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Cocoa (*Theobroma cacao* L.) Somatic Embryos Tolerate Some Ice Crystallization during Cryopreservation

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Abstract

Secondary somatic embryos (SSEs) of cocoa, a recalcitrant tropical, seed-producing species, were cryopreserved using a vitrification approach and Differential Scanning Calorimetry (DSC) was employed to optimise sucrose preculture and Plant Vitrification Solution 2 (PVS2) incubation. The objective of the study was to evaluate the influence of sucrose preculture and PVS2 dehydration on water content of SSE that will enable it to survive cryostorage. SSEs were precultured for 3 or 5 days on media containing 0.5 M or 0.75 M sucrose and cryoprotected in loading solution (2 M glycerol and 0.4 M sucrose in medium) for 20 min before they were dehydrated with cold PVS2 for 0 - 90 min. Thermal analysis revealed the occurrence of ice crystallization in the SSEs with the extent declining with increasing PVS2 exposure. Maximal survival of SSEs was promoted by preculture on 0.5 M sucrose medium and dehydration with PVS2 for 45 - 60 min, which was characterised by small ice crystallization. Exposure of SSEs beyond 60 min leads to excessive dehydration as characterized by no change in the thermograms. Based on these findings, preculture of SSEs on 0.5 M sucrose medium and dehydration with cold PVS2 for 60 min has been adopted for the successful cryopreservation of cocoa germplasm.

Keywords

Differential Scanning Calorimetry, Sucrose Preculture, Cocoa Somatic Embryo, Vitrification, PVS2

1. Introduction

Due to losses caused by diseases, pests, bushfire, and native forest clearance, the

conservation of cocoa (*Theobroma cacao* L.) germplasm has become of paramount importance to breeders of the crop. Cocoa is conventionally propagated using seeds which are considered recalcitrant and therefore cannot be stored using conventional freezer-based storage protocols. In the absence of an efficient shoot tip based micropropagation regime for cocoa, secondary somatic embryos (SSEs) [1] have been used to cryopreserve cocoa using the encapsulation-dehydration method [2] with genotype-dependent success. The encapsulation-dehydration of somatic embryos is relatively labour-intensive and the asymmetrical position of some embryos at encapsulation can lead to non-uniform drying during their desiccation over silica gel. To address these limitations we adopted the vitrification approach which has proved successful in cryopreserving other tropical species [3]-[9].

Successful cryopreservation depends on the ability to reduce intracellular ice formation during storage of tissue in liquid nitrogen (LN) and thawing of the material [10]. The use of Differential Scanning Calorimetry (DSC) to analyse thermal behaviour of plant materials has revealed a strong relationship between the presence of freezing water or melting transitions and cellular damage at sub-freezing temperatures [11] [12] [13] [14]. Evidence of freezing and melting during cryopreservation have been observed in winter-hardy buds [15], shoot tips of garlic and mint [16] and shoot tips of tropical species, e.g. *Parkia speciosa* [17]. Plant tissues that survive cryogenic temperatures usually have a water content of below 0.4 g H_2O g^{-1} dry matter (dm) [12] [18] with higher contents being manageable if the cooling rate is increased above about 100˚Cs^{-1} [18] [19]. Water content in tissue can be classified as frozen and un-frozen or as osmotically active and inactive [17]. Water content in tissue less than 0.4 g H_2O g^{-1} dm is referred to as un-freezable or un-frozen water and is molecularly incapable of reorganisation into a crystalline structure within a practical time frame. Tissue having this low water content will vitrify with further cooling and glass formation may be detected [10]. In the course of rewarming, devitrification and recrystallization sometimes occur [10]. If on rewarming the molecular motion increases to a critical point and/or if the rewarming is not sufficiently rapid, devitrification and recrystallization occur, resulting in lethal intracellular ice-damage on return to higher sub-zero temperatures [20]. These exothermic events (devitrification and recrystallization) occur in materials containing 0.2 - 0.5 g H_2O g^{-1} dm [21] or hydrated materials treated with cryoprotectants [11] [17]. Cryopreserved materials with such water content risk the formation of ice causing injury if they are warmed slowly as occurs in DSC experiments [20] [22] [23].

