Plant Cell Rep (2009) 28:883–889
DOI 10.1007/s00299-009-0691-5

CELL BIOLOGY AND MORPHOGENESIS

Influence of freezable/non-freezable water and sucrose on the viability of *Theobroma cacao* somatic embryos following desiccation and freezing

Jong-Yi Fang · Moctar Sacandé · Hugh Pritchard · Andy Wetten

Received: 18 November 2008 / Revised: 29 January 2009 / Accepted: 19 February 2009 / Published online: 10 March 2009

Abstract Encapsulated cocoa (*Theobroma cacao* L.) somatic embryos subjected to 0.08–1.25 M sucrose treatments were analyzed for embryo soluble sugar content, non-freezable water content, moisture level after desiccation and viability after desiccation and freezing. Results indicated that the higher the sucrose concentration in the treatment medium, the greater was the extent of sucrose accumulation in the embryos. Sucrose treatment greatly assisted embryo post-desiccation recovery since only 40% of the control embryos survived desiccation, whereas a survival rate of 60–95% was recorded for embryos exposed to 0.5–1.25 M sucrose. The non-freezable water content of the embryos was estimated at between 0.26 and 0.61 g H_2O g^{-1}dw depending on the sucrose treatment, and no obvious relationship could be found between the endogenous sucrose level and the amount of non-freezable water in the embryos. Cocoa somatic embryos could withstand the loss of a fraction of their non-freezable water without losing viability following desiccation. Nevertheless, the complete removal of potentially freezable water was not sufficient for most embryos to survive freezing.

Keywords Freezable water · Non-freezable water · Desiccation · Freezing · Sucrose · Cocoa · *Theobroma cacao* · Somatic embryo · Cryopreservation

Communicated by J. Bennett.

J.-Y. Fang (✉)
Department of Tropical Agriculture and International Cooperation, National Pingtung University of Science and Technology, No. 1 Shueh Fu Road, Neipu, Pingtung 91201, Taiwan
e-mail: jyfang@mail.npust.edu.tw

M. Sacandé · H. Pritchard
Seed Conservation Department, Royal Botanic Gardens, Kew, Wakehurst Place, Ardingly, Haywards Heath, West Sussex RH17 6TN, UK

A. Wetten
School of Biological Sciences, University of Reading, Whiteknights, PO Box 221, Reading, Berkshire RG6 6AS, UK

Introduction

The recalcitrant nature of cocoa (*Theobroma cacao* L.) seed with regard to low temperature storage means that cryopreservation of tissue-cultured germplasm represents the most attractive backup for vulnerable field collections of the species. With a view to this, an encapsulation and dehydration-based cryopreservation procedure was developed that involves the pretreatment of encapsulated cocoa somatic embryos with sucrose, followed by desiccation and liquid nitrogen storage (Fang et al. 2004). Recovery rates of between 25 and 72% were obtained depending on the genotype tested. For effective running of cryo-genebanks recovery rates of at least 50% from the frozen propagules are desirable. Further improvement of the procedure is therefore needed and this will be greatly assisted if the stages at which the injuries occur in the embryos are identified.

The desiccation stage in an encapsulation and dehydration procedure is crucial, as the presence of excess moisture may expose the cells to mechanical, chemical and osmotic stresses upon freezing (Wolfe and Bryant 2001; Benson 2008). However, excessive water extraction from cells can lead to desiccation damage as manifested by crystallization of salts and proteins in the cytoplasm, membrane lipid peroxidation and phase transition from liquid-crystalline to gel phase, disfunctionment of

membrane proteins and/or membrane collapse following mechanical stress (Crowe et al. 1989, 1992; Hoekstra et al. 1992; Varghese and Naithani 2002). The length of desiccation treatment to achieve optimal moisture contents in cryopreserved materials should therefore be controlled carefully. For many species this optimization has been achieved empirically by selection of the treatment which provided the highest explant post-thaw survival. For species such as cocoa for which the somatic embryo-based regeneration can be protracted and asynchronous, this empirical approach is time-consuming and requires a great number of explants.

