the application of tissue culture technology to the preservation of plant genetic resources has greatly evolved in recent years. However, the maintenance of large collections in conventional *in vitro* storage systems requires continual, expensive manipulation of tissue, as most cultures need sub culturing at regular intervals to prevent loss of viability. In addition, the risks of contamination and somaclonal variation increase with time (20). Even under slow growth conditions, *in vitro* conservation of ginger requires subculturing at 12 month intervals, and can ensure only medium-term storage (13).

Cryopreservation is a strategy for long-term conservation of germplasm (32) and its use for vegetatively propagated species is being explored (33). Storage of clonal materials in liquid nitrogen (LN) as a base collection is the goal of many gene banks (34), but for efficiency and reliability each species requires the development of an appropriate cryopreservation protocol. The most relevant strategy for the long term conservation of vegetatively propagated species is the cryopreservation of shoot tips, a genetically stable plant material. For ginger, only recently has it been reported that shoot cryopreservation through encapsulation dehydration had a success level of 40-50% (13). A simple, efficient and effective cryopreservation protocol would facilitate long-term storage and much wider utilization of plant germplasm (4). Encapsulation dehydration (En De) (12), encapsulation-vitrification (En Vi) (38) and vitrification (37) are among the newly developed techniques for cryopreservation of plant germplasm (10). Here we report the successful cryopreservation of *in vitro* grown shoots of ginger (*Zingiber officinale* Rosc.) based on these three techniques and the genetic stability of cryopreserved plants using RAPD and ISSR markers.

MATERIALS AND METHODS

Plant material

In all the experiments shoot buds (0.5-1.0 mm in length) of ginger, consisting of the apical dome with 3-4 leaf primordia, were used. *In vitro* stock cultures of ginger cv Maran were maintained on MS medium (26) that contained 3% sucrose and 7% (w/v) (Hi Media) agar at pH 5.8 with 1 mg l^{-1} BA and 0.5 mg l^{-1} NAA (proliferation medium). Sub-culturing was performed every 4 weeks and cultures were incubated under a 16 h photoperiod provided by cool white fluorescent light (50-60 µmol m^{-2} s^{-1}) and at 25ºC. These culture conditions were maintained in all experiments unless otherwise stated.

Cryopreservation procedures

Encapsulation dehydration procedure: Ginger shoots consisting of the apical dome with 3-4 leaf primordia were pipetted with 4% sodium alginate solution and dispensed drop wise in liquid MS medium containing 0.1 M calcium chloride. After encapsulation, beads (each

containing one or two shoots) were left for 30 min in the calcium chloride solution to ensure polymerization of the calcium alginate. The beads were then subsequently precultured in liquid MS medium containing 0.1, 0.3, 0.5, 0.7, 0.9 and 1.0 M sucrose for 24 h each. The precultured beads were placed on sterile filter paper in Petri dishes (90 mm) and dehydrated in a laminar flow cabinet (at room temperature and humidity) for periods of 0-10 h to determine the optimal dehydration time. For each dehydration time, half of the beads were then transferred to proliferation medium and the other half were placed in 2 ml cryotubes (15 beads to a vial), which were immediately plunged into liquid nitrogen and kept for 24 h. The beads of a control group that had received no sucrose or dehydration treatment were similarly dealt with; half being cooled and the other half left uncooled. The water content of the beads was determined as follows: total fresh weight of 15 beads with three replicates, was measured after sucrose preculture and during the dehydration period (0-10 h). After the final period of dehydration, the beads were oven-dried at 85ºC for 24 h to determine the dry weight. Bead water content on each period was calculated from these values and expressed as the percentage of water weight over fresh weight.