The amount of desiccation tolerated in propagules to be cryopreserved is species-dependent. In some species water that readily freezes are removed completely before maximum survival could be achieved as in the case of oil palm embryo [5] while *Landolphia kirkii* zygotic axes showed superior survival with some residual freezable water [24]. Kaczmarczyk *et al.* [25] reported survival of cryopreserved potato shoot tips in spite of ice crystal growth in the tissue. Other

studies [26] [27] have also confirmed survival of cryopreserved tissues in the presence of intracellular ice if those ice crystals were small in size so that they do not destroy cells.

Thermal analysis was applied to cocoa somatic embryos after preculture and cryoprotection to provide important information about the cryoprotective treatments required to prevent lethal ice formation and ensure survival after re-warming.

The objective of the study was to evaluate the influence of sucrose preculture and PVS2 dehydration on water content that allow somatic embryo to survive in liquid nitrogen.

It was also to determine if survival can be achieved if ice crystallization occurs in a tropical species.

2. Materials and Methods

2.1. Preparation of Plant Materials

The study was done at the University of Reading, Plant Science. Secondary somatic embryos (SSEs) were generated from floral tissue of cocoa clone AMAZ 15 using the protocol of Maximova *et al.* [1]. The SSEs were pre-cultured for 3 or 5 d on embryo development (ED) media containing 0.5 M or 0.75 M sucrose. Embryos were then treated with 0.5 ml loading solution (LS) (2 M glycerol and 0.4 M sucrose prepared in ED medium) for 20 min before they were dehydrated with ice-cold PVS2 (23) for 45 - 90 min. PVS2 dehydrated SSEs were placed in 1.2 ml cryovials containing a further 1 ml PVS2 and plunged into LN where they remained for at least 1 h. Cryovials were re-warmed in a 42˚C water bath for 3 min. PVS2 was pipetted out and the SSEs were flushed out of the cryovials into an excess of cation-free deloading solution (DLS) (1.2 M sucrose in ED medium free of all cation nutrients) in a 9 cm Petri dish for 20 min [1]. SSEs were blotted on sterile filter paper and plated on ED medium and maintained in the dark at 25˚C.

2.2. Recovery Assessment

Samples were deemed to have survived if eight weeks after removal from LN and culturing there was cotyledon regrowth sufficient to provide explants for tertiary somatic embryo initiation or emergence of a whole embryo from the cryo-stored SSE. Ten up to fifteen SSEs was used for each treatment and replicated three times. Data collected was binomial, survival or non-survival after cryo-storage.

2.3. Thermal Analysis

Cooling and warming cycles of PVS2 dehydrated SSEs were performed using a Q2000 DSC with LN cooling systems (TA Instruments New Castle, Delaware, USA), calibrated for temperature scale and enthalpy using indium. One individual cryoprotected SSE was blot-dried on a filter paper and placed into a pre-weighed T Zero aluminium pan (TA Instruments, Switzerland) having her-

metic lid. It was then placed in a die and hermetically sealed with a press. The pan was weighed to obtain fresh weight (FW). The pan containing the SSE was then loaded into the auto sampler of DSC Q2000. The dry weight of the embryos used ranges from 0.8 to 2.12 mg. Scans were performed from 25˚C to −70˚C with a cooling and warming rate of 5˚C min^{-1} for SSEs. In the thermal analysis each treatment was replicated three times.

Temperatures and energies of crystallization were calculated from heat flow data using TA Universal Analysis software. Onset temperatures for ice crystallization and melting were calculated from the intersection of the baseline and the tangent to the steepest part of the transition peak. Enthalpy of the ice crystallization and melting was calculated from the area enclosed by the transition peaks and a projected linear baseline.

2.4. Water Content Determination

After thermal analysis, lids were pierced and pans were oven-dried at 100˚C for 24 h to determine sample dry weight. The total water content and water fraction of the embryo were obtained. The total water was converted to g H_2O per g dry weight ($g \cdot g^{-1}$) by dividing the total water in g by the dry weight of sample in g. Given that 334.5 J is released when 1 g of water converts to ice and the same amount of energy is consumed to melt 1 g of ice to water [29]. The area under the endotherm curve was used to obtain the percentage frozen water using the formula:

$$\text{Percentage frozen water} = \left[\Delta H_f / (\rho * 334.5)\right] 100$$

where ΔH_f is enthalpy of melt in Jg^{-1}; ρ is water fraction, 334.5 is the energy released when 1 g of water is converted to ice in Jg^{-1}.

