VIABILITY OF *ELATERIOSPERMUM TAPOS* (PERAH) EMBRYO AFTER STORAGE IN LIQUID NITROGEN

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Received January 2006

NASHATUL ZAIMAH, N. A., BENSON, E. E. & MARZALINA, M. 2007. Viability of *Elateriospermum tapos* (perah) embryo after storage in liquid nitrogen. Moisture content is the most critical factor in ensuring success of a cryopreservation protocol. Moisture content relates to the composition of the seed and embryo. A precise level of desiccation must be achieved so that it is low enough to facilitate cryopreservation without killing the seed or embryo. Germplasm for *Elateriospermum tapos* has never been assessed for desiccation tolerance, or tested for cryogenic storage. In this study, a TZ (2,3,5-triphenyl-2H-tetrazolium chloride) test was used for rapid estimation of viability of embryos. The objective was to ascertain the levels of desiccation that this species can tolerate and the levels of moisture content at which cryopreservation can be done. Data obtained suggested that embryos could tolerate desiccation to about 17% on fresh weight basis. Embryos at 56% moisture level were not able to withstand immersion in liquid nitrogen. Desiccation plus application of a vitrification solution containing 30% glycerol, 15% ethylene glycol and 15% dimethyl sulfoxide for 10 min at 0 °C showed high survival for embryos stored in liquid nitrogen.

Keywords: Cryopreservation, 2,3,5-triphenyl-2H-tetrazolium chloride, TZ, desiccation, moisture content

NASHATUL ZAIMAH, N. A., BENSON, E. E. & MARZALINA, M. 2007. Kebolehhidupan embrio *Elateriospermum tapos* (perah) selepas disimpan dalam nitrogen cecair. Kandungan lembapan merupakan faktor kritikal dalam menentukan kejayaan sesuatu protokol krioawetan. Kandungan lembapan juga berkait rapat dengan komposisi biji benih dan embrio. Proses pengeringan perlu dilakukan sehingga mencapai satu tahap yang selamat demi memastikan protokol krioawetan dapat dijalankan tanpa menyebabkan biji benih atau embrio mati. Germplasma *E. tapos* belum pernah dikaji untuk mengetahui tahap pengeringannya dan tidak pernah pula diuji untuk penyimpanan menggunakan kaedah krioawetan. Dalam kajian ini, ujian tetrazolium menggunakan 2,3,5-triphenyl-2H-tetrazolium klorida digunakan untuk menyukat kebolehhidupan embrio *E. tapos*. Ujian ini dapat menentukan tahap pengeringan dan aras kandungan lembapan yang selamat digunakan dalam pembentukan protokol krioawetan bagi *E. tapos*. Keputusan yang diperoleh menunjukkan embrio *E. tapos* tahan kepada pengeringan sehingga 17% kandungan lembapan. Pada kandungan lembapan 56%, embrio didapati sensitif dan tidak tahan disimpan dalam cecair nitrogen. Pengeringan dan penggunaan larutan vitrifikasi yang mengandungi 30% gliserol, 15% etilena glikol dan 15% dimetil sulfoksida selama 10 minit pada 0 °C memberikan kemandirian yang tinggi bagi embrio yang disimpan dalam cecair nitrogen.

INTRODUCTION

Elateriospermum is a monotypic genus occurring in Peninsular Malaysia, peninsular Thailand, Sumatera, Java and Borneo. *Elateriospermum tapos*, locally known as perah, is fairly common in Peninsular Malaysia and grows in lowland and hill forests up to 600 m. The timber is hard and suitable for medium to heavy construction. The tree is deciduous and is also cultivated as an ornamental. Fruits of this tree explode and scatter seeds which are edible. Large seeds (usually 3–4) indicate that they belong to the recalcitrant group of seeds.

Recalcitrant seeds have high water content and cannot be dried without damage. They are also sensitive to desiccation and low temperatures and usually have a short (a few weeks to a few months) viability period. Germplasm of recalcitrant seeds can be conserved via cryopreservation of their excised embryos (Bajaj 1985). Zygotic embryonic axes are highly regenerative and their smaller size offers easier handling and storage (Kendall *et al.* 1993). They are also more tolerant to dehydration and freezing. However, for these seeds or embryos to survive at very

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low temperatures, they must have reduced intra- and extracellular moisture content before freezing. To achieve this, two methods are available, namely, encapsulation-dehydration and vitrification. Encapsulation-dehydration was first described by Fabre and Dereuddre (1990) for cryopreservation of *Solanum* shoot tips. Today, this technique has been widely applied mainly to several tropical fruit species such as *Coffea canephora* (Hatanaka *et al.* 1994) and *Camellia sinensis* (Janeiro *et al.* 1997).

In vitrification, drying of cells is achieved by using a highly concentrated cryoprotective solution which can partially dehydrate tissues and facilitate the formation of a stable glass-like state in plant tissues prior to immersion in liquid nitrogen. The most common vitrification solution used is Plant Vitrification Solution 2 (PVS2) (Sakai *et al.* 1990). It consists of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide in Murashige and Skoog (MS) medium supplemented with 0.4 M sucrose. Several tropical species have been successfully cryopreserved using this solution, namely, *C. sinensis* (Kuranuki & Sakai 1995), *Ipomoea batatas* (Towill & Jarret 1992) and *Saccharum* sp. (Paulet *et al.* 1993).