Encapsulation vitrification procedure: Shoots were encapsulated following the above procedure and precultured in 0.1 M sucrose for 24 h. and osmoprotected with 0.3 M sucrose for 16 h, followed by treatment with 2 M glycerol with sucrose (0.4 M, 0.6 M, 0.8 M, 1.0 M, 1.2 M, 1.4 M 1.6 M or 1.8 M) for 3 hours on a shaker at 60 rpm at 25ºC. The beads were then placed in a 2 ml cryotube and then osmotically dehydrated with 1.5 ml PVS2 at 25 ºC for 40 min. The encapsulated beads in the cryotube were suspended in 1.0 ml fresh PVS2 [15% (w/v) DMSO, 15% (w/v) ethylene glycol and 30% (w/v) glycerol] in liquid MS medium with 0.4 M sucrose solution (37) prior to plunging into LN and storing for 24 h.

Vitrification procedure: Naked shoots (0.5-1.0 mm) were precultured in 0.3 M sucrose for 72 h and then immersed for 20 min at room temperature in five cryoprotectant mixtures containing: 5% DMSO; 10% DMSO; 5% glycerol; 10% glycerol; or 5% DMSO + 5% glycerol. Further, the shoots were treated with loading solution (2 M glycerol + 0.4 M sucrose) for 20 min at 25ºC. Naked, precultured and osmoprotected shoots were then dehydrated in 20 ml PVS2 in a 50 ml Erlenmeyer flask on a rotary shaker (60 rpm) at 25 ºC for various periods of time (0-60 min). After dehydration shoots were suspended in 1 ml of PVS2 in cryotubes and plunged into LN where they were kept for 24 h. Control replicates were transferred directly to MS + BA (1 mg l^{-1}) and NAA (0.5 mg l^{-1}) and placed in the culture room as described in *in vitro* culture.

Warming and regrowth

Rewarming was performed by rapidly immersing the cryotubes in a waterbath at 40ºC for 1 min for all three treatments. For encapsulation vitrification and vitrification treated samples, PVS2 was drained from the cryotubes and replaced twice at 10 minute intervals with 1.2 M sucrose solution (dilution solution). The rewarmed explants were then placed directly on MS + BA (1 mg l^{-1}) and NAA (0.5 mg l^{-1}) medium in Petri dishes and maintained at the same culture conditions as stock explants. Treated controls were processed in the same way except that they were not stored in liquid nitrogen.

Viability and plant growth

Shoot formation was expressed as a percentage of the total number of shoots forming normal shoots 8 weeks after plating. Fifteen shoots were used in each experiment with three replicates.

Genetic stability analysis

RAPD (42) and ISSR (44) profiling of was done to test the genetic stability of recovered plants after cryopreservation. DNA was isolated from control and randomly selected cryopreserved plants (2). Molecular profiling was done using 6 random operon primers (Operon Technologies, Alameda, CA, USA) and 2 ISSR primers (X,Y), IS 1 [$(CT)_7$ TG] and IS 8 [$(GA)_6$ GG] (Genei, Bangalore, India), which were previously identified to detect polymorphism in ginger. All PCR reagents were obtained from Genei, Bangalore, India and the reactions were performed in a PTC-200 DNA engine (MJ Research, USA). The PCR reaction was done in a final volume of 25 µl containing 30-40 ng of genomic DNA, 30 p moles of primer, 150 µM of dNTPs, 2 mM $MgCl_2$ and 1U of Taq DNA polymerase. The PCR was performed at 95ºC for 2 min, then 30 cycles for 95ºC for 1 min, 40^{o}C for 1 min and 72ºC for 1 min followed by a final extension step of 72^{o}C for 15 min.

Statistical analysis

Eight weeks after the shoots were planted on recovery medium their response was assessed in terms of the percentages of buds that had grown into shoots. Each experiment was repeated at least twice and consisted of three replicates per treatment, with 15 samples in each replicate. Results were analyzed by analysis of variance (ANOVA), following arcsin transformation, with the least significant difference (LSD) or Duncan's multiple range tests (DMRT), using the SPSS software.