2.5. Data Analysis

Genstat statistical software edition 12 was used to analyse the data. Survival proportion data were analysed using logistic regression analysis [17] to investigate the factors that contribute significantly ($P < 0.05$) to survival of the embryos. Logistic regression was used in preference to the often-used arcsine transformation or ANOVA which assumes normality. Thermodynamic data on cooling and warming were analysed using general linear regression.

3. Results

3.1. Water Content Analysis of Somatic Embryos

Water content was determined as freezable (active water) and unfreezable (inactive water) as fractions of total water contained in the embryos. The average proportion of freezable water as compared to total water, in the SSEs not precultured on sucrose, was 84.9%. On preculturing on 0.5 M sucrose for 3 and 5 d the freezable water declined to 69% and 55.8% respectively (**Figure 1(a)** and **Figure 1(b)**). Preculturing on 0.75 M sucrose for 3 and 5 d led to a further decline

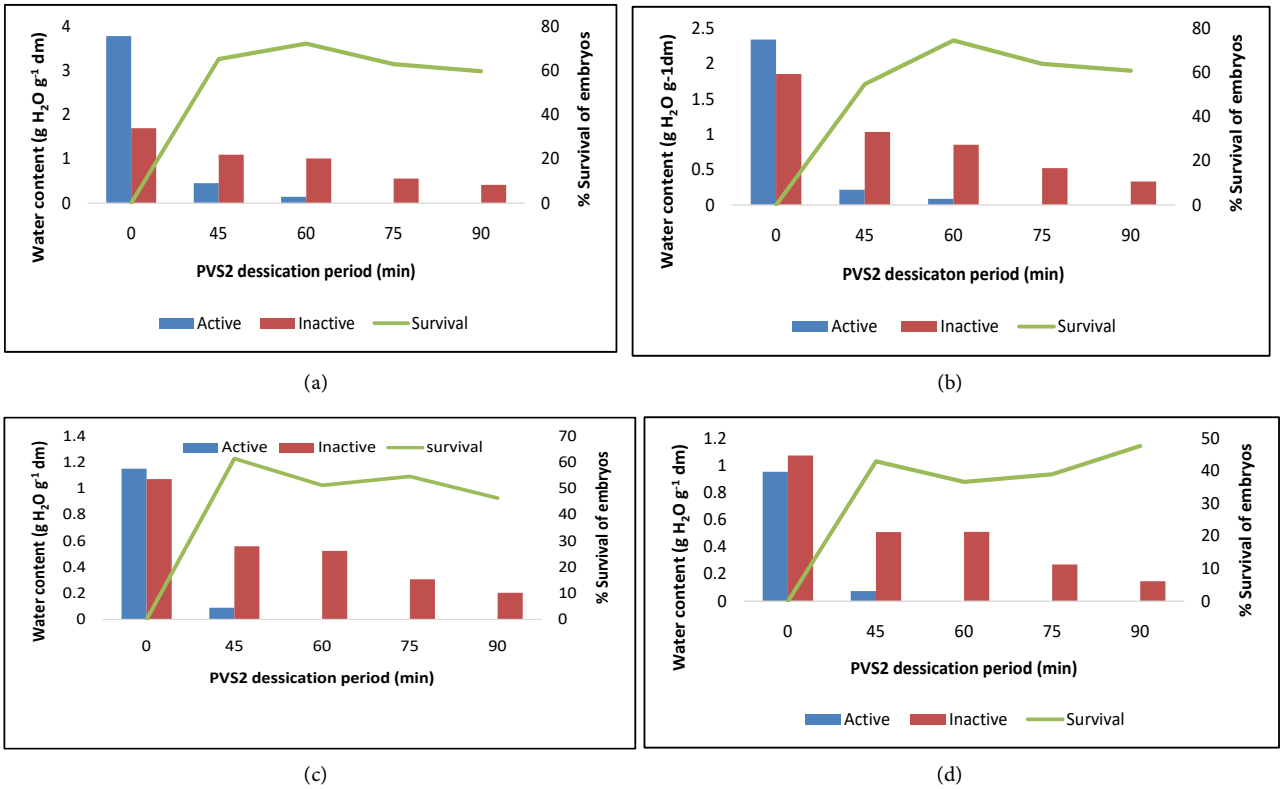

Figure 1. Water content of embryo and its survival after precultured on (a) 0.5 M sucrose for 3 d, (b) 0.5 M sucrose for 5 d, (c) 0.75 M sucrose for 3 d, (d) 0.75 M sucrose for 5 d and exposure to PVS2 for different periods. Author published embryo survival data in CryoLetters 33(6) [28] and it is used here to show how DSC was applied to get that data.

