OPTIMIZATION OF CRYOPRESERVATION FOR *STERCULIA CORDATA* ZYGOTIC EMBRYOS USING VITRIFICATION TECHNIQUES

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NADARAJAN, J., STAINES, H. J., BENSON, E. E., MARZALINA, M., KRISHNAPILLAY, B. & HARDING, K. 2007. Optimization of cryopreservation for *Sterculia cordata* zygotic embryos using vitrification techniques. Vitrification is a physical process by which a concentrated aqueous solution solidifies into stable amorphous glass at a sufficiently low temperature. This process eliminates the potentially damaging effect of intra- and extracellular crystallization in cryopreservation of plants and animal cells. *Sterculia cordata* embryos were cryopreserved using a vitrification technique. The embryos were precultured for three days on MS media supplemented with sucrose (0.35, 0.55 and 0.75 M) and treated with loading solution (LS) and plant vitification solution 2 (PVS2) for one to three hours before storing in liquid nitrogen. Their germination was assessed before and after cryopreservation. Multiple logistic regression analysis showed that the sucrose pregrowth medium, LS and PVS2 pretreatments were not detrimental to the germination of embryos. However, sucrose and LS pretreatments alone were not sufficient for successful cryopreservation in contrast to the high germination (≥ 80%) achieved with PVS2 treatment.

Keywords: Logistic regression analysis, tropical forest seed, intermediate-recalcitrant seed

NADARAJAN, J., STAINES, H. J., BENSON, E. E., MARZALINA, M., KRISHNAPILLAY, B. & HARDING, K. 2007. Pengoptimum krioawetan bagi embrio zigot *Sterculia cordata* menggunakan teknik vitrifikasi. Vitrifikasi merupakan proses mengubah larutan akues pekat menjadi kaca amorfus yang stabil pada suhu rendah. Proses ini mengelak kerosakan yang terjadi akibat pengkristalan intrasel dan luar sel semasa krioawetan sel tumbuhan dan haiwan. Embrio *Sterculia cordata* dikrioawet menggunakan teknik vitrifikasi. Embrio tersebut diprakultur selama tiga hari pada medium MS yang ditambah sukros (0.35, 0.55 dan 0.75 M) dan dirawat dengan larutan muatan (LS) serta larutan vitrifikasi tumbuhan (PVS2) selama satu hingga tiga jam sebelum disimpan dalam nitrogen cecair. Percambahan embrio dinilai sebelum dan selepas krioawetan. Analisis regresi logistik berganda menunjukkan bahawa prarawatan media sukros, LS dan LVS2 tidak memudaratkan percambahan embrio. Bagaimanapun, prarawatan sukros serta LS tidak menunjukkan keputusan krioawetan yang baik berbanding dengan rawatan PVS2 yang menghasilkan percambahan tinggi melebihi 80%.

INTRODUCTION

Cryopreservation is an important conservation method to preserve tropical forest tree germplasm not only in base genebanks but also in active germplasm repositories that support biotechnologically-based initiatives including micropropagation and tissue culture (Krishnapillay 2000). Therefore, the investigation of different cryopreservation methods that present different storage stresses, ranges of tolerance and cryoprotective modes of action is important. This may extend the cryopreservation protocol options available for conserving physiologically and genotypically diverse tropical tree seeds. Our previous study investigated cryopreservation of *Sterculia cordata*, an intermediate/recalcitrant seed producing species using a desiccation technique (Nadarajan *et al.* 2006). The present study explored the possibility of using a vitrification technique for cryopreservation of this species.

The process of vitrification is now becoming one of the main approaches in the cryopreservation of plant germplasm for long-term conservation (Engelmann 2004). Vitrification refers to

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the physical process by which a concentrated aqueous solution solidifies into stable amorphous glass at a sufficiently low temperature without ice crystallization (Sakai 2004). Complete vitrification eliminates the potentially damaging effect of intra- and extracellular crystallization and produces high levels of cell survival (Benson 2004). Benson *et al.* (1996) confirmed this in the differential scanning calorimetry (DSC) profiles for PVS2 treated ribes apices, which showed no evidence of ice nucleation on cooling (at -10 °C/min).

