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Full Length Research Paper

Importance of structural integrity of somatic embryos for long-term cryopreservation of cocoa (*Theobroma cacao* L.) germplasm

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Cryopreservation is a reliable means for the long-term conservation of plant genetic resources. It is of particular interest for cocoa (*Theobroma cacao* L.) whose seeds are recalcitrant to conventional storage methods and field collections susceptible to disease infestations. However, the encapsulation-dehydration procedure previously developed for cocoa somatic embryos has resulted in poor survival after retrieval from liquid nitrogen. To examine the causes of such failure, cocoa somatic embryos following each treatment step of the encapsulation-dehydration procedure were examined using a combination of confocal scanning laser microscopy and transmission electron microscopy. Results showed that the parenchyma's cells of the hypocotyl and radicle were the major sites of injury possibly due to their large size and non-cytoplasmic nature, whereas the shoot meristem and provascular strand were well preserved throughout the treatments. In general, cell deformation and/or disruption was observed following the sucrose preculture, desiccation and freezing steps, with the extent of damage increasing at each treatment step. Post-thaw regrowth of injured embryos was possible through the proliferation of surviving cells, but these embryos often failed to convert into plantlets. The present study suggests that the maintenance of structural integrity of somatic embryos at each treatment step is essential for the successful cryopreservation of cocoa germplasm.

Key words: Cocoa somatic embryos, confocal scanning laser microscopy, cryopreservation, structural integrity, transmission electron microscopy.

INTRODUCTION

Cryopreservation is considered to be the most reliable means for the long-term conservation of cocoa (*Theobroma cacao* L.) germplasm. Since 2004, the University of Reading (U.K.) has launched a cryopreservation program to preserve its ca. 600 cocoa accessions which were previously maintained under structures at the International Cocoa Quarantine Unit. Cocoa cryopreservation has been performed using an encapsulation-dehydration procedure developed for the somatic embryos (Fang et al., 2004). The procedure, nevertheless, revealed two problems: The post-thaw recovery rate was highly genotype-dependent (that is, ranged from 25 to 72% depending on the genotype tested), and the atypical morphology of the recovered embryos (e.g. fasciated cotyledons, stunted hypocotyls, blackened radicles, adventitious somatic embryos) (Fang et al., 2004; Quainoo and Wetten, 2006). The cryogenic procedure has obviously generated injuries to the stored embryos. Understanding of the nature and location of the injuries would greatly assist with its optimization.

Cocoa somatic embryos used for cryogenic storage

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were at the early-cotyledonary stage of development, measuring approximately 2 to 3 mm in length and up to 1 mm in diameter. Cryopreserved plant specimens of this size were generally studied using light microscopy in the past (Gonzalez-Arnao et al., 1993). A light microscopic analysis requires, however, that the specimens be physically sectioned during processing. This operation is not only time-consuming but may also cause additional damages to the examined samples. The present study has adopted a confocal scanning laser microscopy (CSLM) system to assess embryo integrity instead of light microscopy. The CSLM offers observation of thin sections in thick, intact specimens, through a process known as optical sectioning. It can also provide information on cell cohesiveness, and tissue may be viewed at different levels by focusing (Brooker, 1991; Lapsley et al., 1992). A number of studies have reported the application of CSLM in the analysis of fresh fruit and vegetable tissues (Lapsley et al., 1992; Braselton et al., 1996; Dolores Alvarez et al., 2000). To our knowledge, this is the first report describing the application of CSLM in the examination of cryopreserved plant specimens.

In the present study, cocoa somatic embryos following the different stages of the encapsulation-dehydration procedure (that is, encapsulation, sucrose preculture, desiccation and freezing) were analyzed gross structurally using CSLM and ultrastructurally using transmission electron microscopy (TEM) for the presence, location and extent of damage. A morphological examination of the embryos recovered from cryopreservation was also conducted in parallel.