in freezable water to 51.8% and 47.0% respectively (Figure 1(c) and Figure 1(d)). Exposure of the embryos precultured on 0.5 M sucrose for 3˚d to PVS2 for 45 and 60 min reduced the freezable water to 29.2% and 12.5% respectively (Figure 1(a)). Equally, the freezable water content in relation to the total water content of embryos precultured on 0.5 M sucrose for 5 d and exposed to PVS2 for 45 and 60 min were 17.3% and 9.4% respectively (Figure 1(b)). Freezable water was not detectable following extended exposures of PVS2 to 75 and 90 min (Figure 1(a) and Figure 1(b)). The same pattern was seen in embryos precultured on 0.75 M sucrose for 3 and 5 d except that freezable water was not detectable earlier, when the embryos were exposed to PVS2 for 60 min (Figure 1(c) and Figure 1(d)).

The average proportion of unfreezable water in relation to the total water increased to 31.0%, 70.8% and 87.5% when embryos precultured on 0.5 M sucrose for 3˚d were exposed to 0, 45 and 60 min PVS2 (Figure 1(a)). Further exposure of PVS2 for 75 and 90 min led to an average of 100 %. A similar pattern was seen in embryos precultured on 0.5 M sucrose for 5 d (Figure 1(b)). Embryos exposed to 0.75 M sucrose for 3 and 5 d recorded similar increases in the proportion of unfreezable water however, 100% was achieved at a shorter PVS2 exposure period, 60 min (Figure 1(c) and Figure 1(d)).

3.2. Thermal Properties of Cocoa Somatic Embryos during Cooling and Warming

During the cooling and warming cycles, the onset temperatures for ice nucleation and melting and the enthalpy of ice crystallization and melting were measured (Table 1). The interactions of sucrose, preculture period and PVS2 exposure time significantly ($P < 0.05$) influenced the onset temperatures of ice crystallization and melting. The onset temperature for ice crystallization and melting decreased with increasing PVS2 exposure time (Table 1).

The occurrences of ice crystallization in the embryos were not significantly ($P > 0.05$) influenced by sucrose concentrations and preculture period but were influenced by PVS2 exposure times. When preculture treatment was 0.5 M sucrose for 3 d ice crystallization occurred in embryos exposed to PVS2 for 45 and 60 min during the cooling cycle (Table 1). Extending the PVS2 exposure time beyond 60 min inhibited the occurrence of ice crystallization. The same pattern was obtained in the warming cycles during which ice melting occurred (Table 1). Enthalpy of crystallization and melting significantly decreased with increasing sucrose concentration and preculture period as well as PVS2 exposure time (Table 1). When the preculture period was increased to 5 d a similar pattern was observed during the cooling and warming cycles (Table 1).

Increase of the sucrose concentration from 0.5 M to 0.75 M led to a decline in the size of the first order transitions. Only SSEs exposed to the control treatment and 45 min PVS2 exhibited ice crystallization and melting (Table 1). Embryos exposed to PVS2 beyond 45 min became excessively dehydrated and therefore did not record any transitions within the temperature range studied. The enthalpies of ice crystallization and melting were significantly lower in SSEs exposed to PVS2 for 45 min than those of the control treatment (Table 1). The extension of the preculture of embryos to 5 d on 0.75 M sucrose did not bring any significant change in the occurrence or onset temperature of thermal events although their absolute size was reduced (Table 1).

4. Discussion

4.1. The Effect of Sucrose-Supplemented Culture Media on Thermal Properties and Water Content of SSEs

Sucrose has often been used to aid tropical crop species' acclimation to low temperature storage [13] [21] [30] [31]. In cocoa being a crop recalcitrant to such low temperature storage, there was the need to explore sucrose preculture in enhancing somatic embryo survival during cryo-storage. Preculturing the somatic embryos on sucrose-supplemented media led to a rapid loss of water from the embryos. Embryos kept on 0.75 M sucrose for 5 d exhibited browning, a possible indication of excessive dehydration (though these tissues can show high rates of embryogenesis when apparently necrotic). The enthalpies of ice crystallization and melting of embryos, cultured on these sucrose-supplemented media, were progressively reduced in size with increasing sucrose concentration

Table 1. Effect of sucrose preculture and PVS2 exposure on cocoa somatic embryo cooling and warming thermodynamic properties Means and standard errors of the mean are displayed (n = 3).

