Recovery, histological observations and genetic integrity in coconut (Cocos nucifera L.) embryogenic calli cryopreserved using encapsulation-dehydration procedure

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Abstract: Encapsulation-dehydration protocol was evaluated for the long-term conservation of coconut embryogenic calli (EC). Encapsulated EC were pretreated in a sucrose medium (either 0.5 M or 0.75 M) for different durations (24, 48 and 72 h) followed by desiccation in silica gel (8, 12, 16 and 20 h) prior to storage in liquid nitrogen. Survival and regrowth of EC following desiccation alone or desiccation and freezing were recorded after four and eight months, respectively. Histological studies and scanning electron microscopy (SEM) were carried out to evaluate the structural changes of cryopreserved samples. Recovered samples from cryopreserved and non-cryopreserved EC were tested for genetic fidelity at 11 simple sequence repeat (SSR) marker loci. Highest survival (45 %) and recovery (25 %) rates were achieved by pre-treating EC on 0.75 M sucrose for three days followed by dehydration for 20 hours, prior to liquid nitrogen immersion. Ultra-structural studies showed no aberrations or damages in the exterior regions of cryopreserved ECs. Massive damages at the interior regions after cryopreservation suggested that osmotic and cryo injuries occur in the internal regions of EC. DNA banding patterns at SSR marker loci showed no evidence of somaclonal variations in tested material confirming that encapsulation-dehydration have not caused any genetic variation in EC.

Keywords: Cryo injuries, genetic fidelity, histology, in vitro, simple sequence repeats.

INTRODUCTION

Conservation of coconut germplasm is important to facilitate future breeding programmes. Conventional storage of coconut as seeds is impossible because of the large size of the seed, recalcitrant nature and lack of a dormancy period (Engelmann, 1997). Currently, coconut germplasm is conserved in field genebanks, which are frequently threatened by adverse weather conditions and pest and disease attacks (Nguyen et al., 2015). Moreover, field gene banks require large areas for the maintenance of plantations and the process is highly labour intensive, thus very costly. Hence, development of in vitro culture techniques for conservation and exchange of coconut germplasm is highly desired.

Cryopreservation, which is a method of conserving plant tissues at an ultra-low temperature, usually in liquid nitrogen (LN; -196 ºC) is an important technique for the long-term storage of plant germplasm in many problematic species such as non-orthodox seed species, vegetatively propagated plants and rare, endangered plant species. At this temperature, metabolic processes and growth activity of cells completely cease and thus plant materials remain stable for an unlimited period with minimal space and maintenance (Engelmann, 1997).

The early attempts of in vitro conservation of coconut germplasm focused on ex-situ conservation of zygotic embryos and pollen (Assy-Bah & Engelmann, 1992a; Frison et al., 1993) as the source of explant with subsequent efforts using plumular tissues (Malaurie et al., 2006). Immature embryos have been cryopreserved...
by pre-growth desiccation (Assy-Bah & Engelmann, 1992b), resulting in survival rates of 10 – 43 %. It is reported that mature embryos can be cryopreserved by vitrification (Sajini et al., 2011; Cueto et al., 2013), rapid dehydration (N’Nan et al., 2012), pre-growth desiccation (Assy-Bah & Engelmann, 1992a), and droplet vitrification (Cueto et al., 2013) and a maximum of 40 % success rate (recovery) for the modified desiccation protocol has been reported (Sisunandar et al., 2010). However, coconut is an open pollinated heterozygous plant, thus genetic characters of the zygotic tissues may vary from the mother palm. Conserving somatic embryos or EC will enable the conservation of superior palms with true-to-type genetic makeup. Furthermore, cell and callus line maintenance, continuous supply of standard stock cultures for physiological and biochemical experiments are considered as beneficial outcomes of cryopreserved in-vitro cultures (Poobathy et al., 2013).