Using a relatively straightforward calorimetric method and a limited number of cocoa somatic embryos, the amount of non-freezable water in the somatic embryos was determined. This parameter, together with the water content of the desiccated embryos prior to cryopreservation, was used to ascertain the relationship between the moisture status of the embryos and their desiccation and freezing tolerances. The influence of sucrose treatment on the water properties of somatic embryos was also evaluated.

Materials and methods

Plant material

Using floral explants from cocoa plants maintained at the University of Reading Intermediate Cocoa Quarantine Unit, somatic embryos were initiated from staminodes as described previously (Li et al. 1998; Maximova et al. 2002). Experiments were conducted using early-cotyledonary stage secondary somatic embryos of the genotype SPA4 (Forastero group). Somatic embryos were encapsulated within 3% alginate beads prior to the following analyses.

Sucrose treatment and soluble sugar analysis

Sucrose treatments were conducted by stepwise culture of somatic embryos in a 0.3 M sucrose-enriched medium for 3 days then on 0.5, 0.75, 1 or 1.25 M sucrose medium for another 4 days. The basal medium consisted of DKW basal salts, 100 mg l^{-1} *myo*-inositol, 2 mg l^{-1} thiamine-HCl, 1 mg l^{-1} nicotinic acid, 2 mg l^{-1} glycine and 2 g l^{-1} Phytagel (Li et al. 1998). The pH of the medium was adjusted to 5.7 before autoclaving. A control set of embryos was maintained on the basal medium which was supplemented with 0.08 M sucrose.

Individual somatic embryos were used to measure the soluble sugar contents. Sugar extraction was carried out according to Buitink et al. (2000). Following sucrose treatment, somatic embryos were carefully removed from the alginate beads and ground with 1 ml of 80% ethanol in a microcentrifuge tube. The samples were kept at 76°C in a water bath for 15 min to extract the soluble sugars and to inactivate enzymes. Subsequently, the ethanol was evaporated and the dried extract was dissolved in 1 ml distilled water. After centrifugation at 13,000 rpm for 1 min, 20 µl of the supernatant was sampled and analyzed using high performance liquid chromatography (Waters-746 HPLC). The separation of sugars was achieved by a Bio-Rad Aminex HPX-42C column (300 mm × 7.8 mm) heated to 80°C. The mobile phase was nanopure water and sugars were identified by comparison with retention times for the sugar standards: fructose (18.21 min), glucose (15.65 min), sucrose (13.51 min), raffinose (12.13 min) and stachyose (11.01 min). Quantification was achieved using integration of elution peaks and comparison with known amounts of the external standards. Between 10 and 15 samples were assessed for each sucrose treatment.

Thermal analysis of cellular water

Calorimetric properties of cellular water were measured for individual somatic embryos using differential scanning calorimetry (DSC-7, Perkin-Elmer, USA) following a method modified from Sacandé et al. (2000). Control and sucrose-treated embryos were first dried to different water contents after which the embryos were extracted from the beads and individually sealed in 10 µl Perkin-Elmer aluminum pans. The samples were cooled down to −50°C at 10°C min^{-1}, held at this temperature for 5 min and then rewarmed to 50°C at the same rate. Embryo cooling and warming thermograms were recorded. Baseline was determined using an empty pan, and all thermograms were baseline-corrected. The temperatures of water crystallization and melting, as well as the transition enthalpies were determined using Pyris Software for Windows (Perkin-Elmer thermal analysis). The warming thermograms were used for the determination of embryo non-freezable water content, as they were less prone to hysteresis than the first recorded cooling thermograms. The enthalpy (ΔH) of the melting transition was determined from the area under the peak. After recording the thermograms, the pans containing the samples were punctured and dried in an oven at 103°C for 17 h, after which they were weighed for the determination of embryo water content (ISTA, 1999). All the water contents were determined on a dry weight basis [(g fresh weight − g dw) g^{-1} dw]. The DSC was calibrated for temperature with indium (156.6°C) and cyclohexane (−87.06°C) standards and for energy with indium (28.45 J g^{-1}). As much as 7–13 embryos were used for each sucrose treatment.

