CryoLetters **33** (1), 58-68 (2012)

VITRIFICATION-BASED CRYOPRESERVATION OF SHOOT-TIPS OF *Pinus kesiya* ROYLE EX. GORD.

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Abstract

The present investigation was aimed at developing a protocol for long-term preservation of germplasm of *Pinus kesiya* Royle ex. Gord. through vitrification. Some of the critical components affecting explant tolerance to cryopreservation, such as effects of preculture, vitrification solutions, exposure time to vitrification solutions, volume of vitrification solution and its toxicity, washing of vitrified tissues after thawing, were analysed. The results showed that shoot regrowth of *P. kesiya* shoot-tips was considerably affected when exposed to cryoprotectants for longer periods of time (> 10 min). Among different vitrification solutions studied, maximum survival (76%) of shoot-tips was achieved with mVSL (using 0.6 ml of the solution) in MS basal medium containing 4.0 mg l^{-1} N^6-benzyladenine (BA).

Keywords: regrowth, thawing, germplasm, LN, *Pinus kesiya*, BA- N^6-benzyladenine.

INTRODUCTION

Germplasm is the source of variability of plants which provides the building blocks in agriculture, horticulture and forestry. Preservation of germplasm is essential for the maintenance of biodiversity and for averting genetic erosion. Cryopreservation is a means of long-term preservation of germplasm. The slow freezing technique of cryopreservation was the most preferred method in the past, but has largely been replaced by the techniques allowing direct immersion of explants in liquid nitrogen (LN); vitrification is one such promising method. The vitrification method enables tissues to cool to a temperature of LN without ice crystal formation. Vitrification as a possible technique of cryopreservation was first advocated by Luyet (13). In the beginning, vitrification was demonstrated as a practical method for cryopreservation of mouse embryos and human monocytes (19, 29). Later on, vitrification was found to be equally effective for plant species also. Several vitrification solutions have been developed until now to improve the regrowth of vitrified tissues. Among them, plant vitrification solution 2 (PVS2), developed by Sakai *et al.* (23), in conjunction with appropriate preculturing and/or pre-incubation has been most frequently used for the vitrification of plant shoot-tips (9, 10, 11, 14, 21, 23, 32). PVS2 was also found to be useful in encapsulation-vitrification and droplet-vitrification protocols (18, 22). Vitrification methods tend to allow the entire dome of a shoot apex to survive cryopreservation, assist in evading

callus formation and somaclonal variation during regrowth (34). Many unsuccessful cases still exist with tissues and species where limited survival following vitrification has been achieved. Difficulties concerned with vitrification methods are mostly the toxicity and high osmolarity (ca. 8 M) of the vitrification solutions which often narrow optimum incubation periods (28). Poor survivability percentage of tissues in a majority of the cases following vitrification-based cryopreservation are ascribed either to insufficient osmotic tolerance of the specimen, toxicity of vitrification solutions, insufficient penetration of vitrification solutions into the tissues, or inadequate dehydration of the explants. Until now a limited effort has been made to improve vitrification solutions like PVS2 (33) or vitrification solution L (VSL) (28). Development of newer vitrification solutions with low toxicity and high survivability, enhanced penetration or rapid dehydration of explants may improve plant cryopreservation methods.

Pinus kesiya Royle ex. Gord. (Khasi pine) is an important timber yielding conifer of higher altitudes of Northeast India. It has high potential value as producer of high quality resin and its products. Khasi pine is mainly exploited for its timber for making furniture, plywood, planks and construction of houses. Indiscriminate exploitation of this tree species have dwindled its number in nature. To meet the regional timber demand, this pine species is used in afforestation as well as in industrial plantations using conventional propagation methods like cuttings and seeds. The seeds of *P. kesiya* can be stored for a considerable period of time (5-6 yrs) in the refrigerator (at 0-4°C) with a little loss of viability, but propagation of pines from seeds is not reliable in clonal forestry because of genetic variability. Superior genotypes with elite characters like straighter bole, optimal stem form and uniformity, rapid growth rate and high production index (stem: total tree biomass) are crucial for successful plantation and clonal forestry programmes. Clonal propagation provides an excellent method as the importance of clonal forestry is that it offers large genetic gains with desired characters from selected superior individuals than is possible using sexual offspring (15). Thus there is a need to develop a long-term conservation protocol for superior *clonal-germplasm* of this species. Vitrification-based cryopreservation can be an excellent one-step rapid method of preservation for this pine species. Therefore, in the present study, we made a maiden attempt to develop a suitable method for cryopreservation of *in vitro* grown shoot-tips of *P. kesiya* through vitrification. The objective of the study was to identify a suitable vitrification solution and optimize the conditions for successful cryopreservation of *P. kesiya* shoot-tips which can be utilised for future clonal forestry programmes.