RESULTS

Cryopreservation by encapsulation-dehydration

In an initial experiment only encapsulation was used without preculture. For shoots dried to different moisture content ranging from 80-20% (control), no survival was observed after cryopreservation. While in the absolute control, where the cryopreservation step was not used, regrowth of shoot tips ranged from 77 to 97 %.

The regrowth of both non-cryopreserved (-LN) and cryopreserved (+LN) shoots was greatly influenced by the water content of the precultured beads. The initial water content of the control beads was high (97%), but was reduced to 88% by 6 days of culture on increasingly sucrose-rich medium, and to 21% by 6 h of subsequent dehydration in the laminar air flow cabinet. The best results in terms of cryopreserved shoot recovery (around 41%) were obtained by progressively increasing sucrose level together with 6 h of dehydration (Fig.1). No survival after cryopreservation was observed for control shoots dried to different moisture content.

Encapsulation vitrification

To enhance the osmotolerance of ginger shoots in the vitrification solution, the effects of pre incubation with 0.1 M sucrose for 24 h after encapsulation, followed by preculture with 0.3 M sucrose for 16 h were studied. Pre-incubation alone and preculture without pre-incubation gave very low recovery levels. Thus, pre-incubation followed by preculture was an important step for the successful cryopreservation of ginger by encapsulation vitrification (Table 1).

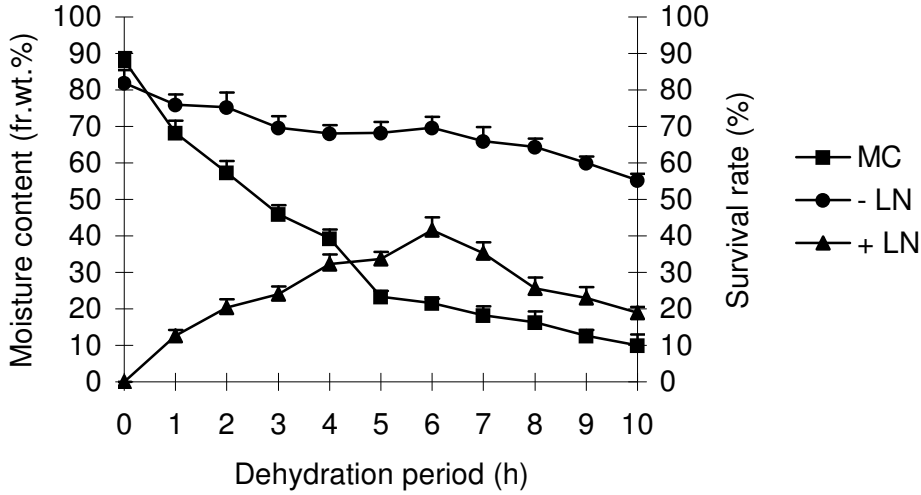

Figure 1. Effect of moisture content (MC; % fresh weight) on recovery of encapsulated ginger shoots without (-LN) and with (+LN) cryopreservation. Shoots were subjected to 6 days of preculture on increasing sucrose concentration medium and then to various desiccation periods, before and after rapid cooling in liquid nitrogen. Assessment was made after 2 months of treatment. Fifteen encapsulated shoots were treated in each of three replicates. Bars indicate the standard errors. No survival after cryopreservation was observed for control shoots dried to different moisture content and the data is not presented.

Table 1. Effect of a 1-day pre-incubation in 0.1M sucrose for 24 h and preculture in 0.3 M sucrose for 16 h on percentage shoot formation from encapsulated vitrified ginger shoots. The beads were then osmoprotected with 2 M glycerol and 1.6 M sucrose for 3 h at 25℃ and then dehydrated with PVS2. Fifteen shoots were treated in each of three replicates.