Assessment of embryo moisture content and embryo survival following desiccation and freezing

Desiccation and freezing of somatic embryos were conducted as per Fang et al. (2004). Desiccation was carried out by placing control and sucrose-treated embryos over silica gel for 4 h. For freezing, desiccated embryos were immersed in liquid nitrogen for 1 h and thawed in a 35°C water bath for 5 min. Embryo recovery following desiccation and freezing took place in a 0.3 M sucrose medium for the first 3 days, then in a basal medium containing only 0.08 M sucrose. Two replicates of ten embryos were used for each sucrose treatment and their survival was recorded after two months. Survival data were arcsine transformed prior to ANOVA (SAS Institute Inc., Cary, NC, USA). Treatment means were ranked using Duncan multiple range test and difference tested at 5% probability.

The water contents of embryos following various sucrose treatments and 4 h desiccation were determined gravimetrically after drying in an 80°C oven for 24 h. Three to five embryos were used for each sucrose treatment.

Results

Soluble sugars analysis

As expected, it was found that the higher the sucrose concentration in the treatment medium, the higher was the amount of soluble sugars accumulated within the embryos (Table 1). The four soluble sugars identified were glucose, fructose, sucrose and stachyose. Sucrose represented the major sugar component and the extent of its accumulation was proportional to the concentration of sucrose in the medium. In contrast, no obvious relationship was found between the amount of glucose, fructose and stachyose in the sample and the concentration of exogenous sucrose. There was a great variation in the concentration of glucose, fructose and stachyose among the individual embryos examined.

Thermal analysis of cellular water

During the cooling scan, somatic embryos were first supercooled, followed by an exothermic peak corresponding to the crystallization of water. On rewarming, an endothermic peak corresponding to the melting of ice was observed. A selection of warming thermograms of 0.5 M sucrose-treated embryos dried to various moisture contents is presented in Fig. 1. As no transition was detected in the dry sample (0.31 g H_2O g^{-1}dw), this was interpreted as the presence of only a negligible amount of lipid in the embryos. The magnitude of the crystallization and melting peaks decreased with the moisture content of the embryos.

The amount of non-freezable water in somatic embryos subjected to the various sucrose treatments was estimated from the ΔH of the melting endotherms versus the moisture content relationships (Fig. 2). The areas under the endothermic peaks corresponding to the amounts of energy in J g^{-1}dw were determined and then plotted against the measured moisture contents of the samples. The results showed single linear regressions with the least-squares best fit. The intersection between the regression lines and the x-axis indicates the moisture content below which only non-freezable water remains. These water contents were 0.61, 0.46, 0.28, 0.35 and 0.26 g H_2O g^{-1}dw for the control, 0.5, 0.75, 1 and 1.25 M sucrose treatment, respectively. Treatment effects ($P < 0.05$) were analyzed by comparing the regression lines, which indicated that the non-freezable water content following sucrose treatments of 0.75, 1 and 1.25 M were not significantly different. The control treatment had the highest non-freezable water content (Table 2).

Embryo moisture content and embryo survival following desiccation and freezing

Embryo post-desiccation and post-freezing survival, embryo moisture content after desiccation determined gravimetrically, and embryo non-freezable water content following various sucrose treatments are compiled in Table 2. With the exception of 0.75 M sucrose treatment, the moisture content of 4 h-dried embryos in the control

Table 1 Soluble sugars accumulated in cocoa somatic embryos after 7 days of treatment in a 0.08–1.25 M sucrose-enriched medium

Sucrose treatment (M)	Soluble sugar concentration (mg g^{-1}dw)				
	Glucose	Fructose	Sucrose	Stachyose	Total
0.08 (Control)	2.38 (2.38)	0 (0)	4 (1.14)	2.81 (2.81)	9.20
0.5	0 (0)	0.31 (0.31)	6.23 (0.67)	0 (0)	6.54
0.75	0 (0)	0 (0)	6.49 (0.81)	0 (0)	6.49
1	0 (0)	0.65 (0.58)	9.86 (0.99)	0 (0)	10.51
1.25	0 (0)	0.50 (0.38)	11.82 (1.17)	0.24 (0.19)	12.56

