

# Cryopreservation of cocoa (*Theobroma cacao* L.) somatic embryos for long-term germplasm storage

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## Abstract

Cryopreservation using encapsulation-dehydration was developed for the long-term conservation of cocoa (*Theobroma cacao* L.) germplasm. Survival of individually encapsulated somatic embryos after desiccation and cryopreservation was achieved through optimization of cryoprotectants (abscisic acid (ABA) and sugar), duration of osmotic and evaporative dehydration, and embryo development stage. Up to 63% of the genotype SPA4 early-cotyledonary somatic embryos survived cryopreservation following 7 days preculture with 1 M sucrose and 4 h silica exposure (16% moisture content in bead). This optimized protocol was successfully applied to three other genotypes, e.g. EET272, IMC14 and AMAZ12, with recovery frequencies of 25, 40 and 72%, respectively (but the latter two genotypes using 0.75 M sucrose). Recovered SPA4 somatic embryos converted to plants at a rate of 33% and the regenerated plants were phenotypically comparable to non-cryopreserved somatic embryo-derived plants.

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**Keywords:** Cocoa; Cryopreservation; Encapsulation-dehydration; Somatic embryos; Sucrose

## 1. Introduction

Cocoa is an economically important tropical tree crop with increasing world production and consumption [1]. Present conservation of its germplasm in field genebanks requires large inputs in land and labour. In addition, more than 25% of global production is lost annually due to diseases [2], thus making a durable conservation scheme essential. The recalcitrant and chilling-sensitive characteristics of cocoa seeds make them unsuitable for prolonged storage. Maintenance in the form of tissue culture is impractical because the *in vitro* cocoa plants demand particularly labour intensive subcultures due to their high production of polyphenols and ethylene. Moreover, the risk of somaclonal variation is likely to increase with extended culture duration [3]. Cryopreservation in liquid nitrogen (LN) therefore offers great potential for the secure, long-term conservation of this crop.

The only cryopreservation protocols available for cocoa are designed for immature zygotic embryos [4] and embryogenic callus [5]. Storage of these explants is of reduced in-

terest as seeds are genetically heterogeneous and callus is prone to somaclonal variation [3]. Moreover, the production of somatic embryos (presently the only reliable means of micropropagation in this crop) varies greatly depending on the genotype and individual floral explants [6,7]. The use of somatic embryos rather than potentially non-embryogenic callusing explants for cryopreservation therefore represents a more efficient routine.

Encapsulation-dehydration is a vitrification-based procedure and comprises the gradual osmotic and evaporative dehydration of plant cells prior to LN exposure. It has been utilised for the cryopreservation of somatic embryos and shoot tips of a range of tropical species [8,9]. Vitrification allows tissue survival by avoiding ice formation and can be achieved by elevating cell viscosity with exposure to highly concentrated cryoprotectants and dehydration followed by rapid cooling [9,10]. Low desiccation and cold tolerance are major problems for successful cryopreservation of tropical species, unlike explants of temperate origin that can gain increased cryopreservation tolerance following cold acclimation [11,12]. Among tropical species artificial cryoprotection by the means of abscisic acid (ABA) and sugar incubation is generally required [8,10]. The present paper describes the establishment of a cocoa-specific cryopreservation protocol

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in which embryo survival was monitored under a series of encapsulation, preculture and dehydration regimes. Factors affecting the success of somatic embryo cryopreservation are discussed.

## 2. Materials and methods

### 2.1. Plant material

Somatic embryos were initiated from floral explants collected at the University of Reading Intermediate Cocoa Quarantine Unit as described previously [6]. Secondary somatic embryos were randomly harvested from the cultures and used in all trials. They were maintained inside 9 cm Petri dishes containing 25 ml embryo development (ED) medium comprising DKW basal salts,  $100 \text{ mg l}^{-1}$  myo-inositol,  $2 \text{ mg l}^{-1}$  thiamine-HCl,  $1 \text{ mg l}^{-1}$  nicotinic acid,  $2 \text{ mg l}^{-1}$  glycine,  $30 \text{ g l}^{-1}$  sucrose,  $1 \text{ g l}^{-1}$  glucose, and  $2 \text{ g l}^{-1}$  Phytigel, pH 5.7 [6]. Early-cotyledonary stage embryos of the genotype SPA4 were used throughout the experiments unless otherwise specified.