Pre-incubation	Preculture	Shoot formation (%) ± SE
+	+	81.4 ± 0.44
+	-	23.8 ± 0.49
-	+	31.4 ± 0.34
-	-	0 ± 0

The highest percentage of shoot formation (66%) (Figure 2) was obtained when precultured shoots were treated with 2 M glycerol and 1.6 M sucrose for 3 h at 25ºC, resulting in elongated shoots following cryopreservation when post-cultured for one month (Fig. 6a-c). Pretreatment with sucrose solution alone produced less than 24 % shoot formation.

Vitrification

Cryopreserved ginger shoots turned light brown within one day of warming, but upon culture on recovery medium surviving buds turned green within 10 days. Successfully recovered buds resumed growth within 2-3 weeks. The first sign of shoot growth was evident by leaf development (4 weeks after cryopreservation) and was followed by shoot development without intermediate callus formation (Fig. 5 a-f).

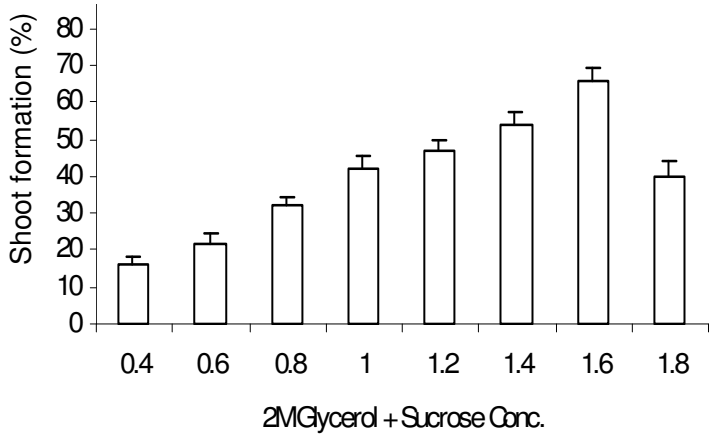

Figure 2. Percentage of shoot formation after 8 weeks of culture for encapsulated-vitrified shoots of ginger treated with different sucrose concentrations. Encapsulated, pre-incubated and precultured shoots were treated with 2 M glycerol + sucrose at 25°C for 3 h before being dehydrated with PVS2 for 40 min at 25ºC. Fifteen shoots were treated in each of three replicates. Bar indicate standard errors.

In the vitrification protocol, an optimum dehydration with vitrification solution (PVS2) is the key step for the production of high levels of recovery and growth after cryopreservation. Properly dehydrated shoot tips treated with PVS2 solution showed less injury during dehydration, increased vitrification and thereby avoided the risk of intracellular freezing upon rapid cooling with LN. To optimize the exposure time to PVS2 solution, the excised shoots were treated with PVS2 for 0-60 min at 25°C prior to plunging into LN (Fig. 3). Shoot formation depended upon the length of the exposure to the PVS2 solution. The highest shoot formation (about 95%) was obtained when the shoots were dehydrated with PVS2 for 40 min at 25ºC before immersion into LN.

Vitrification with a cryoprotectant mix containing 5 % DMSO + 5 % glycerol resulted in 80 % post thaw recovery (Fig. 4).