This paper presents results of a preliminary study on the evaluation of the viability of *E. tapos* embryos using 2,3,5-triphenyl-2H-tetrazolium chloride (TZ) test before and after cryopreservation.

MATERIALS AND METHODS

Plant materials

In this study fruits of *E. tapos* were collected from a tree located within the grounds of the Forest Research Institute Malaysia. The fruits were immediately brought to the laboratory for processing. They were washed in Teepol to remove dirt and soil and then rinsed under running tap water. By using a pair of secateurs, the seeds were separated and rinsed in tap water. Finally, they were immersed for 20 min in a solution containing 1% (w/v) Benlate, 1% (w/v) Thiram and 3.5% (w/v) boric acid. The seeds were then blotted dry on absorbent paper and placed overnight on a bench. The endocarp of the seed was then removed after which the seed was sterilized further in 10% (v/v) commercial bleach (Chlorox) for 10 min, followed by three rinses in 70% ethanol and three rinses in sterile distilled water. The embryo was then excised with its associated cotyledon material and placed on sterile, moistened filter paper in a sterile Petri dish.

Moisture contents of all samples were determined on fresh weight basis using oven drying at 103 ± 2 °C for 17 hours as recommended by International Seed Testing Association (ISTA 1985).

Encapsulation/dehydration

For cryopreservation, encapsulation/dehydration method was performed using the modifications of Matsumoto and Sakai (1995). Fifteen embryos (5 embryos × 3 replicates) were initially precultured for one day on solid MS (Murashige and Skoog 1962) medium containing 0.3 M sucrose. The aseptically excised embryos were then suspended in sterilized alginate solution containing 3% (w/v) sodium alginate and 0.4 M sucrose, prepared in MS basal medium (pH 5.8). Using a pipette, droplets of the mixture were dispensed into a calcium solution (comprising 100 mM calcium chloride and 0.4 M sucrose). The encapsulation around the embryos was allowed to polymerize in the calcium solution for 30 min after which the embryos were dispensed into conical flasks containing cryoprotectant solution (0.8 M sucrose and 1 M glycerol). The embryos were then incubated for 16 hours at 25 °C. Following incubation, the beads (polymerized embryos) were removed from the medium and blotted dry with sterile filter paper. The beads were then spread evenly on sterile filter paper and desiccated in a laminar air flow cabinet for up to eight hours. After each drying period, the beads were withdrawn for determination of moisture content and viability (tetrazolium test) and also for storage in liquid nitrogen.

Cryoprotectant toxicity trial for PVS2 solution

Another set of 15 embryos were transferred into 1.8 ml cryotubes and exposed sequentially to step-wise addition of PVS2 solution designed by Sakai *et al.* (1990), consisting of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15%

(w/v) DMSO in MS medium (50%, 60%, 80%, 100% and 50–100% sequence). All exposures were for 5 min. The PVS2 solution was then removed and replaced with sucrose unloading solution (1.2 M sucrose in MS medium) and the embryos were maintained in this solution for 5 min. The embryos were then placed in clean Petri dishes flooded with 1% (w/v) tetrazolium chloride and incubated in the dark overnight. Toxicity test was performed at room temperature (25 °C) and on ice (0 °C).

Sensitivity to PVS2 solution

Fifteen encapsulated embryos (5 embryos × 3 replicates) were suspended into 1.8 ml cryotubes with 0.5 ml fresh PVS2 solution at 25 °C and 0 °C for 5, 10 and 15 min. Each embryo was assessed for tetrazolium salts staining. To determine the optimum time of exposure to PVS2, the embryos were dehydrated with PVS2 for different periods of time and at different temperatures (25 °C and 0 °C) prior to direct plunge in liquid nitrogen.

Storage in liquid nitrogen

Embryos were placed in 1.8 ml cryotubes and directly immersed into liquid nitrogen for 24 hours. The embryos were then assessed for viability using tetrazolium salts.

Tetrazolium test

The test applied followed the description contained in ISTA (1996). It was done immediately after the cryoprotectant toxicity trial for PVS2 solution and sensitivity to PVS2 experiment. The embryos were cut longitudinally and placed in 1% tetrazolium salt solution and allowed to soak in the dark overnight. The embryos were assessed according to the extent of staining on the tissue. Scoring number 1 meant slight staining in approximately one-third of the tissue, 2, in over half of the tissue and 3, in the majority of the tissue. Embryos that scored 3 showed healthy tissues and will most likely germinate.

RESULTS AND DISCUSSIONS

The average moisture content of *E. tapos* seeds in this study was 53%. The moisture content of embryos without associated cotyledon material was 56%. The moisture content profile of *E. tapos* seeds and embryos showed that they belong to a recalcitrant group of seeds.