Values in brackets represent SE, $n = 10$–15

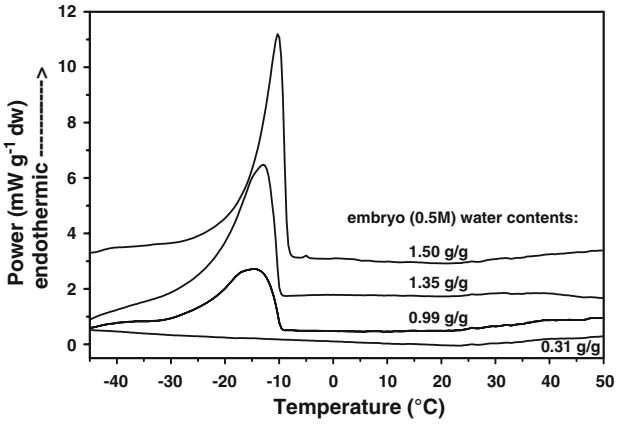

Fig. 1 Representative warming thermograms of 0.5 M sucrose-treated cocoa somatic embryos dehydrated to different moisture levels

and other sucrose treatments were comparable or inferior to the level of non-freezable water.

There was a significant difference in terms of embryo survival following desiccation between the different sucrose treatments. Only 40% of the control embryos survived desiccation, but survival was significantly improved if the embryos were treated with sucrose at concentrations higher than 0.5 M. In terms of embryo survival after freezing, 0.75–1 M sucrose-treated embryos had a significantly higher survival than those incubated with 1.25 M sucrose. No survival was recorded for the control embryos after freezing.

Discussion

In order to develop an efficient cryopreservation procedure for any plant species, a great number of explants are conventionally required for the testing of parameters affecting post-thaw survival. This posed a problem while working with cocoa, as somatic embryos of the species can only be regenerated after lengthy subculture cycles and, since embryo formation is highly asynchronous, the availability of sufficient embryos for cryopreservation testing at a given time is low. In the present study, the adoption of a calorimetric method allowed us to study the relationship