Cryopreservation of EC has experimented in several other perennial commercial crops such as rubber (Zhou et al., 2012), cocoa (Fang et al., 2004) and oil palm (Suranthran et al., 2012). Encapsulation-dehydration procedure, which is developed based on the technology for the production of artificial seeds can be successfully applied to calli because of the protected covering formed around the delicate calli tissue providing the protection against dehydration and freezing damage. This technique has been successfully applied in several species (Feng et al., 2013; Barraco et al., 2014) and reported for coconut plumule cryopreservation with 60 % survival and 20 % shoot development (reviewed in Welewanni et al., 2017).

No studies have been reported for coconut cryopreservation using EC, which may be indeed important for conservation of coconut germplasm. The objective of this study is to determine the amenability of coconut embryogenic calli (derived from unfertilised ovary explants) to cryopreservation technique, assessment of regenerated plants for their genetic fidelity and ultrastructural changes leading to the identification of an optimum cryopreservation protocol for coconut EC by encapsulation dehydration method.

**METHODOLOGY**

**Plant material**

Unfertilised ovaries were excised from female flowers of 4-stage (which is the inflorescence that will open in four months considering the most recently opened inflorescences as 0 stage; Perera et al., 2007) inflorescences from mature palms of the improved coconut hybrid CRIC 65.

**Initiation and multiplication of embryogenic callus through ovary culture**

Ovary derived calli were raised using the method described by Bandupriya et al. (2017) with the modification that, instead of CRI 72 medium, modified Eeuwens Y₃ medium (Eeuwens, 1976) was used. Well-developed embryogenic calli (Figure 1a) from the third or fourth cycle of sub-culturing were used for cryopreservation studies.

**Cryopreservation by encapsulation-dehydration**

Dissected embryogenic calli parts (2–3 mm; Figure 1b) were pre-cultured on the same modified Eeuwens Y₃ medium for 3 d to screen for microbial contamination. Encapsulation was carried out by suspending the EC parts in Eeuwens Y₃ medium (devoid of CaCl₂) containing 3 % (w/v) Na-alginate (2 % viscosity, SIGMA) and 4 % sucrose. This was followed by dropping each EC using a sterile pipette into 0.1 M CaCl₂·2H₂O solution prepared in Eeuwens Y₃ medium supplemented with 4 % sucrose and allowed to polymerise for 45 min at room temperature to form beads (4 – 5 mm in diameter, Figure 1c). Osmoprotection was carried out by pretreating the EC containing beads in liquid Y₃ medium containing either 0.5 M or 0.75 M sucrose for different durations (24, 48 and 72 h). The beads were then subjected to dehydration for 8, 12, 16, or 20 h inside glass jars by placing 20 pretreated beads on top of 40.0 g of dried silica gel separated by a filter paper. Upon dehydration (Figure 1d), half of the beads were cultured into the standard recovery medium and the other half of the beads of each treatment was transferred to 2.0 mL sterilised cryo-tubes and directly plunged into LN at least for 2 h. Thawing was performed by immersing the cryotubes in a water bath at 40 °C for 3 min. The water content of the beads (on fresh weight basis) was determined by drying them in an oven at 80 °C until a constant weight was obtained.

**Plant regeneration**

Each alginate bead was cultured for regeneration in a small glass vial containing 15 mL somatic embryo induction medium [modified Eeuwens Y₃ medium containing 95.0 µM 2,4-D and 2.7 gL⁻¹ Glutamine (3G)] for 4 wk. Plant regeneration protocol described by Bandupriya et al. (2017) was followed for the regeneration of plantlets.
Assessment of survival and regrowth

The survival was assessed after 4 months of in vitro culture as the percentage of calli manifesting new tissue growth as indicated by any sign of growth such as swelling, development of tissue and/or callus formation. The recovery of calli (indicated by the ability of calli to produce somatic embryos and/or grow into shoots or plantlets following desiccation alone or desiccation and freezing) was assessed at least after 8 months in culture.