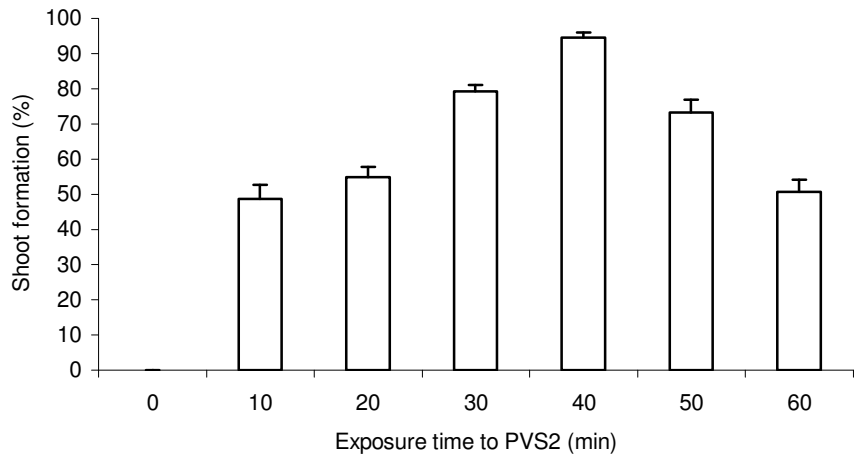

Figure 3. Effect of exposure time to PVS2 solution at 25ºC on ginger shoots cooled to -196^0 C by vitrification. Shoots were dehydrated with PVS2 solution before immersion into LN. Approximately 15 shoots were tested in each of three replicates. Bar indicates standard error. Data taken at 8 weeks

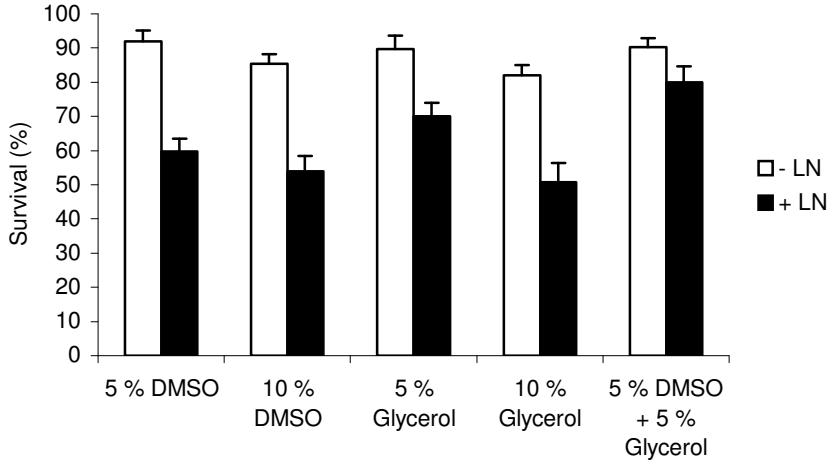

Figure 4. Effects of preculture and cryoprotectants on regeneration of shoots after exposure to liquid nitrogen (+LN) and without (-LN) during the vitrification procedure. Approximately 15 shoots were tested for each of three replicates. Data taken after 8 weeks of culture.

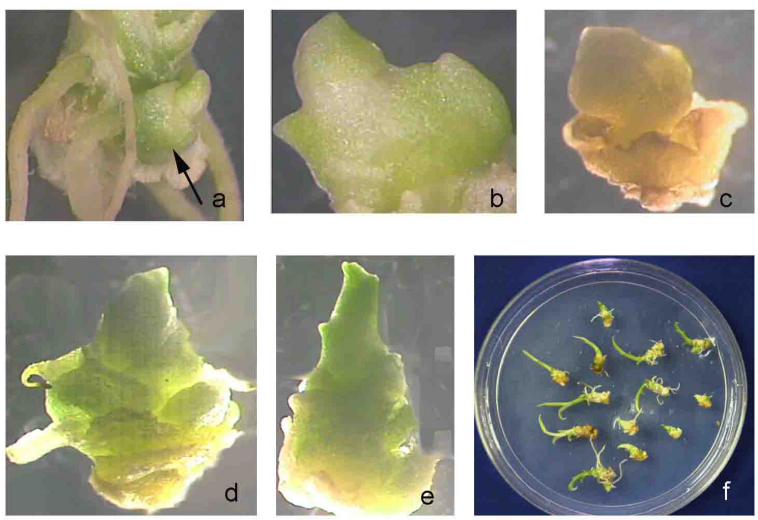

Fig. 5 a-f) Shoot development from vitrified shoot buds of ginger cooled in LN a) Bud of ginger (arrow) from which explant were obtained. b) Excised shoot bud c) 3 days after recovery. d) 14 days after recovery. e) One month after recovery. f) Two months after recovery.