Sucrose Preculture (M/days)	PVS2 exposure time (min)	Thermal cycle	Thermal event	Onset (˚C)	Enthalpy ($J \cdot g^{-1}$)
0.5/3d	**0**	Cooling Warming	Ice N Ice M	−18.90 ± 0.28 −17.57 ± 1.46	185.50 ± 1.33 192.45 ± 1.07
0.5/3d	**45**	Cooling Warming	Ice N Ice M	−29.73 ± 0.88 −46.34 ± 2.06	41.24 ± 0.33 36.10 ± 0.42
0.5/3d	**60**	Cooling Warming	Ice N Ice M	−37.98 ± 1.04 −48.64 ± 0.51	26.78 ± 0.54 22.30 ± 0.48
0.5/3d	**75**	Cooling Warming	No event observed No event observed	NA* NA	NA NA
0.5/3d	**90**	Cooling Warming	No event observed No event observed	NA NA	NA NA
0.5/5d	**0**	Cooling Warming	Ice N Ice M	−20.76 ± 0.93 −21.44 ± 0.43	162.90 ± 1.19 150.23 ± 5.10
0.5/5d	**45**	Cooling Warming	Ice N Ice M	−30.65 ± 1.27 −48.42 ± 1.66	32.30 ± 1.17 30.77 ± 0.61
0.5/5d	**60**	Cooling Warming	Ice N Ice M	−39.07 ± 1.48 −50.51 ± 2.91	18.35 ± 0.57 15.19 ± 1.59
0.5/5d	**75**	Cooling Warming	No event observed No event observed	NA* NA	NA NA
0.5/5d	**90**	Cooling Warming	No event observed No event observed	NA* NA	NA NA
0.75/3d	**0**	Cooling Warming	Ice N Ice M	−22.42 ± 0.61 −26.07 ± 0.275	157.10 ± 2.77 116.83 ± 4.05
0.75/3d	**45**	Cooling Warming	Ice N Ice M	−37.09 ± 0.87 −45.87 ± 0.83	35.10 ± 0.89 25.07 ± 0.72
0.75/3d	**60 - 90**	No event observed	NA* NA	NA NA	NA* NA
0.75/5d	**0**	Cooling Warming	Ice N Ice M	−22.95 ± 0.41 −−25.16 ± 0.28	149.50 ± 1.87 111.27 ± 0.92
0.75/5d	**45**	Cooling Warming	Ice N Ice M	−39.58 ± 0.02 −46.41 ± 0.04	32.57 ± 0.08 23.86 ± 0.04
0.75/5d	**60 - 90**	No event observed	NA* NA	NA NA	NA* NA

*Not applicable due to non-observable event. N = nucleation, M = melting.

and preculture duration. The increase in sucrose concentration evidently drew out more water from the embryo, as reflected in the moisture content values of 12.75 ± 0.153 and 4.196 ± 0.304 $g \cdot g^{-1} \cdot dm$, when the embryos were cultured on the normal media containing 0.08 M and 0.5 M sucrose for 5 d, respectively. As the sucrose concentration was increased from 0.5 M to 0.75 M so were shorter durations of PVS2 exposure were necessary to achieve water content that was not harmful for cryostorage. Soluble sugars like sucrose are noted for stabiliza-

tion of membrane, enzymes and macromolecules and prevention of fusion between liposomes [32]. Sucrose accumulates in tissues and helps to maintain cell viability during cryopreservation by preventing gross freezing injury to cell membranes [33] [34]. Fang *et al.* [35] studied the significance of sucrose preculture of cocoa somatic embryos for cryopreservation success and concluded that sucrose accumulation in the embryos was essential for increasing their desiccation tolerance.