MATERIALS AND METHODS

Plant material

Mature seeds from *Pk-04* genotype of *P. kesiya* as used in our laboratory for somatic embryogenesis work (1) were germinated aseptically on half-strength MS [Murashige and Skoog (16)] basal medium containing 15 g^{-1} sucrose, 8 g^{-1} agar with pH adjusted to 5.8. The cultured seeds were incubated at 25±2°C under a 16 h photoperiod at 50 µmol s^{-1} m^{-2}. Shoot-tips of about 2-3 mm size were dissected from 15 day old *in vitro* raised seedlings and used as explants for vitrification studies.

Preculture

To induce dehydration tolerance in the shoot-tips (explants) a two step preculturing with sucrose was employed following the method of Suzuki *et al*. (25, 27). In the first preculture step, the shoot-tips were precultured on full-strength MS agar medium containing 0.1 M sucrose (2.3-fold the sucrose concentration than the seed germination medium) for 0-15 days at 25±2°C under a 16 h photoperiod (50 µmol s^{-1} m^{-2}) unless otherwise specified. In the subsequent step, the explants were consecutively cultured on full-strength MS agar medium

containing 0.4 M and 0.7 M sucrose for one day each and incubated under same temperature and light regime. These precultured shoot-tips (2-3 mm long) were used for vitrification-based cryopreservation. To test the effect of various types of sugar (sucrose, glucose, fructose and maltose) on preculture and subsequent shoot regrowth of cryopreserved tissues, another set of experiment was carried out using the most suitable vitrification solution.

Vitrification procedure

Precultured shoot-tips were transferred to three types of cryotubes namely polypropylene (Polylab), glass (Borosil) and aluminium (local make-USIC) and five tubes were used for each type. In each of these cryotubes (1.5 ml capacity) five pre-cultured shoot-tips were taken and were directly incubated in 1 ml of vitrification solution for 0-30 min. During the incubation, the vitrification solutions in the cryotubes were replaced by fresh solutions once or twice. The volume of the solution was reduced to 0.6 ml before immersion in LN. Five different vitrification solutions were tested: (i) vitrification solution A (VA), (ii) vitrification solution B [VB (i.e. PVS2)] (23), (iii) vitrification solution C [VC (i.e.VSL)] (28), (iv) vitrification solution D (VD) and (v) vitrification solution E [VE (i.e. modifiedVSL)] (Table 1). After incubation in respective vitrification solutions for desired period of time (0-30 min) at 25±1°C, the cryotubes containing 0.1-1.0 ml of solution and five shoot-tips were plunged into LN and kept for at least 1 h.

Table 1. Composition of various vitrification solution candidates

Candidate solutions	Composition (% w/v)			
	Glycerol	Sucrose	Ethylene glycol	DMSO
VA	30	5	15	15
VB (PVS2)	30	15	15	15
[a]VC (VSL)	20	5	30	10
[b]VD	20	10	25	10
[b]VE (mVSL)	20	5	25	10

DMSO-dimethylsulfoxide; VA: vitrification solution A; VB: vitrification solution B or PVS2 (plant vitrification solution 2); VC: vitrification solution C or VSL (vitrification solution L); VD: vitrification solution D; VE: vitrification solution E or mVSL (modified VSL)
[a] Vitrification solution contained 10 mM $CaCl_2$
[b] Vitrification solutions contained 5 mM $CaCl_2$

Thawing, washing and regrowth

The cryotubes containing shoot-tips were removed from LN and immediately plunged in a water bath at 40°C and thawed for 1-2 min by repeated shaking. Immediately after thawing, the vitrification solutions were decanted from the cryotubes, the shoot-tips were washed using varying concentrations of sucrose solution (0-2 M). Five millilitre of the sucrose solution was added either directly or drop-wise within a period of 20-30 min. While adding drop-wise, the tubes were shaken gently after the addition of every drop. The sucrose solution was drained from the cryotubes and replaced with 1.0 ml of fresh solution and the tubes were shaken slowly and decanted again. The shoot-tips subjected to vitrification solutions only (treated control-C_T) were washed with 0-2 M sucrose in the same manner. The shoot-tips of C_T or vitrified (LN) were cultured on to a gelled recovery medium (MS + 3% sucrose + 4.0 mg l^{-1} BA). Single shoot-tip was cultured in each test tube. The cultures were maintained at 25±2°C under 16 h photoperiod of 150 µmol s^{-1} m^{-2} light intensity. The response of vitrified shoot-tips on recovery medium was observed periodically. The viable shoot-tips were then transferred on to the fresh recovery medium for further development of shoots. The percentage response

of viable shoot-tips (regrowth capacity) was based on the number of explants cryopreserved and the numbers of explants showing shoot regrowth (normal shoot formation) after 30 days of re-culturing *in vitro*.