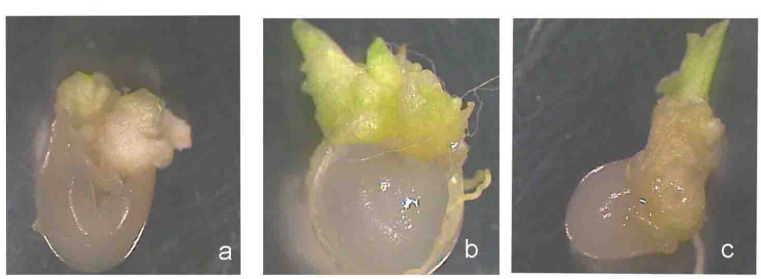

Fig. 6 a-c) Plant regeneration from cryopreserved shoot buds of ginger by encapsulation vitrification. a) Surviving shoot bud 7 days after post culture. b) Elongated shoot with no intermediary callus formation 20 days after post culture. c) One month after post culture.

Genetic stability analysis

The genetic fidelity of the cryopreserved buds as well as the controls were tested by comparing RAPD and ISSR profiles. No variation in the RAPD and ISSR profiles within the cryopreserved and control plants and among storage treatments were observed (Fig 7).

DISCUSSION

Cryopreservation is a strategy for long-term conservation of germplasm (32, 43) more so in ginger where there is no seed set. There is only one preliminary report, from this laboratory, on the cryopreservation of ginger, which reported a success level of 40-50% after encapsulation dehydration of shoots (13). In the present study an efficient cryopreservation technique for *in vitro* grown shoots of ginger was developed with 80% success, based on vitrification procedure. The other two procedures applied, encapsulation dehydration and encapsulation vitrification, gave lower percentages of success. Pregrowth and serial preculture were needed to obtain the best regrowth for all the techniques.