Statistical analysis

Treatments were arranged in a completely randomised design (CRD) including 10 experimental units per treatment with two replicates. Data analysis was conducted using STATA 13.1. Summary of the results was presented as mean, standard deviation (SD), median and interquartile range (IQR) for continuous variables as appropriate, whereas counts are presented as percentages for categorical variables. As normality assumptions were violated, non-parametric statistical tests were used in the analysis in order to eliminate potential bias (Vickers, 2005) and median was used as the measure of central tendency. To compare more than two categories of a continuous variable, Wilcoxon rank sum test was used. To determine the effects of exposure on survival rate and recovery rate, ordinal logistic regression was employed. Survival and recovery rates were categorised into 4 categories based on the percentiles as observed (0 %, 0 – 10 %, 10 – 20 %, and above 20 %). For survival rate, 40 (0 %), 27 (0 –10 %), 12 (10 – 20 %), and 17 (above 20 %) observations were obtained in 4 categories. Similarly, 64 (0 %), 18 (0 – 10 %), 6 (10 – 20 %), and 8 (above 20 %) observations were obtained for the recovery rate. Results are displayed as proportional odds ratios for a one unit increment; whereas one unit is the increment of categorised survival or recovery percentiles by one level to other. Significance level of the treatment effects was indicated at the 95 % level of probability (p ≤ 0.05).

Histological analysis

Cryopreserved and non-cryopreserved EC, treated with 0.5 M and 0.75 M sucrose for 24, 48 and 72 h and dehydrated for 20 h, were subjected to histology studies. EC without any treatment were used as the control. Samples were fixed in FAA solution (50 % ethanol: 10 % formaldehyde: glacial acetic acid = 18:1:1, v/v/v) for 72 h. Dehydration was carried out through a graded alcohol series (50 – 100 % ethanol) and clearing was done using pure xylene. Calli were embedded in paraffin wax and blocks were prepared followed by obtaining 4.0 µm thick sections using a microtome (Sakura, Japan) with steel blades. The sections were double stained with periodic acid Schiff’s reagent (PAS) and protein-specific naphthol blue black (NBB) (Fisher, 1968). The slides were observed under microscope and images were obtained by a camera-fitted light microscope (ZEISS, USA).

Scanning electron microscopy

Samples were selected from cryopreserved and non-cryopreserved EC, pretreated with 0.75 M sucrose for 72 h followed by 20 h dehyrdation, and osmoprotected with 0.75 M sucrose for 72 h without physical dehyrdation by silica gel. EC without any treatment were selected as the control with 3 biological replicates from each treatment. Fresh samples were obtained from the recovery medium, the alginate covering was removed, and fixed on double-sided strips of tape that were affixed onto the sample stub. Samples selected were neither treated nor coated prior to the viewing and the samples were gold coated using a sputter coater (Quorum, SC7620). The samples were viewed in their native state, using scanning electron microscope (ZEISS, Evo LSIS) at saturated humidity at 4 °C at an extra high tension (EHT) value of 10 kV.

SSR marker analysis

Somatic embryos and shoots recovered after cryopreservation together with non-cryopreserved and counterpart controls were used to assess the genetic integrity. DNA was extracted using DNeasy® Plant Mini Kit (QIAGEN®) following the manufacturer’s instructions. SSR primers (Table 1) were selected according to Rivera et al. (1999). PCR reaction mixtures were prepared as described by Bandupriya et al. (2017). Amplifications were performed in thermal cycler (BIO-RAD; MyCyclerTM) programmed at 94 °C for 5 min for initial denaturation, followed by 94 °C for 30 s, annealing temperature depending on the primer used (Table 1) for 30 s and 72 °C for 30 s, annealing temperature depending on the primer used (Table 1) for 30 s and 72 °C for 1 min for 35 cycles followed by a final step of extension at 72 °C for 5 min. PCR samples were electrophoresed on 6 % (w/v) polyacrylamide gel and bands were visualised by silver staining. The genetic polymorphisms of the samples were scored and compared with the control samples and the donor palm.
RESULTS AND DISCUSSION

