Acta Physiol Plant (2013) 35:2629–2634
DOI 10.1007/s11738-013-1284-z

SHORT COMMUNICATION

Cryopreservation of immature *Parkia speciosa* Hassk. zygotic embryonic axes following desiccation or exposure to vitrification solution

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Received: 29 September 2012 / Revised: 29 March 2013 / Accepted: 3 April 2013 / Published online: 12 April 2013

Abstract This work reports on the cryopreservation of immature zygotic embryonic axes (EA) of petai (*Parkia speciosa* Hassk.) for the first time. Two cryopreservation protocols, namely desiccation and vitrification method were tested individually using excised EA. Desiccation of EA to lower moisture content (MC) reduced the survival percentage but a drastic decline in survival percentage (~20 %) was recorded at 16 % MC prior to exposure to LN, rendering the EA to be sensitive to desiccation. Cryopreservation of EA after desiccation, irrespective of the MC, did not result in any survival. On the other hand, post-cryopreservation survival was obtained when the EA were exposed to plant vitrification solution-2 (PVS2) for 75–105 min. The best results were obtained when the EA were exposed to PVS2 for 90 min with an average recovery of 55.5 %. EA recovery into whole plantlets was obtained when the EA were cultured on MS medium supplemented with 2 gl^{-1} activated charcoal and 0.1 mgl^{-1} of the plant growth regulators α-naphthalene acetic acid, 6-benzylaminopurine and gibberellin A_3, each. EA, exposed for less than 75 min and more than 105 min to PVS2, did not show any survival after cryopreservation. The optimization of exposure time is necessary to increase survival. This study has shown that the employment of suitable method is important for conservation using cryopreservation.

Keywords Desiccation · Liquid nitrogen · PVS2 · Recalcitrant seeds · Survival · Tree · Vitrification

Communicated by M. Capuana.

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Abbreviations

EA	Zygotic embryonic axes
LAF	Laminar air flow
LN	Liquid nitrogen
LS	Loading solution
MC	Moisture content
MS	Murashige and Skoog (1962)
PGR	Plant growth regulator
PVS2	Plant vitrification solution-2

Introduction

Parkia speciosa Hassk. (common name: petai) is a tall, heavily branched perennial tree belonging to the family Leguminosae. It is native to South-East Asia and is commonly found wild in lowland forests throughout Malaysia, Thailand, Indonesia and northern India (Roshetko et al. 2008). The tree produces large pods measuring about 35–50 cm in length and 3–5 cm in width. Each pod contains around 10–15 seeds. Traditionally, petai seeds have been used for their medicinal (antioxidant and antimicrobial) (Kawamura et al. 2011) and nutritional quality (Wiradinata and Bamroongrugsa 1994). Commercial petai plantations do not exist; and mostly, the pods are collected directly from the forests. Due to the economic value of the immature seeds, pods are seldom allowed to mature. Thus, the availability of mature seeds as a source for planting material as well as for storage is scarce.

Legumes generally are orthodox in their seed storage behavior; however, petai being a perennial legume, is recalcitrant (Nadarajan et al. 2008). According to Corredoira et al. (2004), long-term storage of recalcitrant seeds in a

germplasm bank is expected to rely on cryopreservation, as other methods cannot retain viability for long. Although cryopreservation is the most likely solution to this problem, yet success is challenged by the fact that recalcitrant seeds are large and are shed at high water content (Wesley-Smith et al. 2001). Hence, often in large-seeded materials, the zygotic embryonic axes (EA) are utilized as they are more amenable to desiccation (Reviewed by Normah and Makeen 2008). In addition, the developmental stage of the seeds plays an important role for successful cryopreservation (Engelmann et al. 1995). However, due to the natural characteristic of recalcitrant seeds with no arrest in their development (as with orthodox seeds), it is difficult to select seeds at a precise developmental stage, even though this is often a critical parameter. Moisture content (MC) of the EA is the most critical factor for a successful cryopreservation protocol, particularly the rate of desiccation (Makeen et al. 2006). Other aspects to be considered are physiological status of the plant materials (Makeen et al. 2005), cryoprotectant treatments (Suranthran et al. 2012) and recovery medium (Al-Zoubi and Noor 2009). Two types of cryopreservation protocols have been highlighted. The conventional technique which involves slow cooling down to a defined pre-freezing temperature, followed by rapid immersion in liquid nitrogen (LN), with freezing done in the presence of ice, while in the newer vitrification-based protocols, cell dehydration is performed prior to freezing by exposure of samples to concentrated cryoprotectants and/or air desiccation. In the vitrification-based protocols an osmotically treated embryo can be rapidly cooled to ultra-low temperature, where ice nucleation is prevented during vitrification, or glass-forming process (Volk and Caspersen 2007). However, vitrification medium formulation and exposure duration are two key factors for successful cryopreservation (Walters et al. 2002).