MATERIALS AND METHODS

Plant material

Secondary cocoa somatic embryos of the genotype IMC14 at the early-cotyledonary stage of development were used. The secondary somatic embryos were initiated according to the procedures of Li et al. (1998) and Maximova et al. (2002). Once initiated, somatic embryos were maintained on the embryo development (ED) medium (consist 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, 30 $g.l^{-1}$ sucrose, 1 $g.l^{-1}$ glucose, and 2 $g.l^{-1}$ Phytagel**,** pH 5.7) (Li et al., 1998).

Cryopreservation by encapsulation-dehydration

Cryopreservation was performed according to Fang et al. (2004). The somatic embryos were subject to the following treatment steps: (1) Encapsulation: The embryos were suspended in calcium-free liquid ED medium containing 3% (w/v) alginic acid (from *Macrocystis pyrifera*, 2% viscosity, SIGMA) after which individual embryos were dropped into liquid ED medium containing 100 mM $CaCl_2.2H2O$. After 30 min polymerization, calcium alginate beads (4 to 5 mm diameter) were rinsed three times with water for 5 s each and blotted on filter paper to remove surface moisture; (2) Sucrose preculture: Encapsulated embryos were cultured on ED medium enriched with 0.3 M sucrose for 3 days then on 0.75 M sucrose medium for another 4 days; (3) Desiccation: Sucrose precultured embryos, supported by a filter paper, were placed inside a 9-cm Petri dish containing 30 g dry silica gel for 4 h; (4) Freezing and thawing: Desiccated embryos were transferred into a 1.8 ml cryotube and plunged rapidly into liquid nitrogen. After 1 h storage, the tube was thawed inside a 35°C water bath for 5 min. All the treatments were conducted in the dark at 25°C. Following each treatment, somatic embryos were first recovered on 0.3 M sucrose medium for 3 days then on ED medium for another 2 days prior to microscopy analyses (except for the encapsulation treatment where the embryos were directly plated on ED medium). A separate batch of cryopreserved embryos was kept on the recovery medium for morphological analysis of their regrowth pattern.

Confocal microscopy analysis

Somatic embryos were first removed from the alginate beads then processed according to the procedure of Braselton et al. (1996) with slight modifications. Somatic embryos were fixed overnight in 3:1 ethanol:glacial acetic acid solution at 4ºC after which they were rinsed three times with water for 15 min each and hydrolyzed in 5 N HCl for 60 min. After hydrolysis, the embryos were rinsed three times with water for 5 min each and stained with Schiff's reagent (Fuchsin-sulphate, SIGMA) for 2 to 3 h. The Schiff's reagent was decanted and specimens were rinsed three times with water for 10 min each. The last change of water was replaced with 70% ethanol for 5 min followed by 3 to 5 changes of 100% ethanol for 5 min each. Embryos were placed in 1:1 ethanol:LR White resin mixture (hard, SIGMA) for 1 h followed by immersion in pure LR White resin for another hour. Embryos were subject to a final change with pure LR White resin and left to stand overnight. The following day, embryos and resin were placed together in a plastic mould and cured in an 80°C oven overnight. Resin-embedded embryos were then trimmed longitudinally using a microtome until approximately half of the embryo was removed and the areas of interest exposed. Samples were placed with oil immersion above a 40X objective and viewed using an inverted Leica TCS SP2 confocal imaging system. The samples were viewed under 500 to 650 nm excitation wavelengths (FITC wide mode) and the laser used was Ar/HeNe. Optical sectioning was possible to a depth of 150 µm. The four areas examined in each embryo included the shoot apex, the outer and inner portion of the hypocotyl (delimited by the provascular strand), as well as the radicle (Figure 1).