In this study, *S. cordata* zygotic embryos were cryopreserved for the first time using a vitrification technique. Our previous work on this species showed that embryos at seed developmental stage 3 (fully matured seeds that have shed from mother trees) have higher rate of germination (Nadarajan *et al.* 2006). Therefore, only embryos at stage 3 were used in this study. The toxicity effect of each cryoprotective treatment was assessed by conducting a germination test at each step before the embryos were introduced to the next successive step of treatment. Germination was also assessed after cryopreservation for all the cryoprotective treatments.

MATERIALS AND METHODS

Seed material

Seeds were collected from mother trees in the Forest Research Institute of Malaysia (FRIM). Seeds were surface sterilized (10% v/v hypochlorite solution, Domestos, Lever Bros.) for 10 min, rinsed once in 30% (v/v) ethanol for 1 min and then rinsed three times in sterile water. The embryos were excised in a laminar airflow cabinet. They were then surface sterilized with 0.3% (w/v) boric acid, rinsed once in 30% (v/v) ethanol for 1 min and rinsed three times in sterile water.

The vitrification procedure consisted of two steps, namely, dehydrating specimens by a loading solution (LS) containing 2 M glycerol and 0.4 M sucrose for one, two and three hours at 25 °C, followed by exposure to a highly concentrated plant vitrification solution 2 (PVS2) then transferred directly into liquid nitrogen (Matsumoto & Sakai 2003). The whole experiment was carried out in three phases. The first phase consisted of sucrose pregrowth treatment, second phase, LS treatment and the third phase, PVS2 treatment.

Experimental procedures

Phase I: Sucrose pregrowth treatment

The excised embryos were precultured for three days on solidified MS medium (Murashige & Skoog 1962), supplemented with three different (0.35, 0.55 or 0.75 M) sucrose treatments. At the end of these treatments, 10 embryos were selected for moisture content test, 10 for toxicity test and 10 more for cryostorage for each treatment.

Phase II: Treatment with LS

The effects of increasing exposure time to the loading solution were evaluated for the embryos after pregrowth in sucrose. Loading solution (comprising 0.4 M sucrose and 2 M glycerol) was applied to the embryos for one, two and three hours at 25 °C. Ten embryos were used for the toxicity trial and a further 10 were tested for germination after cryopreservation for each treatment combination. This experiment had nine treatments and 30 embryos were used for each treatment.

Phase III: Treatment with PVS2

After the sucrose pregrowth and LS treatments, the embryos were then treated with PVS2 (30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide (DMSO) and 0.4 M sucrose) for one, two and three hours at 0 °C (Sakai 2004). After these treatments, the vitrification solution was replaced with 0.5 ml liquid MS medium containing 1.2 M sucrose for 20 min. The embryos were then blotted dry on sterile filter paper. The effect of exposure time to PVS2 solution on survival of embryos was investigated before cryopreservation. This experiment had 27 treatments and 30 embryos were used for each treatment.

Cryopreservation

Cryopreservation was carried out by rapid immersion into liquid nitrogen for each treatment. After 24 hours of storage in liquid nitrogen, placing them in a water bath at 38 $\pm$ 2 °C for 10 to 15 min rapidly warmed the embryos. The vitrification solution was then replaced with 0.5 ml liquid MS medium containing

1.2 M sucrose for 20 min. The embryos were then blotted dry on sterile filter paper. The effect of the exposure time to PVS2 solution on survival of embryos was investigated after cryopreservation.

Germination

Germination was assessed before and after cryopreservation for each step. The embryos were placed on standard semi-solid medium with 0.3 M sucrose for one day. They were then placed on standard medium and 0.1 M sucrose, 0.5 mg/l BAP and activated charcoal (1 g/l) were added for growth recovery. The embryos were then incubated in a temperature-controlled growth room at 25 $\pm$ 1 °C with a 12-hour photoperiod and 30 µmol m^{-2} s^{-1} light intensity for two weeks. Germination was defined as embryos that swelled and elongated and eventually grew into complete plantlets. After two weeks the developing embryos were subcultured on MS medium with 0.1 M sucrose and 0.5 mg/l BAP but without activated charcoal and incubated at the same conditions described above.