### 2.2. Cryopreservation procedure

Encapsulation was performed by suspending the embryos in calcium-free liquid ED medium containing 3% (w/v) alginate acid (2% viscosity, SIGMA) after which individual embryos were dropped into liquid ED medium containing  $100 \text{ mM CaCl}_2 \cdot 2\text{H}_2\text{O}$ . After 30 min polymerization, calcium alginate beads (4–5 mm diameter) were rinsed three times with water for 5 s each and blotted on filter paper to remove surface moisture. Groups of 10 encapsulated embryos were then precultured on ED medium enriched with 0.3 M sucrose for 3 days, followed by incubation with higher levels of sugar for another 4 days; or they were precultured on ED medium supplemented with ABA for 7 days. Filter-sterilized ABA was added to the medium after autoclaving. Dehydration was carried out inside 9 cm Petri dishes with 10 precultured embryos placed on top of 30 g dry silica gel separated by a filter paper. Dehydrated embryos were transferred in batches of 10 into 1.8 ml cryotubes which were plunged rapidly into LN. After 1 h storage, the tubes were rewarmed inside a  $35^\circ\text{C}$  water bath for 5 min. For recovery, embryos were plated on ED medium containing 0.3 M sucrose for 3 days and transferred to standard ED medium, except for trials involving ABA where the explants were directly plated on ED medium. Subculture took place every 14 days. The preculture, dehydration and recovery were all conducted in the dark at  $25^\circ\text{C}$ .

### 2.3. Factors affecting embryo survival after desiccation and cryopreservation

Factors tested were: (1) 7 days preculture with ABA (1–40  $\mu\text{M}$ ), alone or in combination with 0.5 M sucrose, and

various drying durations (0–4 h); (2) preculture durations of 1–15 days on 0.5 M sucrose medium; (3) globular (<0.5 mm long), heart/torpedo (0.5–1 mm) and early-cotyledonary (1–1.5 mm) stage embryos following 7 days preculture with 0.5 M sucrose; (4) 7 days preculture on media containing various amounts of sucrose, maltose, trehalose, mannitol and glycerol; (5) 0.5–1.25 M sucrose preculture with the genotypes AMAZ12, EET272, IMC14 and SPA4.

All the experiments were of a completely randomized design with two to three replicates of 10 embryos per treatment. Survival was assessed as the percentage of embryos manifesting new tissue growth. All the cultures were kept for a minimum of 2 months before being regarded as non-surviving. Data were arcsine transformed prior to analysis using the GLM procedure (SAS Institute Inc., Cary, NC, USA).

### 2.4. Alginate bead moisture content determination

Moisture content was determined for alginate beads following 0–1.25 M sucrose incubation and 0–6 h silica exposure. Bead dry weight was measured after 24 h oven drying at  $80^\circ\text{C}$ .

### 2.5. Plant regeneration

The conversion of embryos to plants was conducted under 16 h photoperiod ( $90 \mu\text{mol m}^{-2} \text{ s}^{-1}$  irradiance) at  $22^\circ\text{C}$ . Surviving embryos were placed in 250 ml glass jars containing 40 ml primary embryo conversion (PEC) medium which comprised DKW basal salts,  $100 \text{ mg l}^{-1}$  myo-inositol,  $2 \text{ mg l}^{-1}$  thiamine-HCl,  $1 \text{ mg l}^{-1}$  nicotinic acid,  $2 \text{ mg l}^{-1}$  glycine,  $0.3 \text{ g l}^{-1}$   $\text{KNO}_3$ ,  $0.435 \text{ mg l}^{-1}$  arginine,  $0.187 \text{ mg l}^{-1}$  glycine,  $0.328 \text{ mg l}^{-1}$  leucine,  $0.456 \text{ mg l}^{-1}$  lysine,  $0.51 \text{ mg l}^{-1}$  tryptophane,  $10 \text{ g l}^{-1}$  sucrose,  $20 \text{ g l}^{-1}$  glucose, and  $1.75 \text{ g l}^{-1}$  Phytigel, pH 5.8 [13]. PEC medium was renewed every 20 days until the appearance of plants with true leaves and roots. These plants were then transferred to seed sowing compost and vermiculite (3:1) and incubated inside a propagator whose relative humidity was gradually reduced during the first 2 weeks.