Transmission electron microscopy analysis

Somatic embryos were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 h on a rotary shaker, followed by three rinses with 0.1 M phosphate buffer for 15 min each. They were post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h, then rinsed three times with distilled water for 15 min each. Fixed embryos were dehydrated in 30, 50, 70, 90, and 100% acetone and absolute dry acetone with anhydrous copper sulphate for 20 to 30 min each. They were then immersed in 1:1 100% dry acetone: 99% propylene oxide for 30 min followed by two immersions in propylene oxide for 30 min each. After dehydration, embryos were embedded in Epon resin. They were left on a rotary shaker in 20% resin:80% propylene oxide, followed by 40% resin:60% propylene oxide, 60% resin:40% propylene oxide, 80% resin:20% propylene oxide each overnight and finally three times in 100% resin for a total duration of 7 days. All fixation, dehydration and infiltration steps were carried out at room temperature. Embryos were finally poured into plastic moulds and left for 1 days in a 60°C oven for polymerization. Before TEM analysis, silver and silver/gold sections 0.5 µm thick were cut and mounted on Formvar (0.5%, w/v) coated copper grids (200 mesh, 3 mm). Sections were stained with uranyl acetate for 1 h followed by lead citrate for 10 min. After air-drying, sections were viewed and photographed using a Hitachi

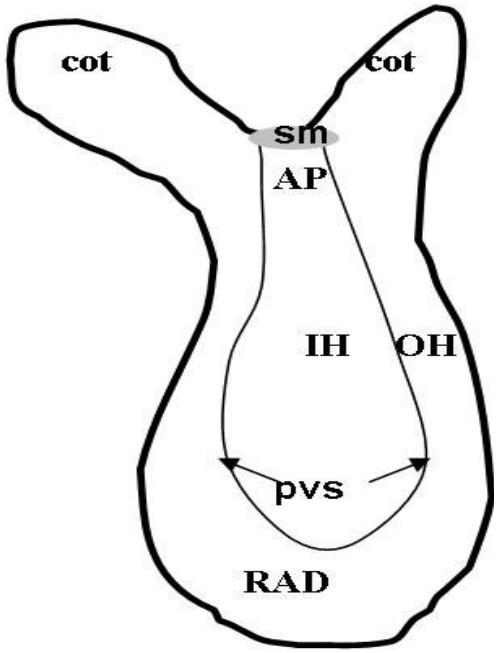

Figure 1. Longitudinal drawing of an early-cotyledonary stage cocoa somatic embryo. The four areas of interest included: The shoot apex (AP), the inner portion of the hycopotyl (IH), the outer portion of the hypocotyl (OH), and the radicle (RAD). sm, shoot meristem; pvs, provascular strand, cot, cotyledons.

H800 (Hitachi Ltd., Japan) TEM operating at 75 kV.

RESULTS

Embryo gross structural integrity

The four areas to be examined under CSLM can be easily identified in each embryo, after spotting the shoot meristem, the cotyledon notch, the radicle and the provascular strand of the embryo. Cells in the shoot meristem and provascular strand were characterized by their isometric shape, small size and prominent nuclei; while the parenchyma's cells of the hypocotyl and the radicle were geometrically-shaped, larger and with small nuclei (Figure 2).

Cell deformation was observed in cocoa somatic embryos at all stages of the cryopreservation cycle, except for the encapsulation. In the encapsulated embryos, cells in the shoot meristem, provascular strand and the inner and outer portions of the hypocotyl showed no abnormalities (Figures 2a to d). Although no obvious cell disruption was observed for sucrose precultured embryos, some stress has occurred in the hypocotyl and radicle as evidenced by the irregularity of cell shape (Figures 2e to h). The subsequent desiccation stage was very stressful to the embryos as cell deformation at the inner portion of the hypocotyl and cell disruption at the outer portion of the hypocotyl was observed (Figures 2i to l). Only those cells located in the shoot meristem and provascular strand appeared untouched (Figures 2i and j). The embryo presented here was one which showed the most severe symptoms of desiccation damage. It should be noted that the extent of desiccation damage varied considerably from one somatic embryo to another. In the frozen embryos, intact cells were interspersed with disrupted cells in the hypocotyl (Figure 2n). Non obvious damage was observed for cells located at the shoot apex and in the provascular strand (Figures 2m to p).