Data analysis

Data analysis was by multiple logistic regression (Sokal & Rohlf 1995). The primary response was the number of germinated embryos from 10 replicates which followed a binomial distribution (either germinated or not germinated). The multiple logistic regression is designed to identify the relative importance of factors and interactions (where appropriate) that significantly reduce (at the 5% significant level for this case) the residual deviance for binomial response variables. Logistic regression was used in preference to the often-used arcsine transformation or ANOVA as these assume normality and require a large number of replicates which were not valid for this experiment.

RESULTS

The initial moisture content of the embryos was approximately 26%. It declined to 24, 21 and 19% after three days of pregrowth on MS media supplemented with 0.35, 0.55 and 0.75 M sucrose respectively.

Phase I: Sucrose pregrowth treatment

The main effect plots showing the number of germinated embryos (from the total of 10) before (Figure 1) and after cryopreservation (data not shown) suggested a quadratic relationship with sucrose concentration. However, multiple logistic regression analysis showed that fitting sucrose and its quadratic was not significant ($G^* = 0.401$, $df = 2$, $p > 0.05$). This suggested that the sucrose concentrations tested in this study produced similar viability before cryopreservation and germination above 80% was achieved (Figure 1). However, adding liquid nitrogen to the model significantly affected the model predicting germination ($G^* = 69.773$, $df = 1$, $p < 0.001$)

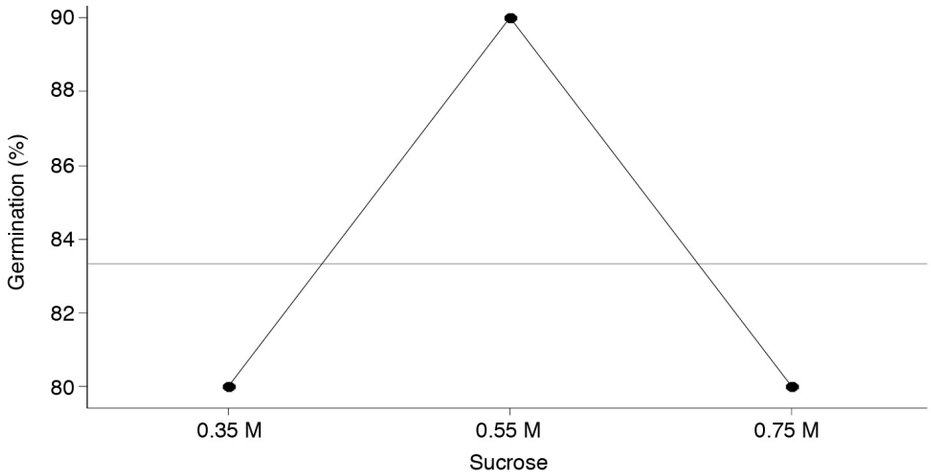

Figure 1 Main effects plot for number of germinated embryos before liquid nitrogen storage for sucrose preculture treatment

showing that there was a significant difference in the germination of embryos before and after cryopreservation. Figure 2a shows the fitted values for germination for the sucrose pregrowth treatment (regardless of concentration) before and after cryopreservation. The fitted value for germination declined from above 90% before cryopreservation to about 3.0% after cryopreservation for the pretreatment showing that this treatment alone was not sufficient to achieve high germination after cryopreservation. Therefore, the LS treatment was added after this pretreatment.