## 3. Results

### 3.1. Combined effect of ABA/sucrose preculture and evaporative drying

By 4 h drying, viability of embryos without a cryoprotective treatment decreased, although it was significantly enhanced after preculture with either ABA or sucrose or a combination of both (Table 1). However, no survival was observed after cryo-storage if ABA was used alone. Furthermore, there was a significant interaction between cryoprotectant treatment and drying duration ( $P < 0.0001$ ). Sucrose preculture, regardless of the level of ABA, in combination

Table 1

Effects of 7 days ABA/sucrose preculture and evaporative drying on SPA4 early-cotyledonary somatic embryo survival after desiccation and cryopreservation

Treatment	Drying duration (h)							
	0		2		4		6	
	–LN	+LN	–LN	+LN	–LN	+LN	–LN	+LN
Control	100 (0)	0 (0)	100 (0)	0 (0)	40 (0)	0 (0)	10 (0)	0 (0)
Sucrose (M)								
0.5	100 (0)	0 (0)	100 (0)	0 (0)	95 (5)	40 (10)	45 (5)	10 (0)
ABA ( $\mu$ M)								
1	100 (0)	0 (0)	100 (0)	0 (0)	75 (5)	0 (0)	25 (5)	0 (0)
10	100 (0)	0 (0)	100 (0)	0 (0)	79 (9)	0 (0)	25 (5)	0 (0)
20	100 (0)	0 (0)	90 (10)	0 (0)	64 (24)	0 (0)	20 (0)	0 (0)
40	100 (0)	0 (0)	95 (5)	0 (0)	65 (15)	0 (0)	20 (10)	0 (0)
Sucrose (M) $\times$ ABA ( $\mu$ M)								
0.5 $\times$ 1	100 (0)	0 (0)	100 (0)	0 (0)	95 (5)	38 (2)	30 (10)	10 (0)
0.5 $\times$ 10	100 (0)	0 (0)	100 (0)	0 (0)	90 (10)	15 (10)	35 (15)	10 (0)
0.5 $\times$ 20	100 (0)	0 (0)	100 (0)	0 (0)	95 (5)	5 (5)	25 (5)	0 (0)
0.5 $\times$ 40	95 (5)	0 (0)	100 (0)	0 (0)	95 (5)	30 (20)	20 (10)	5 (5)

Values represent survival percentage ( $\pm$ S.E.). Factorial analysis:  $P$  (cryoprotectant  $\times$  drying duration) = 0.1087 (–LN);  $P$  (cryoprotectant  $\times$  drying duration) < 0.0001 (+LN). Treatment difference was evaluated at 5% probability. LN: liquid nitrogen.  $n$  = 20–30.

with a drying duration of 4 h, allowed the highest embryo survival after cryopreservation (40%).

### 3.2. Effects of sucrose preculture duration and embryo development stage

The duration of sucrose exposure significantly affected embryo survival following desiccation ( $P$  = 0.0469) and cryopreservation ( $P$  = 0.0104) (Fig. 1). A minimum duration of 7 days was required to obtain high embryo survival (>75%) after desiccation. Significantly higher cryopreservation tolerance was recorded following 7 days preculture compared with other culture durations. Development stage also significantly influenced embryo survival following desiccation ( $P$  = 0.0439) and cryopreservation ( $P$  = 0.0151). Early-cotyledonary stage embryos exhibited greater regrowth compared to the globular stage embryos (Fig. 2).