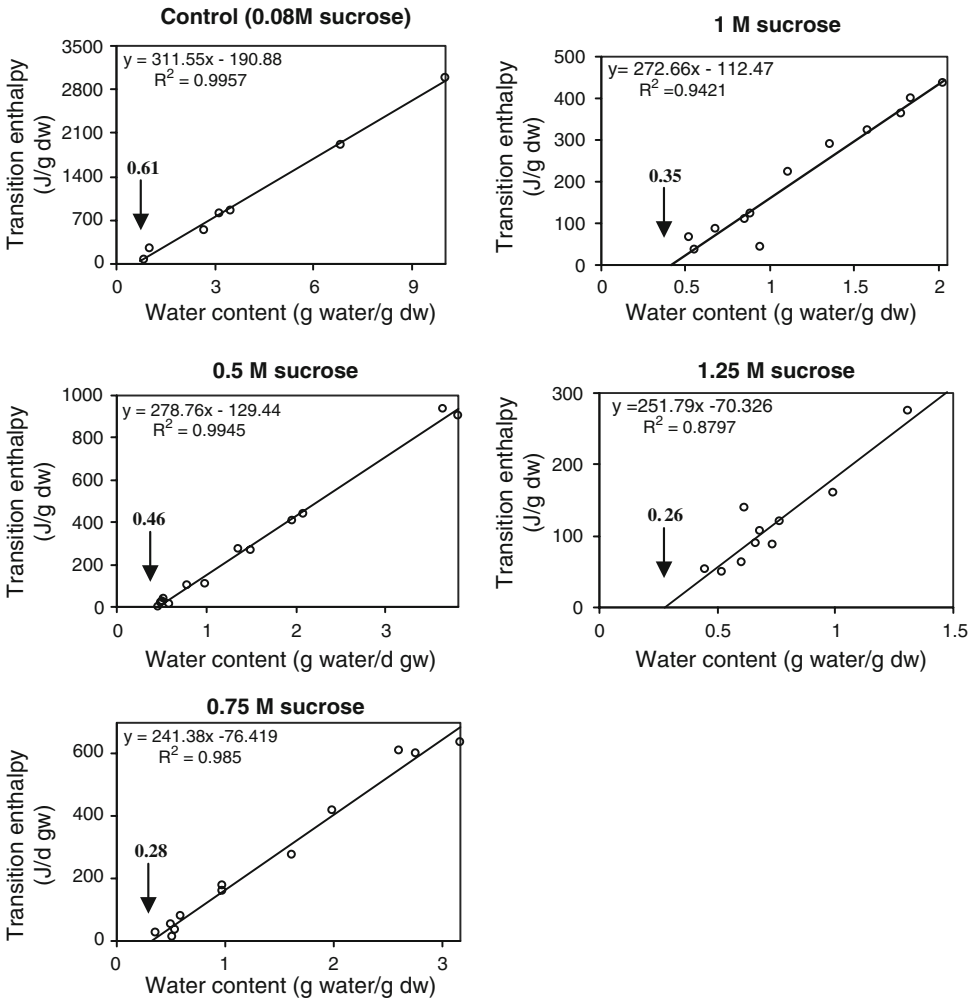

Fig. 2 Non-freezable water contents (*arrows*) of cocoa somatic embryos following 0.08–1.25 M sucrose treatments, $n = 7–13$

Table 2 Embryo water content after 4 h desiccation, embryo non-freezable water content and embryo survival following desiccation and freezing

Sucrose (M)	Embryo moisture content (g H_2O g^{-1}dw)		Embryo survival[1] (%)	
	After 4 h drying	Non-freezable	After desiccation	After freezing
0.08 (control)	0.46 (0.05)	0.61 a	40.0 d	0.0 d
0.5	0.38 (0.08)	0.46 b	90.0 ab	45.0 ab
0.75	0.43 (0.04)	0.28 c	95.0 a	50.0 a
1	0.19 (0.03)	0.35 c	83.3 bc	62.6 a
1.25	0.31 (0.06)	0.26 c	60.0 cd	20.0 bc

Values in brackets represent SE

Data with the same letters in a column were not significantly different at 5% probability

[1] Data were recorded after 2 months. $n = 2 \times 10$

between the amount and presence of freezable/non-freezable water and embryo survival after desiccation and freezing. This approach was particularly useful in the advancement of cocoa cryo-technology, as the moisture control in the explants is often the most difficult parameter to fine-tune in cryopreservation.

It is now widely accepted that desiccation prior to cryopreservation is important for obtaining high post-thaw survival of cryopreserved explants. Improved survival is thought to depend on the reduction of moisture down to a level at which no crystallization would occur in the cells during freezing. For example, post-thaw survival was successfully recorded for carrot somatic embryos when their moisture level was reduced to 16.4%, and for oil palm somatic embryos when these were dried to 0.5 g H_2O g^{-1}dw prior to freezing (Dereuddre et al. 1991; Dumet et al. 1993). In both cases, it was shown by calorimetric studies that the improved survival of the somatic embryos to liquid nitrogen was correlated with the progressive reduction of ice and glass formation during cooling (Dereuddre et al. 1991; Dumet et al. 1993). In the present study, survival of cocoa somatic embryos following freezing was achieved when their moistures were reduced to 0.19–0.38 g H_2O g^{-1}dw depending on the sucrose treatment. On comparing with the thermal analysis data, it can be seen that these moisture contents were in the range where freezable water was negligible or absent, except for the 0.75 M sucrose treatment. The presence of potentially freezable water in the 0.75 M sucrose-treated embryos was apparently not lethal to the explants, since their survival was comparable to the 0.5 and 1 M treatments. A similar situation was observed for the zygotic embryo axes of *Landolphia kirki*, which survived cooling to −80°C having retained a small amount of freezable water (Vertucci et al. 1991). The reason why the presence of some potentially freezable water was not lethal to cocoa embryos in the present study may be because the ice crystals formed upon cooling were not sufficiently large to lethally damage the explants. The location and size of ice crystals formed in 0.75 M sucrose-treated embryos will be investigated in future studies.

In nature, desiccation-tolerant organisms were found to withstand the loss of a substantial proportion of bound water (Crowe et al. 1992), whereas sensitive species were damaged if it was removed (Pammenter et al. 1991; Finch-Savage 1992). Several researchers have found that there is more bound water present in desiccation-tolerant plant tissues than in desiccation-sensitive tissues (Vertucci and Leopold 1987; Vertucci and Stushnoff 1992). Crowe et al. (1990) suggested that the removal of this water may result in profound changes in the physical properties of biomolecules, particularly phospholipids and proteins. This phenomenon has been reported for silver maple in which the removal of all freezable water resulted in damage when the seeds were dehydrated below 32% of their original moisture content, the threshold moisture level below which only non-freezable water remained (Becwar et al. 1983). However, it was observed in the present study that cocoa somatic embryos could withstand the loss of some of their non-freezable water without losing viability.