Effects of moisture content on encapsulated *E. tapos* embryos

Moisture contents of encapsulated embryos, dried in a laminar air flow cabinet for up to eight hours showed reduced moisture contents, i.e. 56 to 17% (Table 1). All polymerized embryos without desiccation (initial moisture content of 56%) did not survive storage in liquid nitrogen. However, beads with reduced moisture contents from 19 to 30% gave good survival rates between 80 and 100% respectively (Table 1). This shows that embryos of certain recalcitrant seeds can be dried to a level below their high moisture freezing limit and they can then be stored in liquid nitrogen (Stanwood & Bass 1978, Chin &

Table 1 Moisture content and recovery of encapsulated embryos of *E. tapos* after desiccation and storage in liquid nitrogen assessed by tetrazolium salt test

Desiccation time (hour)	Moisture content (%)	Survival after desiccation*	Survival after storage in liquid nitrogen*
0 (control)	55.69	0	0
2	46.80	0	20%: 3
4	30.11	100%: 2	20%: 3 40%: 2 40%: 1
6	17.57	0	40%: 2 60%: 1
8	18.84	0	40%: 2 40%: 1

n = 15

* 1 = Slight staining in approximately one-third of the tissue; 2 = staining in over half of the tissue; 3 = staining in the majority of the tissue

Roberts 1980). However, the ranges of acceptable moisture content limits that facilitate storage in liquid nitrogen differ from one species to the another (Dickie & Smith 1995). The water content of encapsulated shoot tips for optimal survival varies among plant species because of their differences in dehydration tolerance but is usually about 20% on fresh weight basis (Withers & Engelmann 1998).

Effects of PVS2 toxicity trial on viability of *E. tapos* embryos

Kinetics of the exposure of explants to the vitrification solutions is critical for explants survival (Towill & Jarrett 1992). Therefore, exposure of the embryos to vitrification solution is usually done in two steps, i.e. by placing the embryos in low concentrations of cryoprotectant to facilitate permeation of individual solutes and then subsequently transferring them to higher concentrations to provide the desired degree of desiccation.

We observed that viability (red staining) was evident throughout the cells of the root and cotyledon tissues. The application of PVS2 solution ranging from 50 to 100% was not toxic within the exposure times tested both on ice and at room temperature (Table 2). All embryos showed high viability (100%). Therefore, in order to confirm which condition is the best, another experiment was conducted in which the effects of different exposure times of PVS2 solution on embryos, on ice and at room temperature, were examined.

Effects of exposure time to PVS2 solution on viability of *E. tapos* embryos

Before immersion in liquid nitrogen, exposure times of 5 to 15 min at 25 °C gave 20 to 40% viability (Table 3). After immersion in liquid nitrogen viability after exposure to PVS2 for 5 min at 25 °C was 80%. Conversely, embryos without treatment in liquid nitrogen at 0 °C had viability ranges from 20–80% after 5–15 min exposure to PVS2 while with liquid nitrogen, 0–100% with maximum survival after 10 min exposure. Viability declined sharply (0%) after 15 min exposure.

Table 2 Survival of *E. tapos* embryos after exposure to PVS2 solution for 5 min at different temperatures

PVS2 concentration (%)	Survival (%)	
	25 °C	0 °C
0 (control)	100	100
50	100	100
60	100	100
80	100	100
100	100	100
50–100 % sequence	100	100

n = 15

Table 3 Effects of exposure times of PVS2 on *E. tapos* embryos at different temperatures

Cryopreservation treatment/time	Survival after exposure to 100% PVS2 solution*		
	5 min	10 min	15 min
Without liquid nitrogen 25 °C	20%: 1 20%: 2	20%: 1 20%: 3	20%: 1
With liquid nitrogen 25 °C	80%: 1	0	0
Without liquid nitrogen 0 °C	80%: 1	20%: 1	20%: 1
With liquid nitrogen 0 °C	60%: 1	100 %: 1	0

n = 15

* 1 = Slight staining in approximately one-third of the tissue; 2 = staining in over half of the tissue; 3 = staining in the majority of the tissue

Application of vitrification solutions at lower temperatures such as 0 °C reduced their toxicity to explants (Matsumoto *et al.* 1994, Huang *et al.* 1995). Our results suggest that *E. tapos* embryos are sensitive to long-term exposure to PVS2 and chilling treatments are required for application of cryoprotectant solutions. In short, PVS2 exposure time of 10 min and at 0 °C were optimum for *E. tapos* embryos in vitrification technique.

In this study viability was measured using tetrazolium salt assay. Viable seeds are seeds that have the potential to produce normal seedlings. Such seeds stain completely, or if only partly stained, the staining patterns indicate that the essential structures are viable (ISTA 1999). For future work, when the cryopreservation protocol for *E. tapos* is developed, thawed embryos will be germinated *in vitro* on MS media and growth will be monitored regularly.

CONCLUSIONS

The present findings demonstrated that *E. tapos* seeds and embryos have a high moisture status, which shows that they are recalcitrant. Embryos of *E. tapos* when coated with alginate can withstand desiccation to about 17% and showed high viability after being kept in liquid nitrogen. In addition, vitrification appeared to be a promising method in conserving this germplasm. The tetrazolium test illustrated that this test can be used for evaluating viability of this species to obtain fast results towards developing a cryopreservation protocol.

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