Studies on the conservation of petai seeds have not been carried out thus far. Currently, only one report on petai cryopreservation is available where shoot tips have been used as the plant material (Nadarajan et al. 2008). Hence, the present study aims to establish a cryopreservation protocol for petai (*Parkia speciosa* Hassk.) using the immature EA as the suitable propagule, following desiccation or vitrification.

Materials and methods

Plant material

Fresh 30 days old immature (green) petai pods were collected from trees located at Bidor, Perak, Malaysia. They were washed under running tap water and drained briefly.

Surface sterilization

Seeds were isolated from the pods and sterilized with 20 % Clorox® (Clorox Co., Broadway, Oakland, CA) (1.2 % sodium hypochlorite) solution for 10 min with rigorous shaking inside the laminar air flow (LAF) cabinet, followed by three rinses in sterilized distilled water. EA were excised and placed on moist filter paper (Whatman no. 4, Whatman Ltd., England) in sterilized Petri dish (15 × 90 mm) to avoid moisture loss.

Desiccation

Surface sterilized embryos were transferred to thin strips of fresh filter paper, approximately 10 × 50 mm in size, with five EA per strip, in Petri dishes under complete aseptic condition. Desiccation was performed by placing open Petri dishes with EA inside the clean bench under the flow of sterile air in the LAF cabinet for 1, 2, 4 and 5 h. A batch of embryos without desiccation (0 h) was treated as control. Temperature was controlled at 27 ± 2 °C and kept stable throughout the experimentation. Upon completion of desiccation, a sample batch of EA was subjected to MC assessment (see "Determination of moisture content") while the remaining EA were either transferred directly to regeneration media (see "Assessment of survival by in vitro regeneration"), or exposed to LN (see "Ultra-low freezing").

Exposure to loading solution and plant vitrification solution-2

The isolated EA, each weighing 10 ± 2 mg, were placed in 2 ml cryo-vials (ten EA per vial) and then loaded with fresh 2.0 M glycerol + 0.4 M sucrose (Nishizawa et al. 1992) solution for 30 min at 25 °C. Murashige and Skoog (1962) (MS) basal medium was used as control, since the loading solution (LS) was prepared using the MS liquid medium. Following the osmo-protecting LS treatment, the EA were subsequently exposed to a concentrated Plant vitrification solution-2 (PVS2) [30 % (w/v) glycerol + 15 % (w/v) EG + 15 % (w/v) DMSO + 0.4 M sucrose (formulated by Sakai et al. 1990)] in MS medium, for 0, 15, 30, 45, 60, 75, 90, 105 or 120 min. The pH was adjusted to 5.7 after all the components were added into MS medium and finally, the volume was made up to 100 ml. The solutions were kept in a refrigerator at 4 °C until use. The MC of the EA was measured after each exposure period (see "Determination of moisture content") and subjected to regeneration (see "Assessment of survival by in vitro regeneration") without or upon direct exposure to LN (see "Ultra-low freezing"), in two groups as mentioned earlier in "Desiccation" section.

Determination of moisture content

The MC of EA was determined following the low constant temperature oven method (ISTA 2005). A sample of EA, which went through simple desiccation, sucrose-preculture desiccation or vitrification following the respective treatments were placed on a pre-weighed aluminum boat. The weight of boat with EA was recorded and subsequently these were placed in oven at 103 °C for 16 h. Upon removal from the oven, the EA were placed in desiccator to cool. After 30 min, the boat with the dried EA was reweighed. The MC was measured in terms of loss of weight and expressed as a percentage of initial fresh weight. The MC of EA was calculated using the following formula (ISTA 2005): MC % = $[(W_2–W_3)/(W_2–W_1)] \times 100$; where W_1 = weight of aluminum boat, W_2 = weight of aluminum boat + EA before drying, W_3 = weight of aluminum boat + EA following drying.