Phase II: Treatment with LS

Main effect plots also indicated a quadratic relationship with LS treatment for embryo germination before cryopreservation (Figure 3). However, multiple regression analysis showed that fitting the full quadratic model involving sucrose and LS treatment did not have a significant effect on the germination of embryos before cryopreservation ($G^* = 4.848$, $df = 5$, $p > 0.05$). This indicated that all LS treatments (1–3 hours) had the same rate of germination (above 70%); therefore, this treatment was not lethal to *S. cordata* embryos. However, adding liquid nitrogen to the model had a significant effect on the germination of embryos ($G^* = 87.146$, $df = 1$; $p < 0.001$) suggesting that there was a significant difference in germination before and after cryopreservation. Germination before cryopreservation was about 85% and it declined to about 20% for cryopreserved embryos after sucrose and LS treatments (Figure 2b). This result suggested that though there was a slight increase in germination after sucrose and LS treatments, it was not sufficient to achieve high germination after cryopreservation. Therefore, PVS2 treatment was added in the next phase of this experiment.

Phase III: Treatment with PVS2

The main effects plot for the germination of embryos also indicated that there was a quadratic relationship for the PVS2 treatment (Figure 4). The multiple logistic regression analysis showed that fitting the full quadratic model involving sucrose, LS and PVS2 treatments and their interactions did not have a significant effect on the germination of embryos before cryopreservation ($G^* = 1.972$, $df = 9$, $p > 0.05$). High germination above 80% before cryopreservation was achieved after PVS2 treatments regardless of the duration of treatment (Figure 2c). Adding liquid nitrogen to the model did not significantly affect the

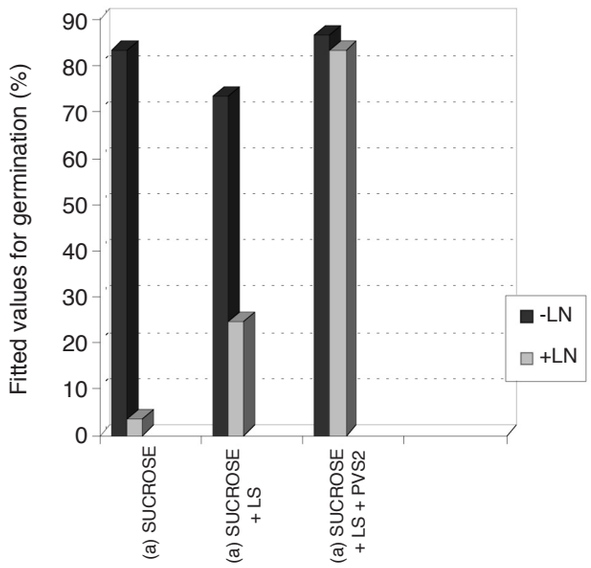

Figure 2 Fitted values for germination of *Sterculia cordata* embryos after (a) sucrose, (b) LS and (c) PVS2 treatments before (-LN) and after cryopreservation (+LN)

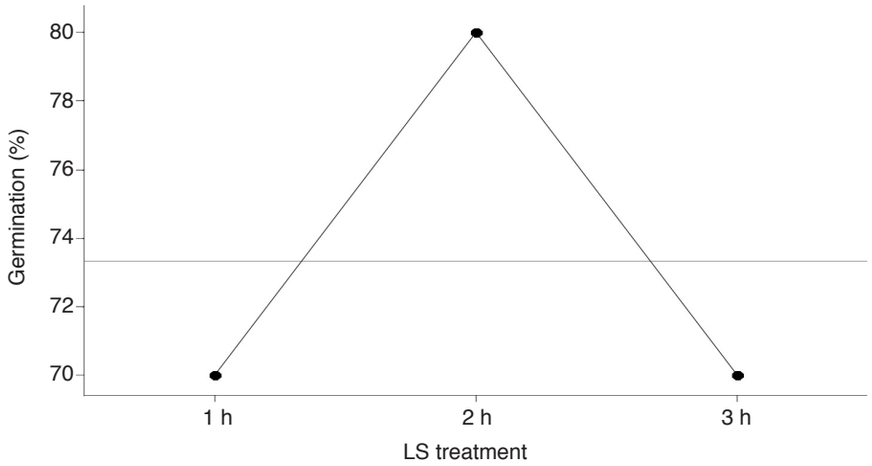

Figure 3 Main effects plot for number of germinated embryos before liquid nitrogen storage for sucrose pretreatment