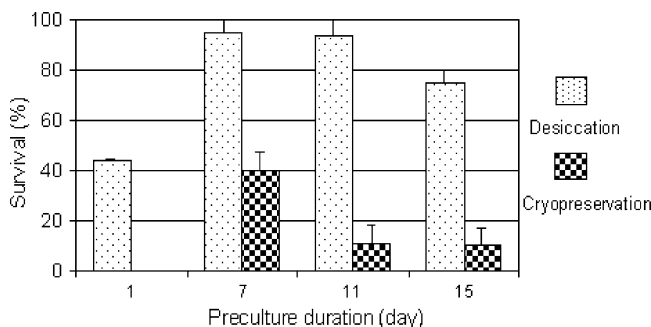


Fig. 1. Effect of sucrose preculture duration on SPA4 early-cotyledonary somatic embryo survival after desiccation and cryopreservation. Bars represent S.E.  $n$  = 20–30.

### 3.3. Effects of constituent sugars and sucrose concentration in the preculture medium

Embryo viability after desiccation was not affected by the type of disaccharides and polyalcohols tested ( $P$  = 0.5417) (Table 2). Nevertheless, a superior cryo-tolerance ( $P$  = 0.0042) was achieved when a high concentration of sucrose was present in the medium, e.g. 0.4 M sucrose supplemented with 0.1 M mannitol/glycerol, or 0.5 M sucrose in isolation. Sucrose was therefore selected as the sole cryoprotectant for the subsequent trial.

Sucrose concentration in the preculture medium significantly affected embryo cryo-tolerance ( $P$  < 0.0001) (Fig. 3). Preculture with 0.5, 0.75 and 1 M sucrose allowed significantly higher survival rates compared to 1.25 M. Differences in genotype performance were also observed: the genotypes AMAZ12 and SPA4 showed significantly greater recovery compared to EET272 and IMC14 ( $P$  < 0.0001) whatever the sucrose concentration tested.

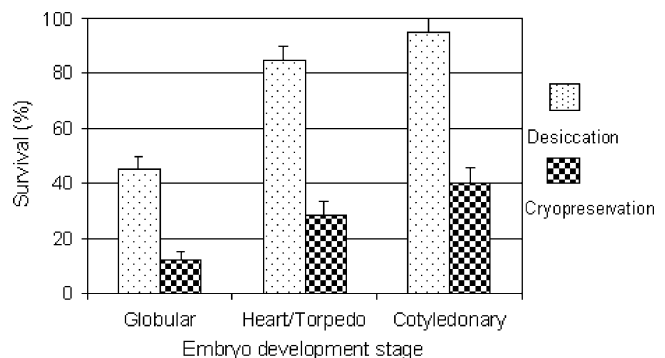


Fig. 2. Effect of development stage on SPA4 somatic embryo survival following desiccation and cryopreservation after 7 days preculture with 0.5 M sucrose. Bars represent S.E.  $n$  = 20–30.

Table 2

Effect of constituent sugars on SPA4 early-cotyledonary somatic embryo survival following desiccation and cryopreservation after 7 days preculture and 4 h evaporative drying

Sugar composition	Survival after desiccation (%)	Survival after cryopreservation (%)
0.25 M sucrose + 0.25 M maltose	90 (10) <sup>a</sup>	0 <sup>c</sup>
0.25 M sucrose + 0.25 M trehalose	90 (10) <sup>a</sup>	0 <sup>c</sup>
0.25 M sucrose + 0.25 M mannitol	89 (1) <sup>a</sup>	10 (10) <sup>bc</sup>
0.25 M sucrose + 0.25 M glycerol	100 <sup>a</sup>	7 (7) <sup>bc</sup>
0.4 M sucrose + 0.1 M maltose	100 <sup>a</sup>	4 (4) <sup>bc</sup>
0.4 M sucrose + 0.1 M trehalose	100 <sup>a</sup>	6 (6) <sup>bc</sup>
0.4 M sucrose + 0.1 M mannitol	95 (5) <sup>a</sup>	30 (10) <sup>a</sup>
0.4 M sucrose + 0.1 M glycerol	100 <sup>a</sup>	14 (4) <sup>ab</sup>
0.5 M sucrose	95 (5) <sup>a</sup>	37 (7) <sup>a</sup>

Values represent mean survival ( $\pm$ S.E.). Survival was ranked using Duncan multiple range test and row data with the same letter (in superscript) were not significantly different at 5% probability.  $n = 20$ –30.