The development of desiccation and freezing tolerance in anhydrobiotic organisms in nature is often associated with the accumulation of soluble carbohydrates in particular disaccharides (Steadman et al. 1996; Sun and Leopold 1997). During drying, the cytoplasm transforms into a glassy state due to the drastic increase of cytoplasmic viscosity. Intracellular glasses exhibit a high molecular packing and slow molecular mobility, which can potentially interact with cytoplasmic components such as salts, organic acids and amino acids (Buitink and Leprince 2004). Sugars are known as good glass formers (Slade and Levine 1991), and have been associated with the stabilization of membrane, enzymes and macromolecules, and with the prevention of fusion between liposomes based on the water replacement hypothesis (Crowe et al. 1988, 2004; Ford and Dawson 1993). The present study showed that control cocoa somatic embryos were only able to partially recover (40%) from desiccation. Higher survival rates (60–95%) were

observed if embryos were treated with sucrose at concentrations higher than 0.5 M prior to desiccation. An increase in endogenous sugar content was detected in embryos after the exogenous application of sucrose. Sucrose represented the major soluble sugar in the embryos and its concentration was directly proportional to the concentration of sucrose in the treatment medium. Although sucrose exerted a protective role towards desiccation damage, it can be clearly seen that the amount of non-freezable water content was not affected by the sucrose content within the embryos. This finding contrasts with the work of Hitmi et al. (2000) in which the non-freezable water content of *Chrysanthemum cinerariaefolium* cells increased proportionally with the sucrose concentration (0.085–0.5 M) in the pregrowth medium and with the duration of sucrose preculture (0–30 days). The increase in the non-freezable water content of the cells was suggested by these authors to result from the interaction of sucrose with intracellular water through hydrogen and multimolecular linkages. We speculate that in the case of cocoa somatic embryos, sucrose mainly worked as an extracellular cryoprotectant and so was unable to raise the non-freezable water fraction in the cells.

The following conclusions could be drawn from the present study: (1) sucrose treatment above 0.5 M was beneficial for increasing cocoa embryo survival after desiccation, and thus after freezing, since the acquirement of desiccation tolerance was a prerequisite for the embryos to survive the subsequent freezing stage; (2) sucrose accumulation within cocoa somatic embryos increased with the sucrose concentration in the treatment medium, but this increase did not alter the water-binding properties in cells, suggesting the extracellular cryoprotective role of sucrose using the present cryogenic approach; (3) cocoa somatic embryos could withstand the loss of a fraction of their non-freezable water without losing viability following desiccation; (4) removal of all potentially freezable water from embryos was not sufficient to achieve high post-thaw survival in cocoa. Factors other than the moisture content of the embryos must be considered in the future while optimizing cryopreservation for this species.

References

Becwar MR, Stanwood PC, Leonhardt KW (1983) Dehydration effects on freezing characteristics and survival in liquid nitrogen of desiccation-tolerant and desiccation-sensitive seeds. J Am Soc Hortic Sci 108:613–618

Benson EE (2008) Cryopreservation theory. In: Reed BM (ed) Plant cryopreservation: a practical guide. Springer, New York, pp 15–32

Buitink J, Leprince O (2004) Glass formation in plant anhydrobiotes: survival in the dry state. Cryobiology 48:215–228

Buitink J, van den Dries IJ, Hoekstra FA, Alberda M, Hemminga MA (2000) High critical temperature above *T*g may contribute to the stability of biological systems. Biophys J 79:1119–1128

Crowe JH, Crowe LM, Carpenter JF (1988) Interactions of sugars with membranes. Biochim Biophys Acta 947:367–384

Crowe JH, Hoekstra FA, Crowe LM (1989) Membrane phase transitions are responsible for imbibitional damage in dry pollen. Proc Natl Acad Sci USA 86:520–523