Embryo ultrastructural integrity

In the encapsulated embryos, cells were generally in good condition with the presence of numerous organelles [e.g. Golgi apparatus, plastids, mitochondria and endoplasmic reticulum (ER)]: Characteristics of an active cell metabolism (Figure 3a). Cells contained either a large or several small vacuoles separated by narrow regions of cytoplasm. Nuclear membranes were well defined enclosing a prominent nucleus and small patches of chromatin attached to the nuclear envelope. The plasma membrane was intact and relatively smooth with only minor undulations. Sucrose preculture resulted in numerous ultrastructural changes in the cells including, for example, the differentiation of plastids into amyloplasts

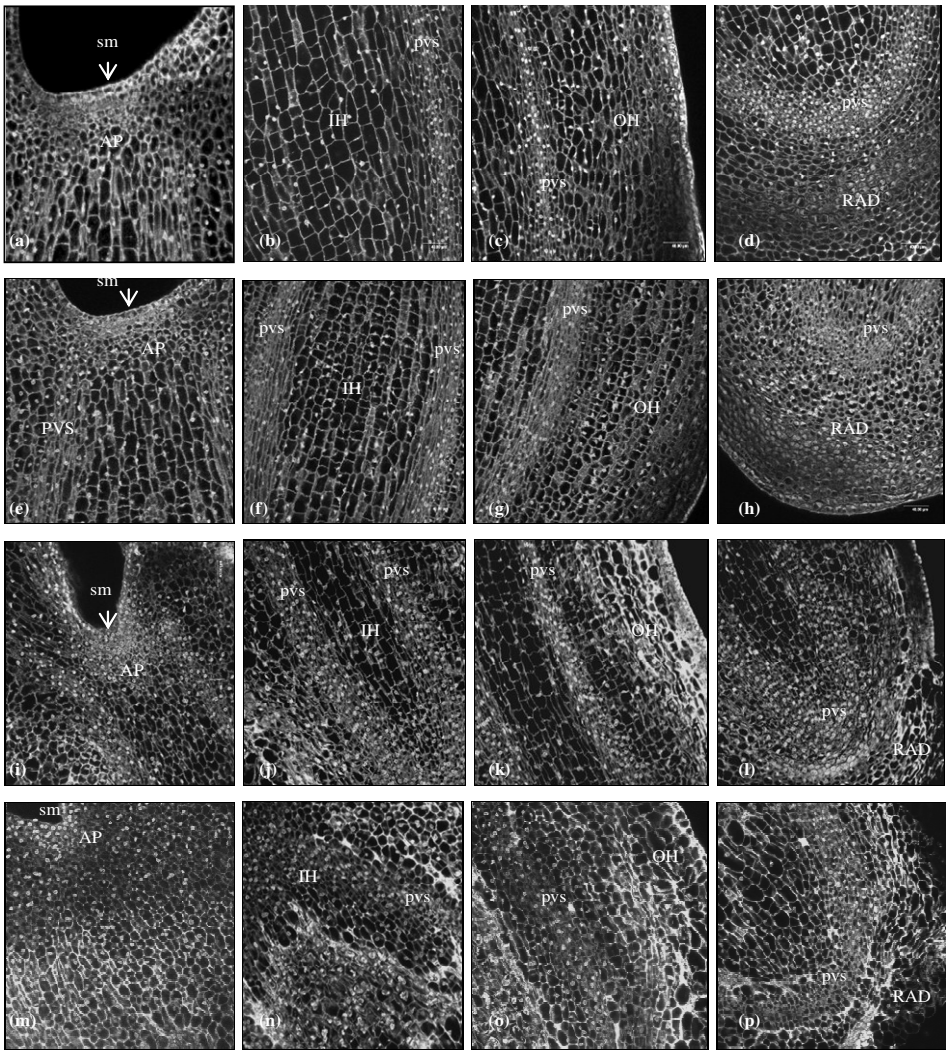

Figure 2. Longitudinal confocal scanning laser micrographs of cocoa somatic embryos following an encapsulation-dehydration cycle: Encapsulation (a-d), sucrose preculture (e-h), desiccation (i-l), and freezing (m-p). The encapsulated embryo contained intact cells at the shoot apex (a), inner and outer portion of the hypocotyl (b, c), and the radicle (d). The sucrose precultured embryo showed well preserved cells in the shoot meristem and provascular strand (e), but stress in the parenchyma's cells of the hypocotyl (f,g) and the radicle (h). The desiccated embryo contained intact cells only in the shoot meristem and provascular strand (i). Cell deformation was observed in the inner portion of the hypocotyl (j) and cell disruption in the outer portion of the hypocotyl (k) and in the radicle (l). The frozen embryo contained well preserved cells at the shoot apex (m), mixture of injured and intact cells in the inner portion of the hypocotyl (n), and severely disrupted cells in the outer portion of the hypocotyl (o) and in the radicle (p). sm, shoot meristem; pvs, provascular strand; AP, shoot apex; IH, inner portion of the hypocotyl; OH, outer portion of the hypocotyl; RAD, radicle.

containing starch (Figure 3b). Undulations of the plasma membrane and tonoplast were also observed in some of the cells (Figure 3c). Nevertheless, other cell structures, including numerous mitochondria, ER and nucleus appeared intact (Figures 3b and c). Desiccated and frozen embryos contained cells which were severely injured with no discernable organelles (Figures 3d and e). These cells lacked most of the membranous structures and were characterized by disintegration of all cellular contents, except for the starch grains. Nevertheless, intact cells were also found in the same embryos and these cells were characterized by the presence of a well-defined nucleus, mitochondria and starch (Figure 3f).