### 3.4. Moisture content in the alginate bead

Bead water content decreased as the evaporative drying progressed (Table 3). It was reduced to 10–23% after the various sucrose precultures and 4 h drying. In addition, the rate of water loss during osmotic and evaporative treatments was dictated by the concentration of sucrose in the medium. A higher sucrose concentration induced a greater osmotic drying followed by a less extensive evaporative drying, while a lower sucrose concentration caused the reverse.

### 3.5. Recovery and plant regeneration

Surviving embryos resumed growth at a variable pace but started as early as the second week after cryopreservation. Two regrowth patterns were observed (Fig. 4): (1) direct growth by cotyledon proliferation, axis elongation and/or root development, and (2) indirect growth through secondary embryogenesis initiated on healthy or degenerating tissue. In both instances, regrowth began with a slight darkening of the embryo followed by the emergence of new tissue. Embryos that retained their original whitish colour generally failed to regenerate. Very rarely embryos exhibited survival by initiating callus with no further development of

Table 3

Moisture content of alginate beads following different sucrose precultures and evaporative drying durations

Sucrose treatment (M)	Drying duration (h)							
	0		2		4		6	
	dw <sup>a</sup>	fw <sup>b</sup> (%)	dw	fw (%)	dw	fw (%)	dw	fw (%)
0	12.87 (0.39)	93	4.46 (0.15)	82	0.56 (0.06)	35	0.23 (0.03)	18
0.5	3.43 (0.01)	77	0.82 (0.05)	45	0.30 (0.02)	23	0.21 (0.01)	17
0.75	2.03 (0.05)	67	0.48 (0.01)	33	0.18 (0.01)	15	0.18 (0.01)	15
1	1.60 (0.02)	62	0.41 (0.02)	29	0.19 (0.01)	16	0.13 (0.00)	12
1.25	1.12 (0.01)	53	0.38 (0.02)	27	0.12 (0.01)	10	0.13 (0.01)	12

<sup>a</sup> Values represent moisture content on dry weight basis ( $\pm$ S.E.).  $n = 10$ .

<sup>b</sup> Values represent moisture content on fresh weight basis.  $n = 10$ .

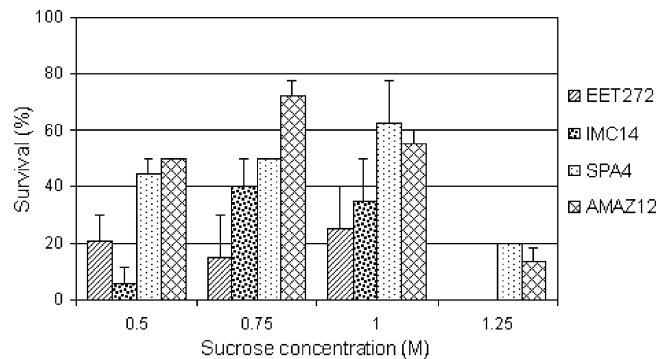


Fig. 3. Post-cryopreservation recovery of four cocoa genotypes following 7 days sucrose preculture and 4 h dehydration. Bars represent S.E.  $n = 20$ –30.

cotyledon or axis; they were thus excluded from the survival data. Surviving embryos of the genotype SPA4 developed into plants at a rate of 33% and no phenotypic abnormalities were observed compared to non-cryopreserved somatic embryo-derived plants (Fig. 5). All the converted plants were successfully weaned and reached 5–8 cm in height with 6–11 leaves within 1 month.