Statistical analysis

Five shoot-tips were tested for each of two replications in every experiment. The experiments were repeated three times. Results are represented as means and standard errors. The data were subjected to analysis of variance and Tukey's (HSD) Multiple Comparison Test ($P<0.05$) using XLSTAT statistical package.

RESULTS AND DISCUSSION

In the present study, some of the critical components such as effect of different vitrification solutions, exposure time in vitrification solutions, preculturing and effect of different sugars on preculture medium, effect of volume of vitrification solution, washing of vitrified tissues after thawing, effect of cryovials on shoot regrowth response, which have a direct bearing on the success of vitrification were studied. Fifteen day old *in vitro* raised shoot-tips were used in the present study as actively growing tissues ensure optimal recovery after cryopreservation (8).

Effect of candidate vitrification solutions

In general, vitrification protocols are suitable for cryopreservation of shoot-tips rather than suspension cells (5, 7). PVS2 is the most extensively used vitrification solution for shoot-tips and was originally developed for the cryopreservation of callus of navel orange (23). The initial growth of callus following cryopreservation in PVS2 was found to be comparatively lower than the untreated control (23). With that perspective, we used different vitrification solutions to achieve higher survivability (shoot regrowth capacity) of the cryopreserved shoot-tips of *P. kesiya*.

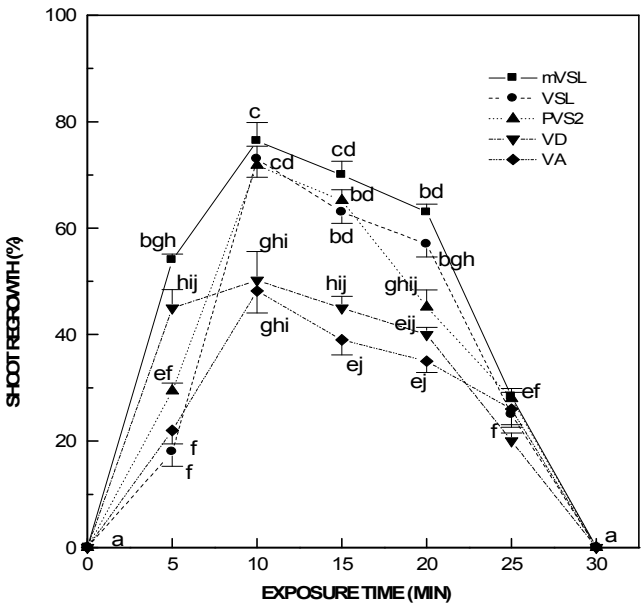


Figure 1. Effect of different vitrification solutions on the survival of *P. kesiya* shoot-tips after exposure to liquid nitrogen (LN). Shoot-tips were two-step precultured and subsequently incubated in vitrification solutions at 25±2°C for stipulated periods prior to immersion in LN. The values are averages with standard error bar. Bars marked by the same letter(s) fall in the same range according to Tukey's Multiple Comparison Test at $P<0.05$.
VA: vitrification solution A;
VD: vitrification solution D;
PVS2: plant vitrification solution 2;
VSL: vitrification solution L;
mVSL**:** modified VSL.

The two-step precultured shoot-tips were directly incubated in the vitrification solution for 0-30 min at 25±1°C and plunged in LN (Fig. 1). Among the vitrification solutions tested,

PVS2, VSL and mVSL resulted in a comparative survival of 71, 73 and 76 % respectively (Fig. 1) as determined by shoot regrowth capacity.