Effect of sucrose pretreatment and dehydration on the water content of encapsulated EC

Sucrose concentration showed a significant effect on the water content of beads (fresh weight basis) after dehydration ($p = 0.0001$) recording a higher water content in 0.5 M (38–42 %) than in 0.75 M (27–30 %) sucrose (Figure 2). A higher sucrose concentration induced greater osmotic dehydration and the values are comparable with the water content recorded for encapsulated coconut plumules after sucrose pretreatment (N’Nan et al., 2008). However, the effect of duration of sucrose pretreatment (24, 48 or 72 h) on the water content of beads after dehydration was not significant ($p = 0.7123$). Generally, a 20–25% water content of beads is optimal for successful cryopreservation (Bian et al., 2002). The optimum water content reduction up to 28 % at 0.75 M sucrose level indicated the need for further reduction of water content to achieve higher recovery rates in further studies. Although water content is an important factor in cryopreservation, it cannot be dissociated with many other parameters such as the material used, the cryoprotectant, and its concentration, pretreatment duration and the method of dehydration, thus it is not possible to detect a specific water content related to the desiccation damage (N’Nan et al., 2008).

Different dehydration durations (8, 12, 16, 20 h) did not show a significant effect on the water content of encapsulated beads ($p = 0.6316$). Similarly, N’Nan et al. (2008) reported no significant differences in the water content for 6 to 16 h of dehydration performed using silica gel. Thus, it could be suggested that the results of the present study is confirmed by the available literature.

Table 1: List of selected primers used in SSR analysis with primer sequence, fragment size and annealing temperature for each primer

| Oligo Name | Sequence | Fragment size/range (bp) | Annealing temperature °C |
|------------|----------|--------------------------|--------------------------|
| CAC08      | F 5’-ATC ACC CCA ATA CAA GGA CA-3’ | 188–210 | 56 |
| R          | 5’-AAT TCT ATG GTC CAC CCA CA-3’ | | |
| CAC20      | F 5’-CTC ATG AAC CAA ACG TTA GA-3’ | 124–133 | 54 |
| R          | 5’-CAT CAT ATA CAT ACA TCA TGC AAC A-3’ | | |
| CAC23      | F 5’-TGA AAA CAA AAG ATA GAT GTC AG-3’ | 170–179 | 56 |
| R          | 5’-GAA GAT GCT TTG ATA TGG AAC-3’ | | |
| CAC65      | F 5’-GAA AAG GAT GTA ATA AGC TGG-3’ | 150–173 | 54 |
| R          | 5’-TTT GTC CCA AAA TAT AGG TAG-3’ | | |
| CNZ04      | F 5’-TAT ATG GGA TGC TTT AGT GGA-3’ | 130–166 | 53 |
| R          | 5’-CAA ATC GAC AGA CAT CCT AAA-3’ | | |
| CNZ10      | F 5’-CCT ATT GCA CCT AAG CAA TTA-3’ | 108–152 | 54 |
| R          | 5’-AAT GAT TTT CGA AGA GAG GTC-3’ | | |
| CNZ12      | F 5’-TAG CTT CCT GAG ATA AGA TGC-3’ | 218–229 | 54 |
| R          | 5’-GAT CAT GGA ACG AAA ACA TTA-3’ | | |
| CNZ21      | F 5’-ATG TTT TAG CTT CAC CAT GAA-3’ | 220–250 | 54 |
| R          | 5’-TCA AAT GTC AAT GTC A9G AAG CCT TTG -3’ | | |
| CNZ29      | F 5’-TAA ATG GGT AAG TGT TTT GTG TGC-3’ | 105–157 | 56 |
| R          | 5’-CTG TCC TAT TTT CCT TTG ATT-3’ | | |
| CNZ40      | F 5’-CTG TGG TAT GCT TTA CGG TGT-3’ | 143–155 | 56 |
| R          | 5’-CTG GCA ACA AAT ACC ATG GTC TGT-3’ | | |
| CNZ44      | F 5’-CAT CAG TCT CAC TCT CAT TTC-3’ | 151–170 | 52 |
| R          | 5’-CAA CAA AAG ACA TAG GTG GTC-3’ | | |
| CNZ46      | F 5’-TGG GTT AGT ATA GCC ATG CAT-3’ | 101–120 | 56 |
| R          | 5’-AAC CAT TTG TAG TAT ACC CCC-3’ | | |
The main cause of injury during cryopreservation is intracellular ice crystal formation. Thus, survival can be increased by reducing the intracellular water content of the cells. Pre-culturing in high sucrose concentrations has been found to cause many cellular alterations, which are favourable for the survival during cryostorage. In most cases, encapsulation-dehydration technique uses sucrose which is a non-toxic substance as a cryoprotectant. Sugars or most importantly sucrose decrease the amount of freezable water content in cells due to its osmotic effect and accumulation of soluble sugars, thus enhances the freezing tolerance (Suzuki et al., 2006; Feng et al., 2013). Increment of total soluble sugar and accumulation of sugar inside cells could help maintain plasma membrane integrity by substituting for water on the membrane surface, thus stabilising proteins under dry and freezing conditions (Crowe et al., 1988). Furthermore, total soluble protein levels in buds increase when the explants are pretreated with high sucrose concentrations. It is considered to be one of the earliest physiological responses of osmotically stressed cells, which may be related to the increased freezing tolerance (Suzuki et al., 2006). The use of high sucrose concentrations facilitate the penetration of a sufficient quantity of sucrose into the cells, and accumulation inside the cells as starch. These accumulated starches could be the remedy for maintaining water output; without producing harmful effects and making the tissue to withstand further desiccation and freezing (González-Arnao et al., 2003).