Morphology of recovered embryos

Following cryopreservation, cocoa somatic embryos

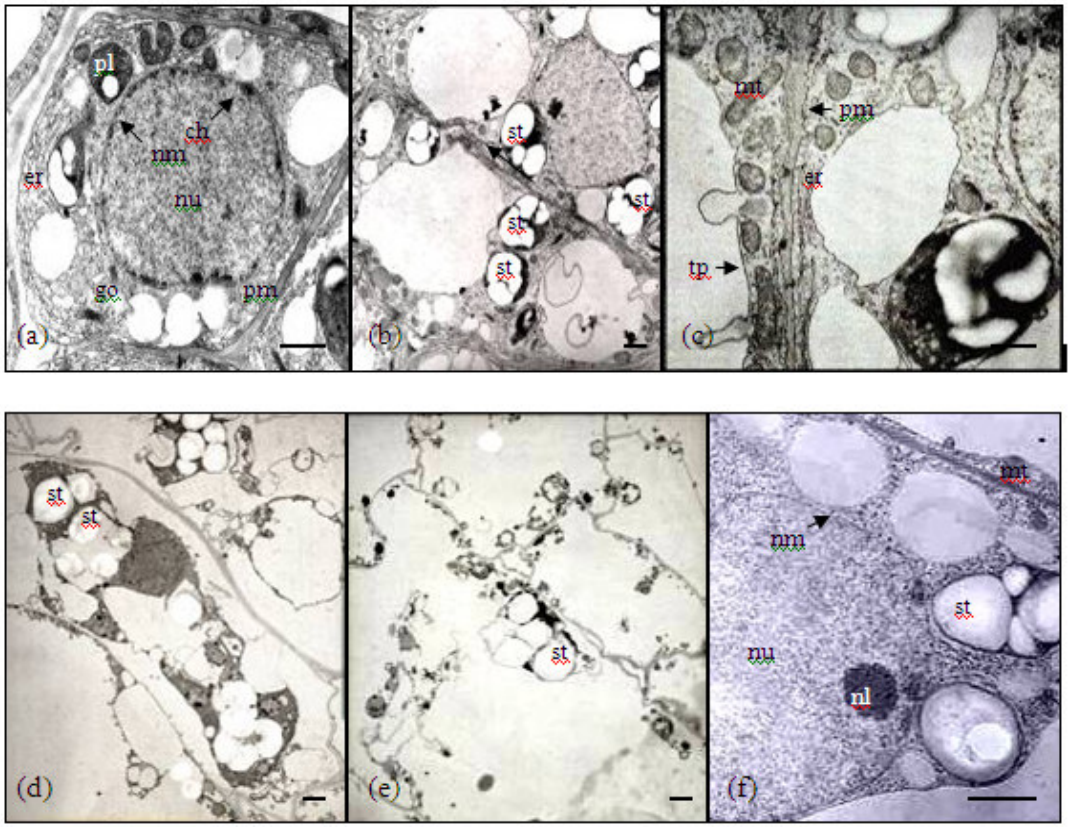

Figure 3. Transmission electron micrographs depicting the ultrastructural integrity of cocoa somatic embryos following a cryopreservation cycle. (a) A meristematic cell showing prominent nucleus enclosing chromatin near the nuclear membranes, numerous plastids, extensive endoplasmic reticulum throughout the cell, golgi apparatus and smooth plasma membrane in an encapsulated embryo; (b) Presence of numerous starch grains in a sucrose precultured embryo; (c) Undulations of the plasma membrane and tonoplast found in cells of a sucrose precultured embryo; (d, e) Severely disrupted cells with only starch present in desiccated and frozen embryos; (f) Well preserved embryo with the presence of nucleus, nucleolus, nuclear membranes, mitochondria and starch. nu, nucleus; ch, chromatin; nm, nuclear membranes; pl, plastids; er, endoplasmic reticulum; go, golgi apparatus; pm, plasma membrane; st, starch grains; tp, tonoplast; nl, nucleolus; mt, mitochondria. Bars = 1 µm.

resumed growth at a variable pace but started as early as the second week after thawing. Embryo regrowth began with the expansion of the cotyledons, followed by the elongation of the hypocotyl (Figure 4a). But it was observed for some embryos that their hypocotyls showed no further elongation during recovery, and eventually became stunted and blackened (Figure 4b and c). Occasionally, adventitious embryos were formed on the degenerating tissue of the hypocotyl (Figure 4d).

DISCUSSION

The integrity of cocoa somatic embryos following the different stages of an encapsulation-dehydration procedure was investigated using CSLM and TEM in the present study. The application of the optical sectioning property of CSLM in the analysis of gross structural changes in cocoa somatic embryos has proven very successful as these relatively large cryo-samples can be processed using a simple approach, and thus may be less prone to damages prior to microscopic examination.

The present study demonstrates that the intensity of cell stress and damage increased as the somatic embryos proceeded through the cryogenic cycle. This means that each stage of the encapsulation-dehydration procedure has contributed to the loss of embryo integrity, except for the encapsulation treatment. To begin with, sucrose precultured embryos were found to contain cells of irregular contour. This may be indicative of osmotic stress as a result of sucrose application. The subsequent desiccation stage was even more stressful to the embryos, but the stress intensity varied greatly from embryo to embryo. It is speculated that the encapsulation and desiccation methods which have been used were responsible for this variation. In fact, it was observed following the encapsulation treatment that the embryos within the beads were mostly off-centred probably