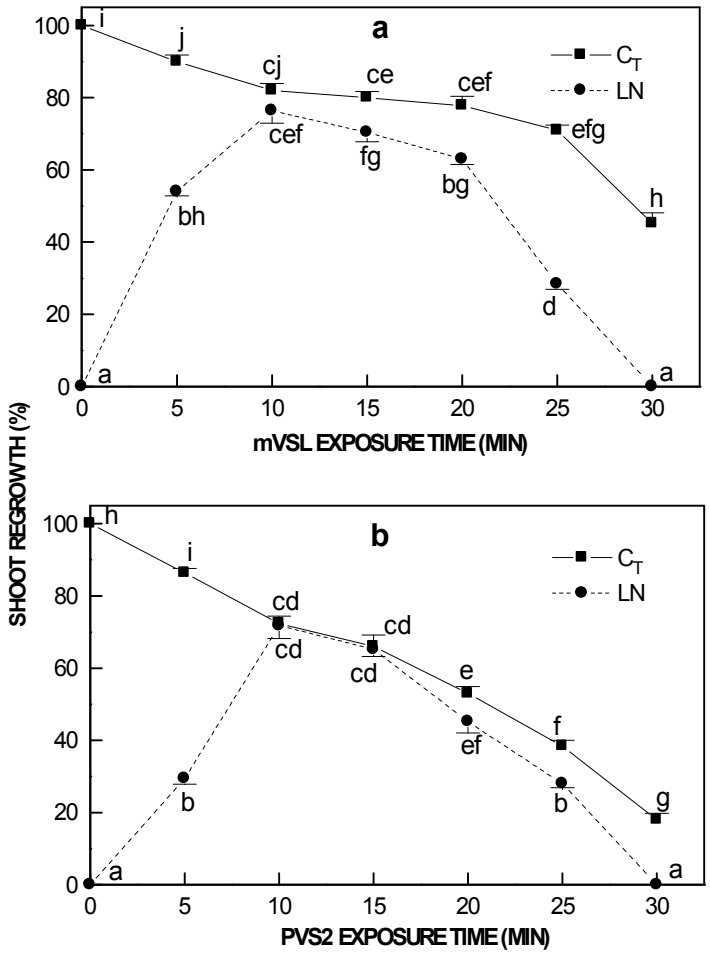


Figure 2. Effect of duration of exposure in (a) modified vitrification solution L (mVSL) and (b) plant vitrification solution 2 (PVS2) on the survival of *P. kesiya* shoot-tips before (C_T) [C_T : treated control (precultured but non-frozen)] and after immersion in liquid nitrogen (LN). The values are averages with standard errors. Bars marked by the same letter(s) fall in the same range according to Tukey's Multiple Comparison Test at $P<0.05$.

The performances of the vitrification solutions mVSL and PVS2 were examined in more detail (Fig. 2), as these solutions exhibited maximum response. Incubation of two-step precultured shoot-tips of *P. kesiya* showed that the shoot regrowth was significantly affected (Fig. 2) with incubation periods in both the vitrification solutions. The shoot-tips thus incubated, when plunged in LN, attained 76-70% shoot regrowth from tissues incubated in mVSL for 10-15 min (Fig. 2a); the survivability declined drastically to about 20% when incubated for 25 min. The incubation of precultured shoot-tips of *P. kesiya* in PVS2 for 30 min decreased the viability to nearly 25% (C_T), while the highest shoot regrowth (72%) following LN exposure was achieved with an incubation period of 10 min (Fig. 2b). Incubation for longer periods with PVS2 resulted in reduced viability both in the C_T and LN exposed shoot-tips of *P. kesiya*. Similar types of incubation dependency of vitrifying solutions were reported by Tanaka *et al.* (30), Suzuki *et al*. (28). Our results showed that the best shoot regrowth obtained (76 and 72%) following cryopreservation were similar for both mVSL and PVS2 respectively at 10 min incubation. Higher survival (54%) of cryopreserved shoot-tips of

P. kesiya at an incubation period of 5 min suggests that mVSL became effective in vitrifying the shoot-tips much faster than PVS2 (30% at 5 min), possibly because of its faster rate of penetration and/or faster dehydration of tissues of the shoot-tips. Our study conforms to the report of Suzuki *et al*. (28) with gentian buds. Higher survival of shoot-tips treated with mVSL (C_T) indicated that it is less toxic for the tissue than PVS2 (Fig. 2a-b). The properties of mVSL, like faster penetration or faster dehydration of tissues and lesser toxicity, perhaps allowed it to have a wider optimal incubation period than PVS2. Time of exposure of plant materials to vitrification solution has been found to be critical for their survival as these treatments subject cells and tissues to osmotic stress and/or chemical toxicity (2, 14, 17, 31). Sakai *et al*. (24) reported that longer exposure periods to cryoprotectant solution affected the survival of nucellar cells of navel orange. However, the effect of exposure time to PVS2 was found to be species-specific and even genotype specific as there are reports of high survival after 3 h exposure to PVS2 solution (6). *Pk-04* genotype of *P. kesiya* showed a considerably higher tolerance range for incubation similar to the report of Suzuki *et al*. (27, 28).

Shoot-tips of *P. kesiya* that survived vitrification-based cryopreservation using mVSL and PVS2 formed normal shoots without callus formation in the regeneration medium (Fig. 3). Evasion of callus formation during regrowth of cryopreserved tissues prevents somaclonal variation and thus can be an effective method for cryopreservation of vegetatively propagated shoot-tips in pines and their subsequent use in clonal forestry.