**Effects of sucrose pretreatment on EC survival and recovery**

Sucrose concentration was the overall significant factor \((p = 0.0245)\) contributing to the survival rate while the duration in sucrose pretreatment was not significant \((p = 0.2173)\). The ordinal logistic regression revealed a higher survival rate in 0.75 M than in 0.5 M sucrose concentration. Similarly, higher survival rates were observed with increased duration of sucrose pretreatment (24 h to 72 h) for both tested sucrose concentrations (Figure 3 a and b). The effect of sucrose concentration was not significant for recovery rate \((p = 0.2594)\), while the effect of sucrose pretreatment duration was significant \((p = 0.0359)\) recording higher recovery rates when the duration is increased (Figure 3 c and d).

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The results revealed that coconut EC can tolerate high sucrose concentrations (0.75 M). Similar results have been reported elsewhere for coconut plumules demonstrating successful survival and recovery upon pretreatment with high sucrose concentrations: 0.75 M or 1 M (N’Nan et al., 2008) and 0.75 M (Bandupriya et al., 2007) through encapsulation-dehydration. Interestingly, date palm (Phoenix dactylifera) EC could not tolerate high sucrose concentrations due to osmotic shock and optimum sucrose concentrations reported for successful pretreatment are ranged between 0.1 – 0.5 M (Subaih et al., 2007).

**Effect of dehydration duration on EC survival and recovery**

Although differences in survival rate did not significantly differ (p = 0.1308), dehydration duration significantly affected the recovery rate (p = 0.0219) after cryopreservation. Ordinal logistic regression for categorised data revealed higher recovery rates when dehydration durations are elevated (Figure 4 a and b). The highest survival (45 %) of EC after cryostorage was achieved by pre-culturing EC in 0.75 M sucrose for 72 h and dehydrating for 20 h, prior to cryostorage. Highest recovery (30 %) of EC was achieved by pre-culturing them in 0.5 M sucrose for 48 h and dehydrating for 20 h and the second highest (25%) recovery was achieved when EC were precultured in 0.75 M sucrose for 72 h and