Crowe JH, Carpenter JF, Crowe LM, Anchordoguy TJ (1990) Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules. Cryobiology 27:219–231

Crowe JH, Hoekstra FA, Crowe LM (1992) Anhydrobiosis. Annu Rev Physiol 54:579–599

Crowe JH, Crowe LM, Tablin F, Wolkers W, Oliver AE (2004) Stabilization of cells during freeze drying: the trehalose myth. In: Fuller BJ, Lane N, Benson EE (eds) Life in the frozen state. CRC Press, Boca Raton, pp 581–601

Dereuddre J, Hassen N, Blandin S, Kaminski M (1991) Resistance of alginate-coated somatic embryos of carrot (*Daucus carota* L.) to desiccation and freezing in liquid nitrogen: 2. Thermal analysis. Cryo Letters 12:135–148

Dumet D, Engelmann F, Chabrillange N, Duval Y, Dereuddre J (1993) Importance of sucrose for the acquisition of tolerance to desiccation and cryopreservation of oil palm somatic embryos. Cryo Letters 14:243–250

Fang J-Y, Wetten A, Hadley P (2004) Cryopreservation of cocoa (*Theobroma cacao* L.) somatic embryos for long-term germplasm storage. Plant Sci 166:669–675

Finch-Savage WE (1992) Embryo water status and survival in the recalcitrant species *Quercus robur* L.: evidence for a critical moisture level. J Exp Bot 43:663–669

Ford AW, Dawson PJ (1993) The effect of carbohydrate additives in the freeze-drying of alkaline phosphatase. J Pharm Pharmacol 45:86–93

Hitmi A, Coudret A, Barthomeuf C, Sallanon H (2000) Role of intracellular water retention strength in freezing tolerance of *Chrysanthemum cinerariaefolium* Vis. cell cultures. J Plant Physiol 157:47–53

Hoekstra FA, Crowe JH, Crowe LM (1992) Germination and ion leakage are linked with phase transitions of membrane lipids during imbibition of *Typha latifolia* pollen. Physiol Plant 84:29–34

Li Z, Traore A, Maximova S, Guiltinan MJ (1998) Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. In Vitro Cell Dev Biol Plant 34:293–299

Maximova SN, Alemanno L, Young A, Ferrière N, Traore A, Guiltinan MJ (2002) Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L. In Vitro Cell Dev Biol Plant 38:252–259

Pammenter NW, Vertucci CW, Berjak P (1991) Homeohydrous (recalcitrant) seeds: dehydration, the state of water and viability characteristics in *Landolphia kirkii*. Plant Physiol 96:1093–1098

Sacandé M, Buitink J, Hoekstra FA (2000) A study of water relations in neem (*Azadirachta indica*) seed that is characterised by complex storage behaviour. J Exp Bot 51:635–643

Slade L, Levine H (1991) Beyond water activity: recent advances in food quality and safety. Crit Rev Food Sci 30:115–360

Steadman KJ, Pritchard HW, Dey PM (1996) Tissue-specific soluble sugars in seeds as indicators of storage category. Ann Bot 77:667–674

Sun WQ, Leopold AC (1997) Cytoplasmic vitrification and survival of anhydrobiotic organisms. Comp Biochem Physiol 117A:327–333

Varghese B, Naithani SC (2002) Desiccation-induced changes in lipid peroxidation, superoxide level and antioxidant enzymes activity

in neem (*Aradirachta indica* A. Juss) seeds. Acta Physiol Plant 24:79–87

Vertucci CW, Leopold C (1987) The relationship between water binding and desiccation tolerance in tissues. Plant Physiol 85:232–238

Vertucci CW, Stushnoff C (1992) The state of water in acclimating vegetative buds from *Malus* and *Amelanchier* and its relationship to winter hardiness. Physiol Plant 86:503–511

Vertucci CW, Berjak P, Pammenter NW, Crane J (1991) Cryopreservation of embryonic axes of an homeohydrous (recalcitrant) seed in relation to calorimetric properties of tissue water. Cryo Letters 12:339–350

Wolfe J, Bryant G (2001) Cellular cryobiology: thermodynamic and mechanical effects. Int J Refrig 24:438–450