Effect of preculturing and sugar species on regrowth response of vitrified shoot-tips

The cryopreservation of gentian axillary buds using two-step preculture and desiccation method was successfully established by Suzuki *et al*. (25, 26, 27). A comparative study of shoot regrowth capacity of *P. kesiya* was carried out with mVSL solution with and without preculturing. The shoot-tips that were precultured in a two-step manner showed a higher survival percentage (76%; Fig. 4) to that of non-precultured shoot-tips (45%; data not shown). In gentian buds, desiccation or osmotic tolerance was induced by a two-step preculture method using sucrose (25, 26, 27). Our result revealed that two-step preculturing with MS medium containing 0.1 M sucrose for 11 days (first step) and subsequent 0.4 and 0.7 M sucrose treatment (1 day each) at the second step resulted in higher survival of shoot-tips after cryopreservation. Again, out of different sugar species tested for two-step preculturing with mVSL, sucrose and glucose showed 76 and 69% shoot regrowth respectively following exposure to LN. However, fructose and maltose showed 14 and 0% shoot regrowth respectively (Fig. 5) in the pre-treatment experiments. Our study revealed that preculture of shoot-tips with sucrose and glucose was effective in increasing shoot regrowth following cryopreservation. Preculturing of gentian buds with sucrose and glucose is an effective way to increase survival in cryopreservation using both desiccation and vitrification methods, while preculturing with fructose and maltose resulted in much lower survivals in the vitrification method than the desiccation method (28). Previous studies also revealed that the two-step preculture of tissues at increased sugar concentration enhances the survival rate of cryopreserved tissues. The first preculture step (with 2.3-fold higher sucrose concentration) entails a temporary ABA increase resulting in ABA-mediated cellular changes; and in the second preculture step the sugar in the medium was loaded into bud cells (27). The differences in the performances of the sugar may develop from the capacity of each sugar to vitrify as such in the desiccated state or upon rapid cooling in LN, and/or the ability of each sugar to be included into the cells in the second step. It is also possible that the results may reflect the abilities of the sugars to elicit signals for inducing dehydration tolerance in the first step (25, 27).

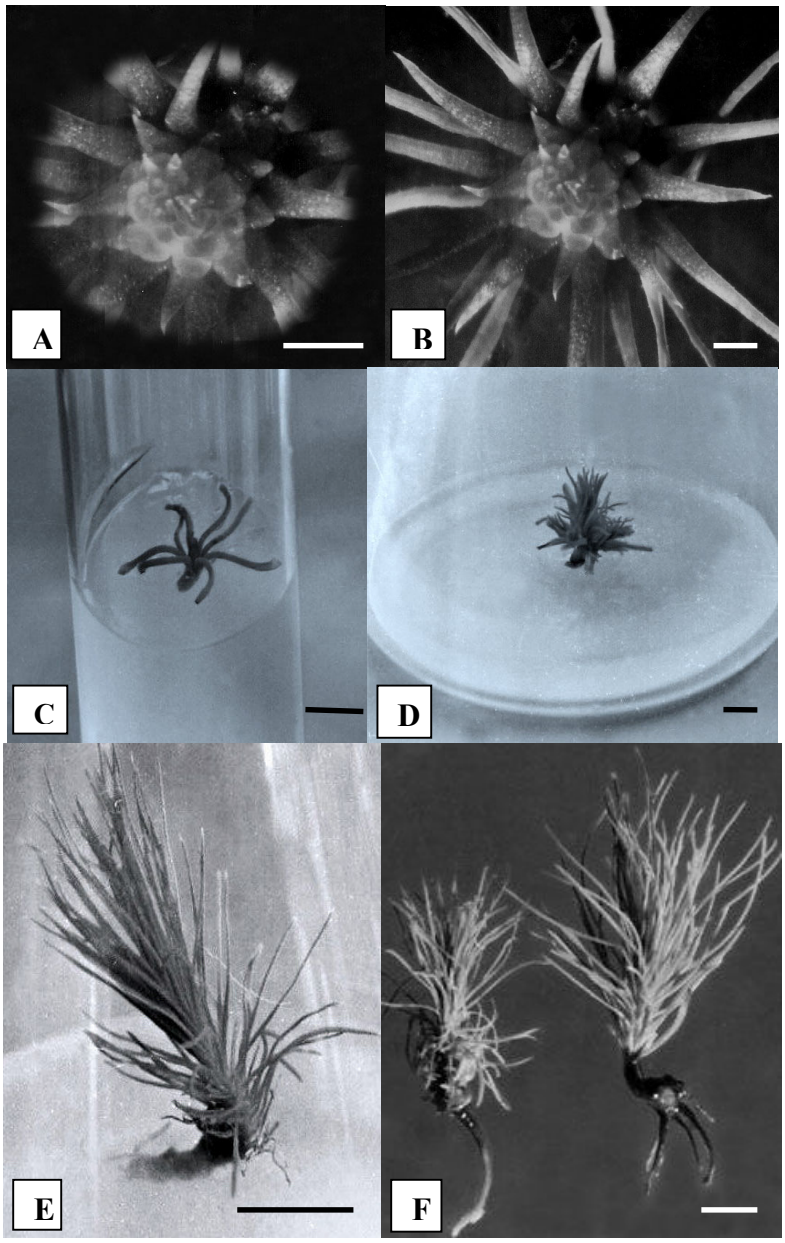


Figure 3. Regrowth of *P. kesiya* shoot-tips cryopreserved using the two-step preculture and mVSL treatment after (A) 15 days (bar: 1 mm), (B) 30 days (bar: 1 mm), (C) 45 days (bar: 1 cm), (D) 75 days (bar: 1 cm), (E) 90 days (bar: 1 cm), (F) 120 days (bar: 1 cm) of re-culturing *in vitro.*