![Figure 3: Effect of sucrose pretreatment duration on survival and recovery of embryogenic calli. Survival % of (a) non-cryopreserved samples (-LN); (b) cryopreserved samples (+LN). Recovery % of (c) non-cryopreserved samples (-LN); (d) cryopreserved samples (+LN). Data correspond to all the cryopreserved (+LN) and non-cryopreserved (-LN) treatments done for two sucrose concentrations. Median within box plots having different letters are significantly different according to two-sample Wilcoxon rank-sum (Mann-Whitney) test at p ≤ 0.05.](image-url)
Cryopreservation of coconut embryogenic calli

Figure 4: Effect of sucrose concentration and dehydration period on survival rates (a) and recovery rates (b) of encapsulated non-cryopreserved (+D/-LN) and cryopreserved (+D/-LN) EC. Encapsulated EC were osmoprotected with 0.5 M and 0.75 M sucrose, and dehydrated for 8, 12, 16 and 20 h durations followed by LN application. Data correspond to the median of all cryopreserved (+LN) and non-cryopreserved (-LN) treatments. Median within box plots having different letters are significantly different according to two-sample Wilcoxon rank-sum (Mann-Whitney) test at p ≤ 0.05.

dehydrated for 20 h, before immersed in LN. Although 0.5 M sucrose level showed the highest recovery, overall better survival and recovery for all the dehydration periods were recorded in 0.75 M sucrose. Therefore, the best treatment combination for further experiments on histology and SSR analysis was determined as preculture of calli in 0.75 M sucrose for 72 h and dehydrating them for 20 h in silica gel. Coconut is an open pollinated plant and palm-to-palm variation is quite high between species, especially in in vitro culture. EC selected for the experiment were from four different donor palms of the same cultivar CRIC65. Unexpected variations in survival may be due to the different genetic makeup of explants. From all the donor palms used in the treatments (donor palm numbers: DT 1662, DT 1680, DT 1713 and DT 50), DT 1680 showed better survival and recovery (Data not shown).

Survival rates observed in the treatments after dehydration and freezing were very low compared to the untreated controls. Some of the cryopreserved and non-cryopreserved EC turned brown and some remained white without any growth, even after two months of culturing in the recovery media. These EC were not dead or vitrified, but turned into rubber like form. Some of the cryopreserved and non-cryopreserved (but dehydrated) calli, which were sub-cultured into a new callus induction medium were able to produce new EC and somatic embryos (Figure 1 e, f and g). Shoots developed from non-cryopreserved (but dehydrated) treatments (Figure 1h) showed no difference in morphology compared to the untreated controls (Figure 1 i and j). Overall somatic embryo and shoot development after dehydration and freezing was very slow. Similar results have been reported in plantlets derived from cryopreserved coconut
embryos and plumules (N’Nan et al., 2008; Sajini et al., 2011). N’Nan et al. (2008) explained that 20% recovery is a commendable achievement in species like coconut which are recalcitrant. The highest recovery (25%) recorded in this study for the best treatment combination is encouraging for further research to develop a reliable cryopreservation technique from coconut EC.

**Histological structure of EC after osmoprotection, dehydration, LN application and comparison with untreated**

In the present study, cells in the untreated coconut EC (Figure 5 a and b) showed similar characteristics to normal meristematic cells as per the description of Perera et al. (2007) for coconut and other species such as date palm (Bagniol et al., 1992) and yam (Barraco et al., 2014). Osmoprotection of cells by sucrose resulted in plasmolysis of certain cells indicating cell reaction during pre-culturing (Figure 5c). Intact plasma membranes of such cells were indicated by the lack of blue staining, corresponding to soluble proteins outside the cells. These results are similar with osmoprotected coconut plumules (N’Nan et al., 2008) and shoot tips of yam (*Dioscorea alata*) (Barraco et al., 2014). N’Nan et al. (2008) reported significant periplasmic space and a certain degree of damage (compact nucleus) in some plumule cells osmoprotected in 0.5 M sucrose, making further development irreversible.