Ultra-low freezing

The remaining EA, following desiccation or vitrification, were placed in sterile polypropylene cryotubes and plunged directly into LN for 1 h in a cryogenic tank (Model 34-XT, USA). The controls, for this experiment, were the EA which were unexposed to LN. However, for vitrification-treated EA, the PVS2 solution was removed from the cryotubes using a Pasteur pipette, and replaced with fresh 100 % PVS2 before direct plunge in LN. The frozen EA in the cryotubes were re-warmed quickly in a 38 ± 2 °C water-bath for 90 s with continuous shaking. EA following thawing were advanced for growth recovery in regeneration medium. EA treated with PVS2 were washed with liquid MS medium containing 1.2 M sucrose for a total period of 20 min, with 5 min intervals at 26 ± 2 °C before transferring to regeneration medium.

Assessment of survival by in vitro regeneration

The cryopreserved EA, after rapid warming, were transferred on top of two layers of sterile filter paper on Petri plate containing semi-solid MS medium, 3 % (w/v) sucrose and 100 mgl^{-1} (w/v) myo-inositol, and 0.75 % (w/v) agar Type A (Sigma Chemical Co., Germany) devoid of plant growth regulator (PGR). The EA cultures were kept in the dark for 7 days prior to exposure to the light and subsequent transfer to regeneration (MS) media supplemented with 2 gl^{-1} activated charcoal and 0.1 mgl^{-1} each of the PGR: α-naphthalene acetic acid, 6-benzylaminopurine and gibberellin A_3 (Suranthran et al. 2011), for 6 weeks. "Emergence" was scored as the percentage of EA showing root and/or shoot formation throughout this period; "survival" comprising "emergence" was scored as the percentage of EA showing noticeable elongation.

In vitro culture conditions

The basal MS medium used, containing 3 % (w/v) sucrose and 100 mg l^{-1} myo-inositol, was solidified with gelrite. PGRs were added to the medium as specified above. The pH of the medium was adjusted to 5.7 using 1 N NaOH or HCl prior to the addition of gelrite. After the addition of 0.8 % (w/v) gelrite (Merck), media was sterilized in an autoclave (Hirayama, Japan) at 121 °C and 1 kg cm^{-2} for 20 min. PGR solutions were filter-sterilized (membrane filtration 0.22 μm) and added to autoclaved basal medium when the temperature dropped to 50 °C. The cultures were maintained at 25 ± 1 °C either in dark or under 16 h photoperiod, which was provided by cool, white fluorescent light (Phillips Lifemax, Thailand) with an irradiance of 50 μmol $m^{-2}s^{-1}$ photosynthetic photon flux density (measured with LI-COR, Lincoln, USA).

Data analysis

A completely randomized design (CRD) was followed for all the experiments. Experiments were replicated five times with 25 samples per replication. The collected data were analyzed statistically using one-way analysis of variance (ANOVA). Significant differences among the treatments means were compared based on Duncan's multiple range test (Duncan 1955) at a P value = 0.05 using SAS®-vers.6.12 (SAS Institute, Cary, NC, USA) (SAS Institute 1999) software package. Survival percentage and MC (percentage) obtained were transformed to arc-sign values before ANOVA was carried out and was converted back to the original scales (Compton 1994).

Results and discussion

Desiccation

Fresh petai EA were high in MC, recording 80–85 %. When the freshly excised EA were cultured in MS medium, 100 % survival was obtained (Table 1). The EA swelled and radicle expansion was observed within 2 days. Shoot emergence occurred at day 4 and fully expanded leaves were seen within 2 weeks. In this study, direct air desiccation using LAF was used to dehydrate the EA since it is one of the easiest and most straight forward desiccation methods. The MC of EA placed in the air current of LAF cabinet decreased gradually from 85 to 13.2 % within 5 h (Table 1). There was a rapid dehydration initially followed by a slower rate of dehydration from 3 to 5 h. Although desiccation resulted in shrunken

Table 1 Influence of desiccation period on moisture content and survival of petai (*Parkia speciosa* Hassk.) embryonic axes prior (−LN) and after (+LN) exposure to liquid nitrogen

Desiccation period (h)	Moisture content (%)	Survival (%)	
		−LN	+LN
0	85.0 ± 1.4[a]	100.0 ± 0.0[a]	0.0 ± 0.0[a]
1	64.9 ± 0.8[b]	100.0 ± 0.0[a]	0.0 ± 0.0[a]
2	39.3 ± 0.6[c]	82.0 ± 0.7[b]	0.0 ± 0.0[a]
3	26.2 ± 0.6[d]	57.5 ± 0.5[c]	0.0 ± 0.0[a]
4	16.0 ± 0.8[e]	19.5 ± 0.6[d]	0.0 ± 0.0[a]
5	13.2 ± 0.6[e]	15.0 ± 1.1[e]	0.0 ± 0.0[a]