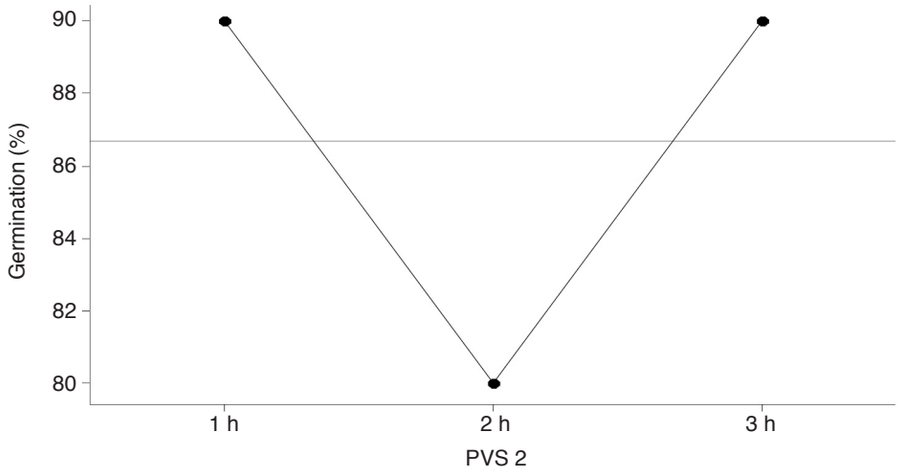

Figure 4 Main effects plot for number of germinated embryos before liquid nitrogen storage for PVS2 pretreatment

model predicting germination of embryos ($G^* = 0.117$, df = 1, $p > 0.05$) suggesting that there was no difference in germination between cryopreserved and non-cryopreserved embryos. High post-cryopreservation germination (> 80%) was also achieved after PVS2 treatment for cryopreserved embryos (Figure 2c). These results suggested that PVS2 was not only non-toxic to the embryos but also increased germination after cryopreservation. Figure 5 shows the cryopreserved embryos growing into normal plantlets after two (a) and four (b) weeks of rewarming.

DISCUSSION

A requirement for successful cryopreservation is often the pregrowth phase involving the culture of plant material on a medium containing cryoprotectants (generally sugars such as sucrose or glucose) followed by the rapid freezing of explants in liquid nitrogen. A high level of sugar accumulated during preculture has been reported to improve survival of cryopreserved materials (Kamata & Uemura 2004). Sucrose partially dehydrates samples through osmotic effects, thereby increasing the concentration

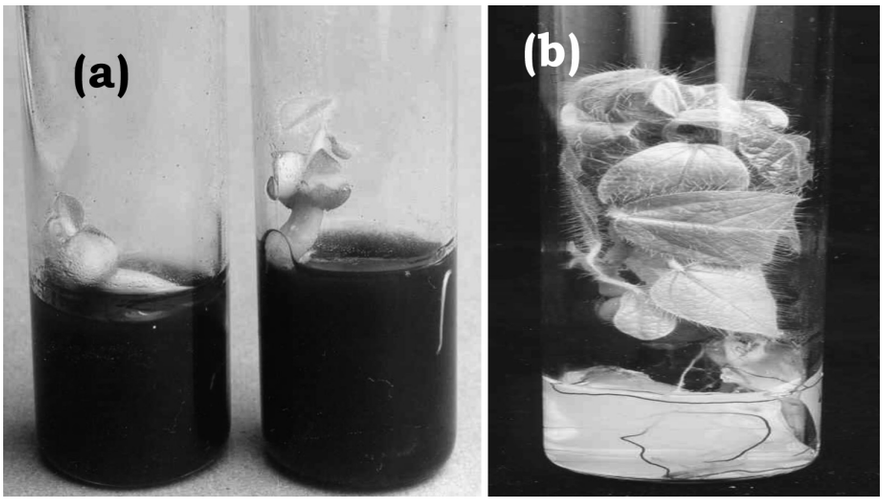

Figure 5 *Sterculia cordata* embryos growing in recovery medium after (a) two weeks and (b) four weeks of germination