The calli showed cellular heterogeneity upon dehydration. However, the intensity of cellular changes varied among samples. Cells were plasmolysed with deformed and retracted nuclei in majority of the samples and the nucleoli were no longer visible. Damaged plasma membrane and cell walls were indicated by the leakage of intracellular soluble proteins in certain cells (Figure 5e). Highly plasmolysed cells were characterised by condensed and poorly stained cytoplasm and slightly visible nuclei. Ration of the area of the nucleus and nucleocytoplasmic decreased significantly (Figure 5f). These results were compatible with the results observed in the research by Bagniol et al., (1992) and Barraco et al., (2014). In cryopreserved samples, the plasma membranes of numerous cells were broken and leakage of intracellular soluble proteins was observed. The nuclei of cells were pyknotic without visible nucleolus (Figure 5 f and g). Most of the treatments showed similar results as described previously, except cryopreserved EC which underwent osmoprotection for 3 days in 0.75 M sucrose concentration and dehydration for 20 h. These samples showed visible nuclei in numerous cells and slightly visible nucleoli in some cells even after cryopreservation (Figure 5h).

**Figure 5:** Histological observations. untreated EC (a, b); osmoprotected, non dehydrated EC (c); osmoprotected in 0.75 M sucrose (d, e), 0.5 M sucrose (f), dehydrated and non-cryopreserved EC; osmoprotected in 0.75 M sucrose for 2 days (g), 3 days (h), dehydrated and cryopreserved EC. Dehydration duration maintained was 20 h. [nucleus (N), nucleolus (n) cambium-like zone (CLZ)] starch (S) plasmolysed cells (PL) ruptured cell membranes (RC) pyknotic nuclei (PN) retracted cytoplasm (C) soluble proteins (SP)].
Non-cryopreserved EC after sucrose pretreatment and dehydration showed accumulation of starch (Figure 5d) as a result of the uptake of sucrose and its partial metabolism to form starch (glucose storage) (Bachiri et al., 2000) indicating the accumulation of sucrose in large quantities in cells subjected to encapsulation/dehydration. According to N’Nan et al. (2008) cryopreserved plumular cells after 8 h dehydration displayed a retracted cytoplasm, nucleus and the presence of starch grains. Furthermore, increased levels of starch have been recorded when the cells had undergone 16 h dehydration.

Barraco et al. (2014) explained osmoprotection using encapsulation-dehydration method and recorded dramatic changes in cellular structure, such as plasmolysis of cells in whole tissue. Shrinkage and intense staining of nuclei can cause pyknotic nuclei and disappearance of nucleoli. This happens due to chromatin contraction and increased protein concentration, respectively which is a reaction to tolerate stress conditions (Barraco et al., 2014). Nevertheless, this is advantageous due to growth arrest of tissues leading to redirecting resources to growth after cryopreservation. However, it may be related to the duration of recovery in standard medium (Barraco et al., 2014).

In contrast, dehydration also causes severe damage to cells especially in outer cell layers (Bagniol et al., 1992; Barraco et al., 2014) and dehydration reported to be the major cause of structural damage rather than freezing during cryopreservation. Considering the results of histological studies, massive structural damages were observed at the interior regions after encapsulation and dehydration of the coconut EC. This might be due to low penetration of the osmoticum. These results were compatible with the observations made on meristems of raspberry (Wang et al., 2005) and shoot tips of yam (Dioscorea alata; Barraco et al., 2014). Barraco et al. (2014) explained the recovery of yam cells into their original morphology in one week after cryopreservation. Therefore, it is necessary to make histological observations regularly after cryopreservation in order to get a better idea on the degree of recovery of cells. However, in this study, cells of EC showed severe cryo injuries during the procedure preventing any growth or callus formation in most of the samples even after six weeks.