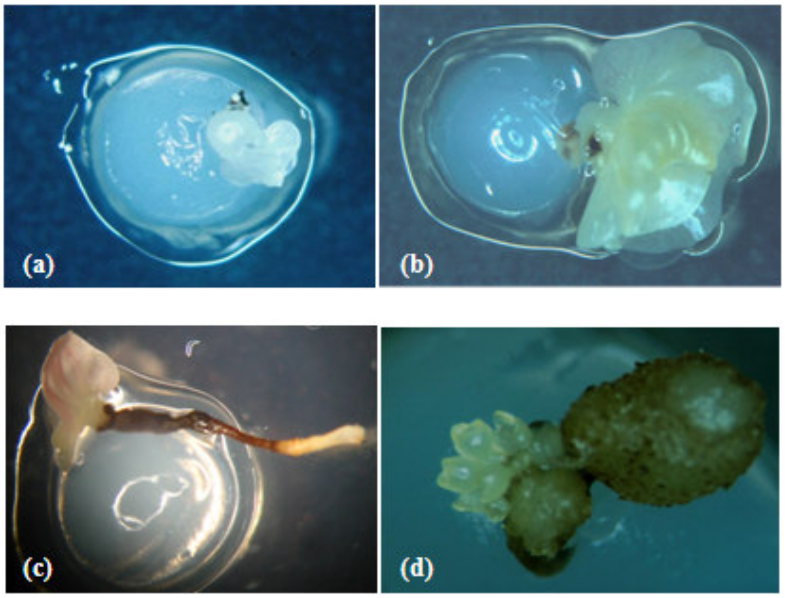

Figure 4. Morphologies of cocoa somatic embryos recovered from cryopreservation. (a) Undamaged embryo resuming normal growth; (b) Embryo with fasciated cotyledons and stunted hypocotyl; (c) Embryo exhibiting cotyledon growth but blackened hypocotyl and radicle; (d) Degenerated embryo showing formation of adventitious embryos.

because of the higher density of the embryos compared to the alginate which made the embryos to gravitate to the underside of the forming bead during the hardening process. As a result, embryos in closer contact with the underlying silica gel may lose water more and faster than those which were further away from the drying agent, and thus the former were more exposed to desiccation stress. An encapsulation method which allows the embryos to be centrally positioned inside the beads can be tested in the future. Or an alternative desiccation system involving saturated salt solutions and evaporative drying could be attempted in order to achieve a more uniform and slower dehydration of the explants. In the frozen embryos, injured cells were found both at the outer and inner portions of the hypocotyl. The damages occurring at the outer portion of the hypocotyl were thought to have initiated following excessive dehydration, whereas those at the inner portion were results of ice crystal damage in insufficiently dehydrated cells. This phenomenon was observed in all the somatic embryos examined, presumably because of the heterogeneous cell composition in embryos at the early-cotyledonary stage of development. Cocoa somatic embryos at earlier stages of development (that is, globular, heart shape), composed of cells of more homogeneous size and type, may be better candidates for cryopreservation. Studies are underway to confirm this hypothesis.

The histological micrographs demonstrated that the cocoa somatic embryos used in the present study were consisted of cells of various sizes and types. It was found that small and densely cytoplasmic cells withstood better the cryogenic treatments than large and vacuolated ones. Indeed, cells located in the shoot meristem and provascular strain were well preserved throughout cryopreservation, as opposed to those in the radicle and hypocotyl parenchyma which were easily injured. Similar observations were also made in other studies. For instance, in highly proliferating meristems of frozen banana apices, highly vacuolated cells were destroyed with the result that only a few layers of meristematic cells localized in the tunica and leaf primordia remained alive following cryopreservation (Helliot et al., 2002). In *Dianthus* shoot tips, most of the subapical tissue was damaged after thawing while most of the surviving cells were located in the apical meristems and primordial leaf tissue (Kartha, 1985). Similarly, cell survival was found to only occur in the meristematic region and lower primordial zone for cryopreserved shoot tips of *Cosmos atrosanguineus* (Wilkinson et al., 2003).