4.2. The Effect of PVS2 on Thermal Properties and Somatic Embryo Survival

Removal of substantial amount of freezable (active) water was achieved when embryos were exposed to PVS2 for 45 and 60 min. Beyond 60 min PVS2 exposure, ice nucleation and melting was not found during cooling and warming. Excessive desiccation happened in higher period of exposure to PVS2. Excessive desiccation correlates with the reduced levels of survival that were observed for treatments with PVS2 exposure in excess of 60 min. Data after cryopreservation show that maximal survival correlates with PVS2 exposure time that brings about a small ice crystallization. Glass formation could not be accounted for at the 25 to −70˚C temperature range the thermal studies were made, however, cryostorage done at −196˚C might have induced ice and glass transition thereby ensuring post-cryo survival of the embryos. The occurrences of ice and glass transitions have been observed in the work of [10]. Such glass formed is unstable and melts on rewarming to produce ice crystals. If the size of the ice crystals is big then they can damage the tissue thereby affecting post cryo-survival.

While both 60 min PVS2/0.5 M sucrose preculture and 45 min PVS2/0.75 M sucrose preculture regimes resulted in small ice nucleation and appreciable post-cryo survival of embryos, the latter combination led to unacceptably low survival of SSEs. The death of such cryostored cocoa embryos may therefore result from excessive preculture-induced dehydration in combination with PVS2 phytotoxicity. Exposure of *Parkia speciosa* shoot tips to PVS2 beyond 60 min led to a decline in survival and excessive desiccation [17], the authors conclude that at a supra-optimal level the cryoprotectant mixture becomes cytotoxic through osmotic stress. PVS2, according to [10], has the ability to limit freezing by reduction of water molecule mobility without forming a glass. Plant cell walls and membranes have been found to be permeable to dimethyl sulfoxide (DMSO) and ethylene glycol (EG) components of PVS2 [14] [36] [37] [38], their entry restricting ice nucleation and growth. 45 min and in some cases 60 min PVS2 exposure appeared to cause ice formation in the embryos but are apparently not sufficiently harmful to be lethal (42.9% ± 7.6% to 74.5% ± 6.4% post cryo survival).

Some authors [18] have demonstrated that it is possible to successfully cryopreserve sub-tropical crop species at water contents within the range of 1.6 - 1.1 $g \cdot g^{-1}$·dm if the cooling rate is sufficiently high (ca. 500˚C·min^{-1}). At −196˚C none of the embryonic axes of *Citrus sinensis* survived cryopreservation at 1.6 -

1.1 $g \cdot g^{-1} \cdot dm$ but when the water content was moderately reduced below 1.1 $g.g^{-1} \cdot dm$ some survival was obtained at −200˚C. A further reduction to 0.6 - 0.44 $g.g^{-1} \cdot dm$ led to a similar amount of survival with a cooling rate of $10˚C \cdot min^{-1}$. Most DSC studies e.g. [5] [18] [24] show that increasing survival of zygotic embryos is correlated with diminishing levels of freezable water. While freezable water had to be removed completely before maximum survival could be achieved in oil palm embryos [5] that has not been the case for all species. Zygotic axes of *Landolphia kirkii,* e.g., that retained some freezable water survived cooling to −80˚C better than those that had been dehydrated to the level of non-freezable water [24]. Cocoa somatic embryos that had been precultured on 0.5 M sucrose were dehydrated to a moisture content of 0.99 $g \cdot g^{-1} \cdot dm$ leading to observation of endothermic transition events [35]. Further desiccation to 0.31 $g \cdot g^{-1} \cdot dm$ resulted in a constant thermogram without any thermal transition. In the present study, cocoa somatic embryos precultured on 0.5 M sucrose for 5 d and dehydrated with PVS2 for 60 and 90 min gave similar thermal profiles to those for encapsulation-dehydrated SSEs [35]. Maximal post-cryo survival was obtained when the water content was 0.941 ± 0.096 $g \cdot g^{-1} \cdot dm$ with 15.19 ± 1.59 Jg^{-1} enthalpy of ice melting. Within-embryo ice formation evidenced in the present study may be responsible for the type of non-meristematic cellular disruption previously reported in cryopreserved cocoa germplasm [39] and needs not be terminally injurious to somatic embryos of recalcitrant tropical species.

5. Conclusion

Preculturing of cocoa somatic embryos on media containing 0.5 M sucrose for 5 days and desiccating the precultured embryo in PVS2 for 60 min retain water content that enables a small amount of ice crystals to form. The ice formed is not harmful to cryostored somatic embryo. DSC scans were performed from 25˚C to −70˚C and that could not capture glass transition; it is therefore recommended further work should use equipment that can scan up to −150˚C.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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