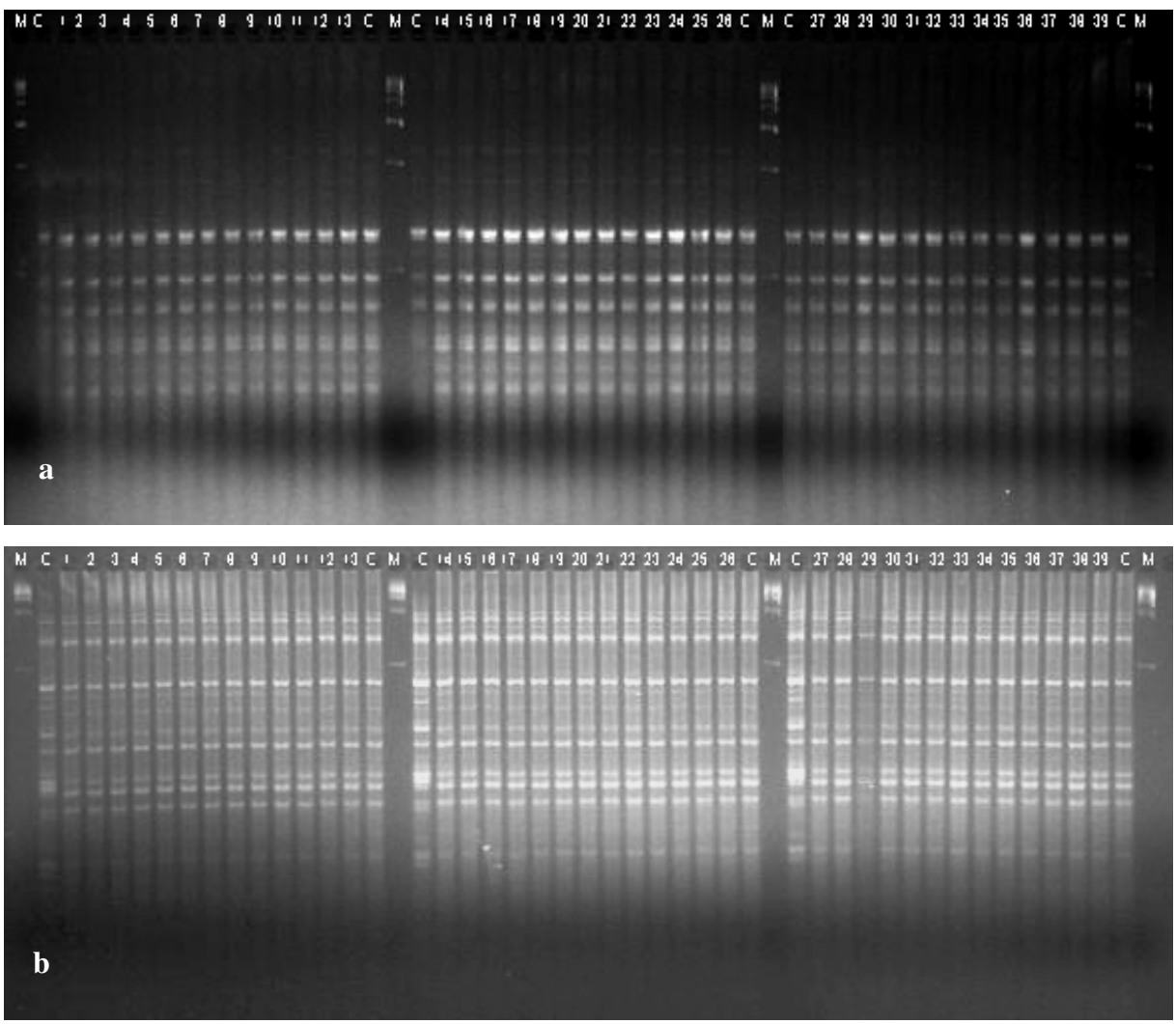

Fig. 7. ISSR (a) and RAPD (b) banding profiles [primer sequence $(CT)_7$ TG-ISSR,OPF-01-RAPD] of DNA samples extracted from cryopreserved plants of ginger M-1 Kb ladder, C-control, 1-13 Encapsulated and dehydrated samples, 14-26 Encapsulated and vitrified samples, 27-39 Vitrified samples.

Cryopreservation by encapsulation dehydration

For some plant species which are sensitive to high sugar concentration, it is necessary to gradually increase sucrose concentration in the preculture medium to enhance shoot tip survival of cryopreservation by encapsulation dehydration (16). Besides its osmotic and colligative effects, it is known that sugars are competitive protein and membrane stabilizers (5). In addition to physical changes, sugar treatments can also induce physiological and metabolic changes leading to cryoprotection. These include alterations in proteins (17, 39), membrane fatty acids (41) and amino acids (7). In ginger, no survival was observed when non-desiccated, precultured explants were frozen in liquid nitrogen. On the other hand, adding a desiccation step to the protocol, removed a significant amount of crystallisable water from the shoot tissue and this facilitated survival. It has been generally observed that bead moisture contents between 15 - 25 % are required for optimal survival after cryopreservation (11), which was also the case in this study. The best survival of encapsulated ginger shoot buds was obtained at 21% bead moisture content. Alginate with adequate preconditioning protects shoot tips from injury during dehydration and cooling as well as during warming (8, 14). The results also indicate that preculture duration and progressive increase of sucrose concentration and optimal bead moisture content are critical for cryopreservation of ginger shoots. Dehydration longer than 10 h resulted in reduction of bead moisture content to less than 10 %, and very low or no recovery. Thus the encapsulation and dehydration method, though easier to use, resulted in low post thaw recovery levels compared to the other two cryopreservation methods.