of intracellular solutes and the tolerance to dehydration (Dumet *et al.* 1993). The decrease in moisture content from 26 to 24, 21 and 19% in the embryos of *S. cordata* after pregrowth in 0.35, 0.55 and 0.75 M sucrose respectively confirmed the dehydration effect of sucrose. High germination was obtained for embryos after sucrose pregrowth treatments before cryopreservation, regardless of the concentrations of sucrose (Figure 2a). This indicated that high levels of sucrose (0.35–0.75 M) were not detrimental to *S. cordata* embryos. Similar observations were reported for *Citrus aurantifolia* embryonic axes (Cho *et al.* 2002). However, preculture of *S. cordata* embryos with sucrose alone did not lead to substantial increases in germination (~3%) after cryopreservation (Figure 2a).

To improve the germination rate, the effects of LS on the embryos were examined. LS treatment for one, two and three hours (after pregrowth in different concentrations of sucrose) was not detrimental to the embryos. However, germination after cryopreservation for this pretreatment was still low (~20%). During LS treatment cells were osmotically dehydrated and considerably plasmolyzed and when subjected to PVS2 solution, the cells successively decreased in cytosolic volume and remained osmotically dehydrated (Matsumoto & Sakai 2003). The high concentration of sucrose caused accumulation of cytosolic stress-responsive solutes during preculture which have osmosis-related protective effect. The protective effect of LS solution after preculture with high concentraions of sucrose may be caused by osmotic dehydration resulting in accumulation of cytosolic stress-responsive solutes which subsequently protects against plasmolysis (Sakai 2000). The presence of a highly concentrated cryoprotective solution in the periprotoplasmic space of plasmolyzed cells may mitigate the mechanical stress caused by successively severe dehydration during sucrose preculture (Jitsuyama *et al.* 1997). These intracellular and extracellular protective effects may minimize the injurious membrane changes during severe dehydration. Thus, LS treatment following preculture with sucrose enriched medium is recommended for successful cryopreservation by vitrification.

To improve germination after cryopreservation, *S. cordata* embryos were treated with PVS2 following pregrowth in high sucrose medium and LS treatment. The PVS2 treatment did not affect the germination of the embryos before cryopreservation. For many species, highly concentrated vitrification solutions were reported to be harmful (Abdelnour-Esquivel & Engelmann 2002). However, higher survival was reported for a number of species after vitrification (Matsumoto & Sakai 2003). In our study, this treatment had a significant effect on the germination of *S. cordata* embryos after cryopreservation. The fitted value for germination increased from 20% without PVS2 treatment to above 80% with PVS2 after cryopreservation regardless of the duration of treatment (Figure 2c). The vitrification-based approach is more suitable for complex organs, such as shoot tips and embryos

that contain a variety of cell types, each with unique requirements under conditions of freeze-induced dehydration. A great advantage of the vitrification technique is that it does not require controlled freezing equipment or sophisticated and expensive apparatus. By precluding ice formation in the cell system, vitrification-based procedures are operationally less complex as compared with traditional controlled rate freezing methods and have greater potential for broad applicability.

Application of an appropriate experimental design and data analysis is very important in any cryobiological investigation. In this experiment though the main effect plots suggested that there were quadratic effects of sucrose pregrowth and LS and PVS2 treatments the multiple logistic regression analysis showed that the quadratic effects were not significant. The multiple logistic regression analysis fitted this experiment perfectly since there were low number of replicates and the data was a binomial distribution. With high recovery rates after freezing, vitrification appears to be a reliable and reproducible cryogenic technique for cryopreserving of *S. cordata* zygotic embryos compared with conventional seed conservation methods.

CONCLUSIONS

From this study it was found that sucrose concentration in pregrowth media, LS and PVS2 pretreatments were not detrimental to the germination of the embryos of *S. cordata*. Sucrose and LS treatments were not sufficient to achieve high germination after cryopreservation. This study also showed that additional PVS2 treatment was necessary to obtain high germination (≥ 80%) of *S. cordata* embryos after cryopreservation. The correct conclusions from these experiments were assisted by use multiple logistic regression analysis.

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