Effect of volume of vitrification solution

The volume of vitrification solution played an important role in regrowth of vitrified tissues of broom grass suspensions (5). In the present study, maximum shoot regrowth was 76% when 0.6 ml of vitrification solution (mVSL) was used. The percentage survivability of shoot-tips decreased with further increase in the volume of the solution (Fig. 6). Suzuki *et al*. (28) used 0.6 ml of vitrification solution for successful regrowth of cryopreserved gentian

buds. In order to increase the heat exchange rates, the volume of the PVS2 solution in the cryotubes is often reduced to 0.5 ml (20) or 250 µl polypropylene straws are sometimes employed (12). Incubation of explants in increased volume of vitrification solution probably favoured greater penetration of tissues at the same exposure time at 25±1°C. This led to either chemical toxicity or excessive osmotic stress of tissues or both, thus reducing the shoot regrowth capacity in C_T beyond 0.6ml of mVSL solution (Fig. 6). The keys to success in cryopreservation by vitrification are to carefully control the dehydration procedures and to prevent injury by chemical toxicity or excessive osmotic stress during treatment with the vitrification solution. Thus, optimizing the time, the volume of vitrification solution and the temperature of exposure to the vitrification solution is most important for achieving a high level of shoot formation (22) during cryopreservation.

Figure 4. Effect of preculture (PC) and the duration of the first preculture step on the survival of *P. kesiya* shoot-tips exposed to mVSL (C_T) and subsequently to liquid nitrogen (LN); PC-LN and C_T indicate two-step preculture (first preculture step using 0.1 M sucrose for the designated period followed by second preculture on 0.4 and 0.7 M sucrose for one day each at 25±1°C) before being incubated in mVSL for 10 min at 25±1°C and immersion in LN. [C_T: treated control (precultured but non-frozen)]. The values are averages with standard errors. Bars marked by the same letter(s) fall in the same range according to Tukey's Multiple Comparison Test at P<0.05.

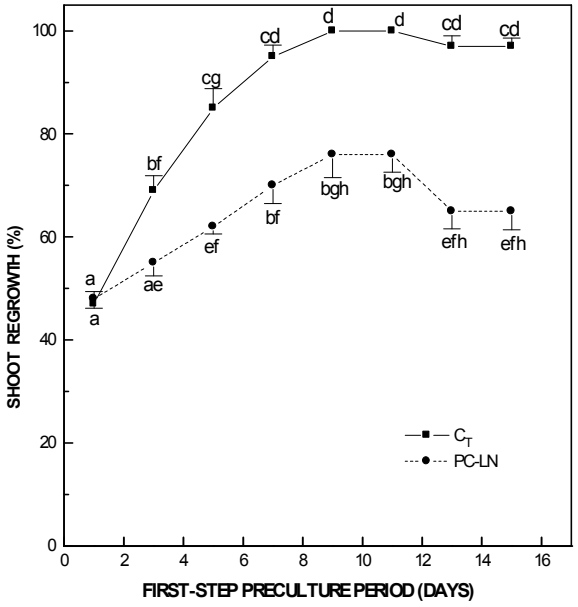
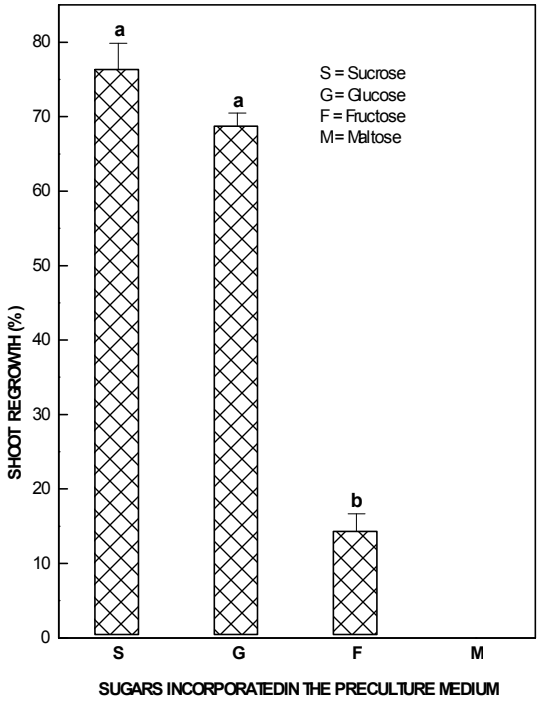


Figure 5. Effect of four different sugar species (*sucrose, glucose, fructose* and *maltose*) in the preculture medium on the survival (shoot-regrowth) of *P. kesiya* shoot-tips containing the designated sugar (0.1 M for 11 days in the first step and 0.4 and 0.7 M for one day each in the second step) before being incubated in mVSL for 10 min at 25±1°C and immersion in liquid nitrogen (LN). The values are averages with standard errors. Bars marked by the same letter(s) fall in the same range according to Tukey's Multiple Comparison Test at P<0.05.