Ultrastructural examination of cocoa somatic embryos also revealed signs of stress and damage throughout cryopreservation. For example, undulations of the plasma membrane were observed following the exposure of embryos to sucrose. These may have served to accommodate the volume reduction caused during the partial plasmolysis. The folding of the plasma membrane and occurrence of vesiculation were thought to represent irreversible membrane loss during the contraction phase of plasmolysis, leading to lysis on subsequent re-expansion from the plasma membrane (Gnanapragasm and Vasil, 1992). Moreover, there was an increased number of starch present compared to the encapsulated

embryos. This may indicate that sucrose preculture induced the synthesis of storage reserves in addition to exercising an osmotic effect. During the subsequent desiccation and freezing steps, two categories of cells were observed: Intact cells with intact nucleus, clearly defined nuclear membranes, abundant mitochondria reflecting active metabolism and presence of ER throughout the cell; and completely disrupted cells with no discernable organelles. This observation is in accordance with the previously reported confocal microscopy data, where the co-existence of these cells was the result of the heterogeneous cell composition in the embryos. Ultrastructural alteration in plant cells following cryo-preservation has also been reported in other studies. For example, preculture of encapsulated sugarcane apices with 0.75 M sucrose showed increased starch synthesis (González-Arnao et al., 1993). After desiccation, the structure of cells was drastically modified: Nuclei were retracted and intensively stained; nucleoli were no longer visible; and the cytoplasm was often detached from the cell wall. These features were later accumulated in cells of cryopreserved samples. In another study by Stewart et al. (2001), cryoprotected and desiccated cassava somatic embryos contained cytoplasm that was often pulled away from the plasma membrane and organelles that were difficult to discern in severely damaged cells. This situation was exacerbated when the tissue was cryostored. Dehydration of non-cryoprotected zygotic axes of *Quercus robur* induced largely necrotic cells in both the root meristems and contiguous parenchyma, and a high degree of vacuolation had occurred in cells of shoot apical meristems (Berjak et al., 1999). Nevertheless, all the axes ultimately germinated indicating that the dehydration stresses applied were not, in themselves, lethal. However, the effects of applied stresses on the excised axes were considered to be cumulative by the authors.

Despite the injuries depicted in cocoa somatic embryos following cryopreservation, their post-thaw regrowth was possible. This is in agreement with the observation of Wilkinson et al. (2003) where tissue regeneration following cryopreservation of *C. atrosanguineus* shoot tips was observed although survival of the entire explants was not achieved. Fukai (1995) also determined that post-cryopreservation regeneration of *Chrysanthemum* shoot tips was originated from the L1 layer and not from the entire meristems by using a periclinal chimaeric cultivar. On the other hand, the presence of injuries has often leaded to aberrant growth from the recovered cocoa somatic embryos. Most of the embryos resumed apical growth but showed stunted hypocotyl and degenerative radicle. This correlates with our microscopy data where the shoot meristems were generally well preserved throughout cryopreservation, whereas damages were mostly manifested in the hypocotyl and radicle. Cryopreservation-derived morphological abnormality was also observed in mature embryos of coconut, which no longer produced haustoria after retrieval from liquid nitrogen, although normal plantlet development did occur (Assy-Bah and Engelmann, 1992). Also, cocoa zygotic axes and pea somatic embryos showed only limited regenerative tissue following cryopreservation, which further led to the formation of callus (Pence, 1991; Mycock et al., 1995). In our study, most of the defected embryos failed to convert into plantlets (data not shown). The maintenance of structural integrity in cocoa somatic embryos throughout cryopreservation is therefore essential since their ability to regenerate into plants has been the prevailing goal in our cryopreservation program.

In conclusion, each stage of the encapsulation-dehydration procedure was found to contribute to the loss of somatic embryo integrity and thus viability (except the encapsulation). The damages were accumulated in the embryos throughout the cryogenic cycle. Despite the injuries, post-thawed embryos were able to resume growth although with low guarantee of plant regeneration. Optimization of the different stages of the procedure is required before cryopreservation can be applied effectively for the long-term conservation of cocoa genetic resources. The CSLM which was proven very successful for cocoa can serve as a fast and effective alternative for the histological examination of other cryopreserved plant systems.

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