## 4. Discussion

A number of authors have addressed the influence of ABA and sugars on somatic embryo desiccation and cryopreservation tolerances [10,14]. Exogenous application of ABA, or the increase in endogenous ABA level under stress conditions (osmotic or water loss) has been associated with synthesis of proteins [15,16] and compatible solutes [14,16], components which play important roles in plant stress tolerance. The accumulation of sucrose inside tissue helps in maintaining cell viability during dehydration and cryopreservation by stabilisation of membranes [17,18] and enhancement of vitrification [9,19]. The present work demonstrated the importance of sugars, especially sucrose, in desiccation and cryopreservation tolerance of cocoa somatic embryos. The superiority of sucrose over other sugars was also reported in cryo-tolerance of oil palm somatic embryo clumps

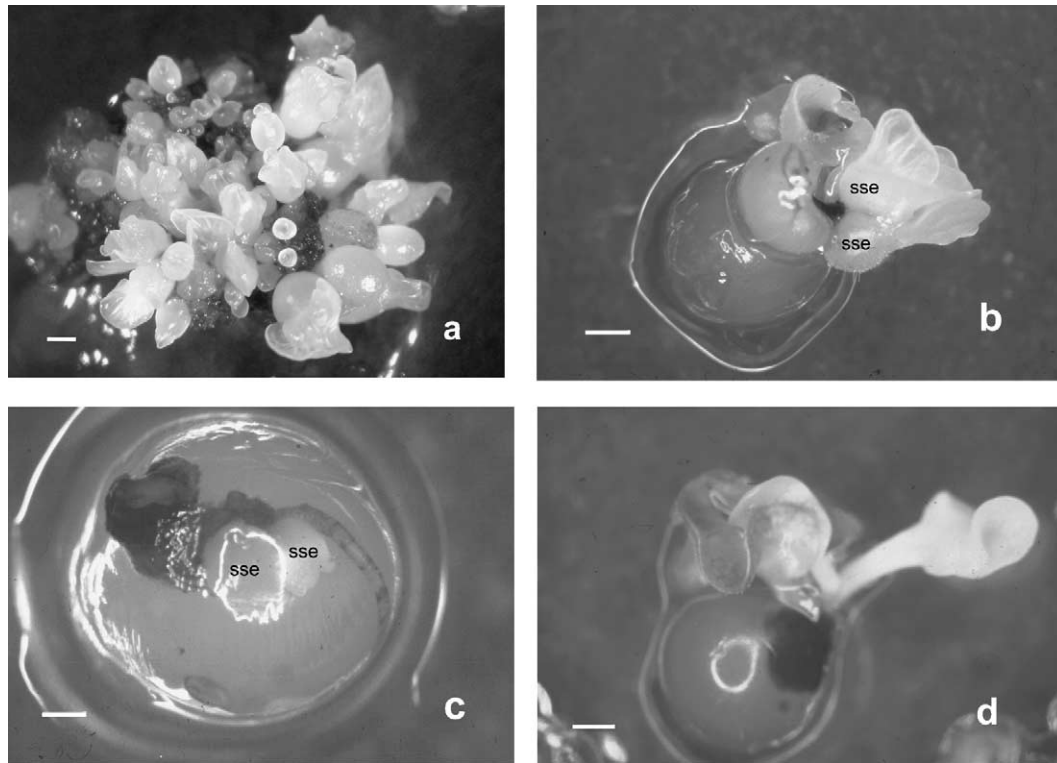


Fig. 4. Regrowth of somatic embryos following cryopreservation. (a) Somatic embryos before cryopreservation. (b) Direct regrowth of an embryo accompanied by newly-produced secondary somatic embryos (sse). (c) Secondary somatic embryos (sse) originated from degenerating tissue. (d) Embryo showing proliferative cotyledons but lacking axis elongation (bar = 1 mm).