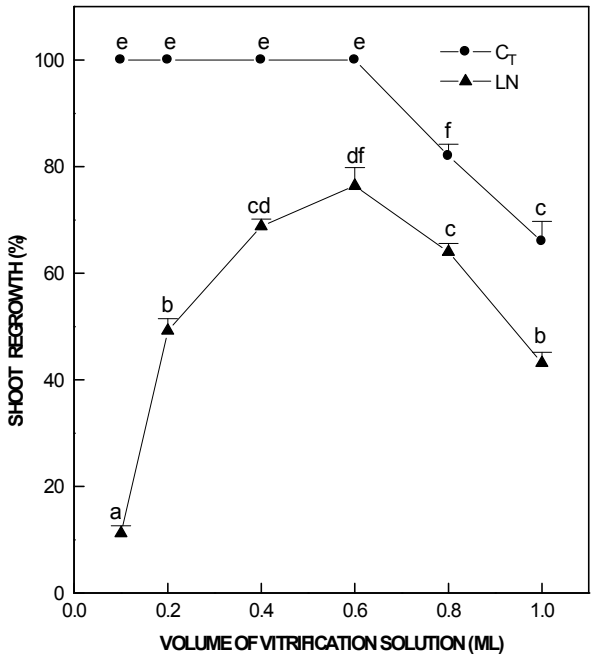


Figure 6. Effect of volume of vitrification solution on the survival of *P. kesiya* shoot-tips after exposure to liquid nitrogen (LN). Shoot-tips were two-step precultured and subsequently incubated in mVSL solution at 25±1°C for 10 min prior to immersion in LN. [C_T: treated control (precultured but non-frozen)]. The values are averages with standard errors. Bars marked by the same letter(s) fall in the same range according to Tukey's Multiple Comparison Test at $P<0.05$.

Effect of washing of vitrified tissues with sucrose solution after thawing

The washing solution has a vital role to play in the regrowth of vitrified tissues. Shoot-tips of *P. kesiya* when washed with 1.2 M sucrose solution showed significantly higher shoot regrowth irrespective of drop-wise or direct addition (Fig. 7). Data for drop-wise and direct washing in the case of C_T was pooled and presented together, as no significant difference was observed in shoot regrowth of the tissues between the two methods of washing (Fig. 7). However, an increase or decrease in the molarity of sucrose other than 1.2 M affected the shoot regrowth percentage. This result is in agreement to that of Sakai *et al*. (23) and Ishikawa *et al*. (5). It is hypothesizable that drop-wise addition, producing a gradual change in osmolarity, can avoid tissue injury.

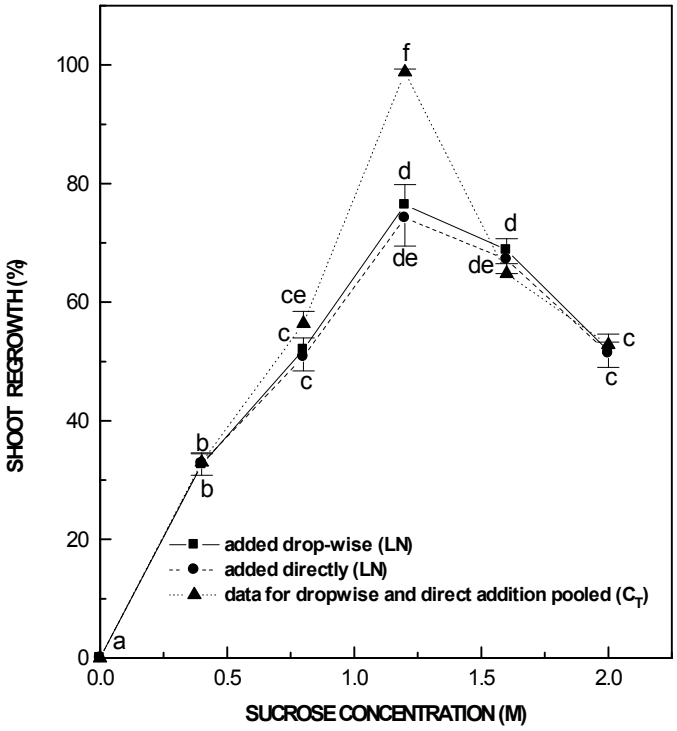


Figure 7. Effect of osmolarity of sucrose solution in the diluents and washing technique on the survival of vitrified shoot-tips after thawing. The shoot-tips were two-step precultured before being incubated in mVSL for 10 min at 25±1°C and immersion in LN. [C_T : treated control (precultured but non-frozen)]. The values are averages with standard errors. Bars marked by the same letter(s) fall in the same range according to Tukey's Multiple Comparison Test at $P<0.05$.