Cryopreservation by encapsulation vitrification

In the present study, to determine the most effective osmoprotection for the encapsulation vitrification method, encapsulated, preincubated and precultured ginger shoots were treated with varying concentrations of sucrose with glycerol. It was found that a higher concentration of sucrose (1.6 M in 2 M glycerol) and a longer period of incubation (3 h) were necessary to increase the osmotolerance of the shoot tips.

The protective effect of osmoprotection might be due to osmotic dehydration, resulting in the concentration of cytosolic stress responsive solutes that were accumulated during preculture with sucrose, or sorbitol enriched medium (35). During incubation in loading solution for 3 h, meristematic cells become plasmolyzed, producing concentrated spherical protoplasts (36). Plasmolysis might mitigate the mechanical stress incurred during severe dehydration (18). In addition to this, sucrose employed during preculture has an osmotic effect by dehydrating encapsulated samples (9, 30, 31, 37). Sucrose is also absorbed by tissues during preculture, resulting in an increase in cytosolic stress responsive intracellular solutes (9, 37). The combination of all these effects reduces, or even suppresses, intracellular ice crystallization during freezing, which is correlated with an increase in survival of cryopreserved samples (9). The recovery level of ginger shoots using encapsulation vitrification (66%) was higher than that of the earlier reported success rate and also higher than the above mentioned encapsulation dehydration method.

Cryopreservation by vitrification

In the vitrification method, shoot tips were exposed to PVS2. Several studies have shown that exposure of less tolerant cells and shoot tips directly to the vitrification solution results in harmful effects due to osmotic stress and or chemical toxicity (1, 21, 25, 29, 40). These harmful effects can be alleviated by adequate preconditioning, such as cryoprotective loading (23) and modification of the typical one step vitrification procedure (i.e., treatment with the PVS2 solution followed by direct plunging into LN). In the present study, a three step vitrification methodology (cryoprotective loading / loading solution / PVS2) was

successful in cryopreserving ginger shoots such that they could be recovered as plants. The preconditioned and cryoprotected shoots were exposed to 2 M glycerol and 0.4 M sucrose for 20 min at 25ºC before being dehydrated with PVS2 for 40 min at 25ºC. Regrowth occurred after vitrification in all treatments applied, ranging from 51% to 80%. Shoots cryoprotected with a mixture of 5% DMSO and 5% glycerol gave the highest regrowth of 80% after vitrification. This is probably due to the positive effects of applying cryoprotective compounds as a mix, leading to more extensive dehydration and penetration of cryoprotective substances in the cells, which in turn avoids the risks of osmotic injuries during vitrification.

No shoots developed on cryopreserved shoots that were treated only with loading solution, but its omission from the pretreatment of shoot apices treated for 40 min with PVS2 resulted in a significant lowering of post-cryostorage recovery. The osmoprotection produced by the loading solution may be explained by the mitigation of injurious effects during the dehydration process with PVS2 by decreasing osmostress, and stabilizing membranes (18).

The PVS2 application time, affording optimal trade off between its toxic and cryoprotective effects on shoot tips, varies widely among different species. Maximum shoot formation levels have been reported following treatment times of 20 min for black currant (3) and persimmon (24), 60 min for poplar (22), and 90 min for mulberry (27) and tea (19). In the present study, maximum post thaw recovery was obtained when the preconditioned shoots were treated with PVS2 at 25ºC for 40 min.

Shoot apices are considered to be the optimal material for long-term preservation because they are genetically stable, capable of regeneration into complete plants without a callus phase and are tolerant to many methods of dehydration (15). In the present study a three step vitrification methodology (cryoprotective loading / loading solution / PVS2) was the most successful in cryopreservation of ginger shoots and recovery of plants.

The genetically stability of shoot apices was confirmed by molecular profiling. The RAPD and ISSR assays performed suggested that no genetic aberrations originated in ginger plants during culture and cryopreservation. In conclusion, these results show that the three step vitrification procedure can be successfully applied for cryopreservation and recovery of genetically stable ginger shoots and thus for the conservation of ginger germplasm.

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