Data represent mean of 5 replicate with 25 explants per treatment. Data expressed as percentage were transformed using arc sine prior to ANOVA and converted back to the original scale for demonstration in the table (Compton 1994). Data for each *column* followed by different *alphabets* are significantly different according to Duncan's multiple range test (Duncan 1955) at $P = 0.05$

embryos, upon culturing they rehydrated quickly, gained their initial size and germinated with no difference as compared to their non-desiccated counterpart. However, this was true only for EA with MC above 40 %. Desiccation to below 40 % MC affected survival, and further desiccation to below 20 % reduced survival to 15 %, indicating that petai embryos were susceptible to desiccation damage at low MC. In nature, tolerance to desiccation in orthodox seeds and embryo is acquired during seed maturation whereby the embryos can lose up to 90 % of its initial MC and thereafter enters a state of metabolic arrest (Bartels et al. 1996). Recalcitrant seeds, on the other hand are intolerant of desiccation even at maturity and often do not survive drying (Chandler and Robertson 1994). In this study the EA of petai desiccated to various MC ranging from 85 to 13.2 % were not able to tolerate LN exposure. However, this complete failure in survival with 85–13.2 % MC upon exposure to LN in the present study could be a common phenomenon, as mentioned by Popov et al. (2006). Failure of EA with high MC is attributed to the formation of ice crystals during the freezing and subsequent thawing which results in cell fatality. The ice crystals are formed due to the presence of free water in the intracellular space (Popov et al. 2006).

According to Engelmann (1997), zygotic embryos are anatomically highly heterogeneous which display differential sensitivity to desiccation depending on the characteristics of their cells. Dussert et al. (2001) has mentioned that in most cases there is indeed a narrow window of hydration levels within which survival is possible, and the optimal water content for cryopreservation usually corresponds to the unfrozen water content, as shown notably with coffee and citrus seeds. However, there may be a limitation in achieving the precise moisture within the hydration window for cryopreservation using the simple desiccation method. The use of more precise desiccation technique using saturated salt solution, which have been successfully utilized in coffee and citrus (Hor et al. 2005; Dussert and Engelmann 2006) may allow the correct moisture corresponding to unfreezable water to be attained accurately.

Another factor related to desiccation sensitivity of petai EA might be the extent of cell shrinkage experienced during dehydration. According to Walters et al. (2002), a partially mature EA containing less fresh mass resulted in a 60 % volume change if dehydration alone is used to protect cells from ice during LN exposure. These severe volume alterations might result in irreversible mechanical injury to cells (Reviewed by Pritchard and Nadarajan 2008) in accordance with the hypothesis that most cells tolerate <50 % change in their volume. This again relates to the rate and method of drying adopted; for example, according to Walters et al. (2008), the usage of slow drying can avoid the mechanical stress resulting in cell shrinkage. Hence, the use of air desiccation method needs to be further investigated before it can be precluded as a method for effective cryopreservation of petai EA. Other research on cryopreservation of embryos, such as Cho (2001), reported that citrus embryos with a MC of 12–17 % were successfully cryopreserved using air drying. Moreover, air desiccation method has been used for the cryopreservation of many species such as large-seeded 18 tree species of *Aesculus*, *Castanea*, *Quercus*, *Juglans*, *Carya*, *Corylus*, and *Fagus* genera (Pence 1990) where survival rates of 40–100 % were obtained using EA as explants.

It appears that tolerance to freezing has to be induced in petai EA before they can be subjected to LN. According to Walters et al. (2008), low survival subsequent to nonlethal desiccation and adequately rapid cooling in LN is frequently reported for EA from immature seeds. Evidently, these EA were found to be deficient in protective mechanisms that are found in other recalcitrant embryos. Employing exogenous protectants to EA may improve survival in these circumstances (Walters et al. 2002). To prevent cellular damage, cryoprotectant solutions both dehydrate and penetrate cells to stabilize proteins and membranes; however, the mode of action of each component may differ with cell type, species, temperature, and other solution components (Reviewed by Volk et al. 2006). However, a physical process by which a highly concentrated cryoprotective solution supercools to very low temperatures and finally solidifies into a metastable glass without undergoing crystallization at a practical cooling rate would be effective in such a situation (Sakai et al. 2008).