[20]. ABA alone did not improve survival of cocoa embryos after cryo-storage. This was also observed in the cryopreservation of *Rubus* meristems [12]. It was suggested that the cryoprotective effect of ABA on alfalfa cells was sometimes counteracted by the presence of cytokinin [21], however, no interaction was apparent between ABA and benzylaminopurine in *Rubus* meristems [12]. We did not test the existence of such interaction as no cytokinin was employed in the

preculture medium. Alfalfa somatic embryos were responsive to ABA treatment for a few days only during their development [21]. Thus, the possibility exists that cocoa somatic embryos would also respond to ABA at a specific stage of development, but this remains to be clarified.

Despite its well-known cryoprotective action, sucrose can have deleterious effects on embryo viability if applied inappropriately. Firstly, direct exposure to high sucrose concentrations during the preculture and direct plating on ED medium (only 0.087 M sucrose) during the recovery resulted in low embryo survival (data not shown). Stepwise preculture and recovery plating, as well as the use of semi-solid instead of liquid medium, were essential for preserving embryo viability. Secondly, an insufficient sucrose exposure led to a decrease in viability following cryopreservation, similar to the case in carrot somatic embryos [22]. Tissue may require a threshold period for metabolic changes associated with improved cryo-tolerance. However, embryos could become sensitive to LN exposure due to continued growth during extended preculture. Furthermore, the sucrose concentration in the preculture medium can dictate the success of embryo cryopreservation. Sucrose concentration affects both its accumulation in the tissue [19] and bead [9] as well as the drying rate and these in turn may influence tissue cryo-tolerance.

Somatic embryos at a late stage of development were less sensitive to water loss than premature embryos. Similar



Fig. 5. Glasshouse weaned plants derived from cryopreserved (right) and non-cryopreserved (left) somatic embryos (bar = 3 cm).

observations were reported in oilseed rape and carrot somatic embryos which acquire a higher desiccation tolerance late in their development [23,24]. In cocoa, somatic embryos were found to lack starch and protein reserves compared with zygotic embryos, but exhibited reserve synthesis after sucrose and ABA were incorporated in the culture medium at a late growth phase [25]. The improved desiccation tolerance of early-cotyledonary somatic embryos in the present study may have derived from the reserve accumulation after a preculture step using sucrose.

Cryopreservation competence of embryos relied only partially on the acquisition of desiccation tolerance as a relatively smaller proportion of desiccation tolerant embryos were recovered after LN storage. Ice crystal formation within cells is considered to be a primary source of cryo-injuries. However, our results indicated that the alginate beads reached a residual water content of 0.1–0.3 g H<sub>2</sub>O g dw<sup>-1</sup> or 10–23% after sucrose preculture and 4 h drying. This was lower or comparable to the range in the somatic embryos of other species in which cells underwent a glass transition at approximately –50 °C during immersion in LN [10,19]. Thus, ice crystals probably did not occur and other factors may have been responsible for embryo damage during cryopreservation.

The regeneration of plants from non-cryopreserved cocoa somatic embryos is generally low (<50%) for the majority of genotypes tested (46% for SPA4, unpublished data). In addition, following cryo-storage, somatic embryos often exhibited root apex damage which further reduced the regeneration frequency (33% for SPA4) as these explants only produce adventitious roots with difficulty. Long-term storage of plant germplasm requires an efficient and reliable plant regeneration system following cryopreservation. Therefore, cell integrity is presently being assessed at the different stages of cryopreservation as a basis for improvement in the conversion process.

In conclusion, cryopreservation of cocoa somatic embryos using encapsulation-dehydration has been achieved for the first time and the protocol initially established using the genotype SPA4 was applicable to three other genotypes. The protocol obviates the use of complex cryoprotectant mixtures and costly controlled-rate freezing equipment thus could be readily implemented in cocoa producing countries. The high desiccation tolerance achieved in somatic embryos may also facilitate the commercial production of synthetic seeds. A better understanding of cryoprotectant actions, embryo developmental stage and recovery pathway may further contribute to the development of cryopreservation of other tropical species with recalcitrant seeds.

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