Effect of cryovials on regrowth response of vitrified shoot-tips

Types of cryovials used were found to affect the vitrification process (3, 4, 5). Among different types of cryotubes used in the present study, the glass tubes and polypropylene tubes were found better for vitrification with 76 and 74% shoot regrowth respectively (Fig. 8**)**. On the contrary, a significant decrease in shoot regrowth (48%) was observed when aluminium cryotubes were used for vitrification of shoot-tips. Glass and polypropylene cryotubes proved to be effective for vitrification as thawing could be accomplished at an optimum rate of heat conductivity as compared to rapid conductivity rate of aluminium tubes. About 25% reduction in the recovery of embryogenic cells in Norway spruce and about 30% in loblolly pine was reported when aluminium envelopes were used instead of polypropylene vials in cryopreservation experiments (4).

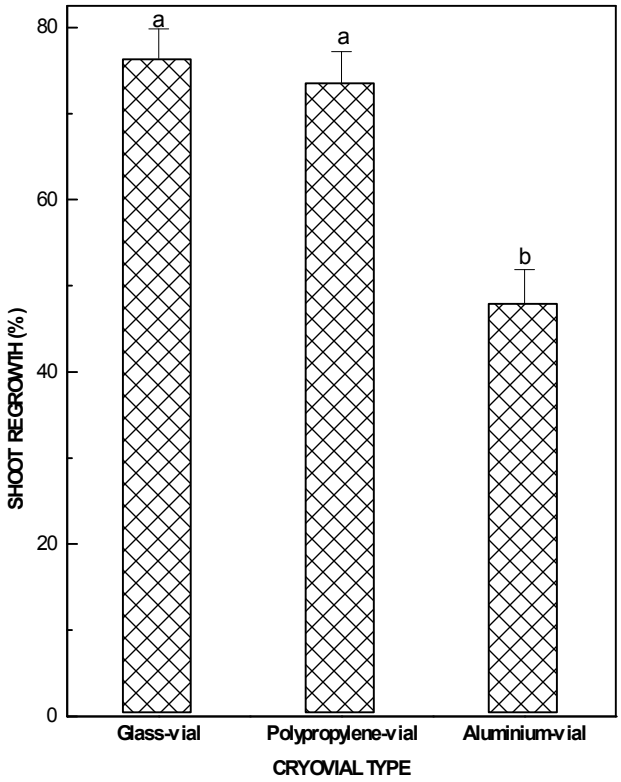


Figure 8. Effect of cryovial types (glass-vial, polypropylene-vial and aluminium-vial) on survival of shoot-tips of *P. kesiya*. The shoot-tips were two step precultured before being incubated in mVSL for 10 min at 25±1^{o}C and immersion in liquid nitrogen (LN). The values are averages with standard errors. Bars marked by the same letter(s) fall in the same range according to Tukey's Multiple Comparison Test at P<0.05.

CONCLUSION

The present study resulted in the development of optimized cryo-protocol for the successful vitrification based cryopreservation of shoot-tips of *P. kesiya*, a timber yielding tree species of Northeast India. Of the different vitrification solutions tested, the best shoot regrowth (76%) was achieved with 0.6 ml of mVSL solution when incubated for 10 min at 25±2^{o}C using glass cryotubes in MS basal medium containing 3% sucrose and 4.0 mg l^{-1} BA. The present investigation also revealed that a two-step preculturing in MS medium containing 0.1 M sucrose for 11 days (first-step) and subsequent 0.4 and 0.7 M sucrose treatment (1 day each) at second-step, prior to vitrification, enhanced the shoot regrowth significantly (76%) compared to that of non-precultured shoot-tips (45%). Thawing of vitrified tissues at 40^{o}C followed by washing (drop-wise or direct) with 1.2 M sucrose solution was found to be optimum for shoot regrowth after cryopreservation. The protocol developed distinctly holds promise in cryopreservation of shoot tips of *P. kesiya* and the technique needs to be tested on

a whole range of germplasm for this species to validate its potential in future cryopreservation experiments and subsequent application in clonal forestry.

Acknowledgements: Laboratory facilities provided by the Department of Botany and other logistics presented by Department of Basic Sciences & Social Sciences, North-Eastern Hill University, Shillong, India are acknowledged. The authors also express their gratitude to Dr. D. Adhikari, Project Scientist (Forest Ecology Laboratory, Department of Botany) and Dr. K. Upadhaya, Assistant Professor, North-Eastern Hill University, Shillong, India for their assistance in statistical analysis.

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Accepted for publication 07/11/2011