Vitrification

The MC of EA, subsequent to exposure to LS, was 70.8 % and had 100 % recovery (data not shown), indicating LS

Table 2 Influence of exposure duration to vitrification solution (using PVS2) on survival of petai (*Parkia speciosa* Hassk.) embryonic axes prior (−LN) and after (+LN) exposure to liquid nitrogen

Exposure duration (min)	Moisture content (%)	Survival (%)	
		−LN	+LN
0	80.8 ± 1.5[a]	100.0 ± 0.0[a]	0.0 ± 0.0[d]
15	62.8 ± 1.1[b]	100.0 ± 0.0[a]	0.0 ± 0.0[d]
30	50.6 ± 1.0[c]	100.0 ± 0.0[a]	0.0 ± 0.0[d]
45	45.7 ± 1.1[d]	100.0 ± 0.0[a]	0.0 ± 0.0[d]
60	43.9 ± 1.2[de]	100.0 ± 0.0[a]	0.0 ± 0.0[d]
75	42.2 ± 1.0[de]	70.0 ± 0.5[b]	25.0 ± 1.4[c]
90	41.2 ± 1.1[ef]	60.5 ± 0.6[c]	55.5 ± 0.7[a]
105	40.5 ± 1.5[ef]	52.5 ± 0.8[d]	45.0 ± 0.6[b]
120	37.9 ± 0.7[fg]	27.5 ± 1.2[e]	0.0 ± 0.0[d]
135	35.4 ± 0.9[g]	0.0 ± 0.0[f]	0.0 ± 0.0[d]
150	35.1 ± 0.7[g]	0.0 ± 0.0[f]	0.0 ± 0.0[d]

Data represent mean of 5 replicate with 25 explants per treatment. Data expressed as percentage were transformed using arc sine prior to ANOVA and converted back to the original scale for demonstration in the table (Compton 1994). Data for each *column* followed by different *alphabets* are significantly different according to Duncan's multiple range test (Duncan 1955) at $P = 0.05$

were not toxic to the embryo. Based on the MC of freshly excised EA (85 %), the LS was able to trigger initial dehydration of the EA due to exposure to hypertonic environment. In addition, other researchers have shown that osmoprotection with 2 M glycerol and 0.4 M sucrose proved to be effective in enhancing the capacity of cells to tolerate the more serve dehydration with PVS2 (Hirai and Sakai 2003; Matsumoto and Sakai 2003; Kobayashi et al. 2006). Upon exposure to PVS2 solution for various periods, the MC of the petai EA declined by 5–12 % during the first 45 min before decreasing at a constant rate (% MC/min) of 1–2 % subsequently (Table 2). After 75 min of exposure to PVS2 with a MC of 42.2 %, the survival percentage of non-cryopreserved EA dropped to 70 %. It was observed that beyond 120 min of exposure in PVS2 no survival was obtained, suggesting that prolonged exposure was toxic to the tissue. Consequently, zero survival was observed during post-freezing. Exposure duration of 75, 90 and 105 min proved to be fruitful in retaining 25, 55.5 and 45 % survival, respectively (Table 2) after cryopreservation. It is a known fact that most living organisms are composed of large amounts of water, hence freezing such organs result in ice-formation. This phenomenon was clearly seen based on the findings of the previous experiment whereby EA with high MC did not survive cryopreservation. Thus, approaches which prevent ice-crystal formation have been explored, leading to the concept of including substances that work as antifreeze, more commonly known as cryoprotectants. One of the most popular concoctions of such substance is the PVS2 by Sakai et al. (1990). Exposure to PVS2 not only osmotically dehydrates cells at nonfreezing temperature, but the vitrification solution subjects cells to osmotic stress, making it very likely for some constituents to enter into cells thus making the cytoplasm more viscous. The highly viscous cytoplasm will vitrify during freezing by phase transition of aqueous solution from a liquid into an amorphous glassy solid, thus avoiding crystallization. However, the penetrative nature of some of the cryoprotectants may also result in toxicity (Matsumoto et al. 1994). Thus, careful determination on the time of exposure is critical.

Petai EA lost more than 50 % of their initial MC upon exposure to PVS2 for 90 min but were able to retain more than 50 % survival after exposure to LN. The survival percentage recorded is commendable as compared to using the direct desiccation method which yielded no survival. However, further improvements can be attempted by manipulating the plant material before cryopreservation. Notably, pre-growth on media containing cryoprotective substances may confer on the tissues increasing tolerance to further desiccation and reducing the heterogeneity of the material (Engelmann 2000; Pence 1995).

Author contribution U.R. Sinniah—conceived the research idea and designed the experiments; S. Gantait—executed the experiments; S. Gantait, U.R. Sinniah—wrote the manuscript.

Acknowledgments The authors are grateful to Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia for providing the research facilities.

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