**SEM observations of cryopreserved and non-cryopreserved EC**

SEM studies indicated the lack of dehydrating and freezing injuries at the exterior regions of the EC (Figure 6b and d). Cryopreserved and non-cryopreserved EC displayed apparent epidermal layers without any significant damages (Figure 6b) and these EC were analogous in morphology to the regular untreated EC (Figure 6a and c). However, there were shrunken cells after sucrose pretreatment (Figure 6e). Similar observations have been reported in scanning electron microscopy studies of the control and cryopreserved protocorm-like bodies of Dendrobium sonia-8, which is a hybrid orchid (Poobathy et al., 2013). The possible reason may be the thorough protection of outermost cell layers through osmoprotection compared to the inner cell layers.

**Assessment of genetic stability in regenerants from cryopreserved and non-cryopreserved EC**

Eleven SSR markers used for the evaluation of genetic stability of cryopreserved shoots and somatic embryos were all informative and generated amplicons. Each tested SSR primer pair produced clear reproducible bands ranging in size from 100 – 250 bp (Figure 7). All the samples scored identical alleles with their respective mother palm and the untreated controls at all SSR loci. The SSR loci CNZ10, CNZ29 and CAC65 scored heterozygous alleles while homozygous alleles were scored at all the remaining loci.

Cryopreservation imposes a series of stresses on plant material causing modifications or alterations in regenerated tissues resulting in possible genetic alterations and changes in allele frequencies (Harding, 2004). Majority of the studies showed genetic stability of cryopreserved material while some showed genetic variations. Studies carried out on Dendranthema grandiflora Tzvelev (Martin & Gonzalez-Benito, 2005) and Rabdosia rubescens (Ai et al., 2012) showed genetic variation after cryopreservation, necessitating the attention for testing the genetic integrity in cryopreserved material.

True-to-type conformity of tissue cultured coconut plants derived from unfertilised ovaries was tested in a previous study with respect to the mother palm and genetic stability at SSR marker loci was confirmed (Bandupriya et al., 2017). Same SSR marker loci were evaluated for the genetic uniformity of the cryopreserved and non-cryopreserved EC and compared with the untreated controls and the mother palm. SSR analysis revealed genetic stability of coconut EC and showed no polymorphisms between the cryopreserved and non-cryopreserved samples. The resulting bands were similar to with their untreated controls and respective mother palm. However, epigenetic changes due to genome methylation or transposable elements and mitotic
changes like aneuploidy could also occur during somatic embryogenesis and they cannot be detected using SSR markers.

Figure 6: Scanning electron microscopic observations of cryopreserved and non-cryopreserved EC: (a, c) surface of the untreated EC. Note the apparent epidermal layers. (a,c bar = 20 μm); (b) surface of the cryopreserved (+LN) and (d, f) non-cryopreserved (-LN) EC after sucrose pretreatment and 20 h dehydration. In b, d - note the presence of apparent epidermal layers with no significant damages (b. bar = 20 μm; d. bar = 30 μm); f - note the shrunken and injured on surface structure (bar = 20 μm); (e) EC pretreated with 0.75 M for 72 h. Note the shrunken epidermal layers due to osmotic effect (bar = 20 μm).

CONCLUSIONS

Commendable survival (45 %) and recovery (25 %) rates were obtained in the treatment combination of 0.75 M sucrose pre-treatment for 72 hours followed by 20 hours dehydration prior to LN treatment. Water content on fresh weight basis ranged between 28 – 30 % and attempts to reduce this further up to about 20 % may have a positive effect on recovery. Ultrastructural studies revealed massive structural damages at the interior regions after encapsulation dehydration of the coconut EC. Observations revealed that majority of the meristematic cells were injured either during the freezing/ thawing step or dehydration step. However, the treatment combination, 0.75 M sucrose pre-treatment for 72 hours followed by 20 hours dehydration and LN application showed better cell structure with less damage. According to the amplification patterns, SSR analysis did not reveal any polymorphisms between cryopreserved samples and untreated controls and with their respective mother palms, suggesting the genetic stability of coconut EC cryopreserved by encapsulation-dehydration method up to plant regeneration stage. It will generate more information when the same analysis is repeated in the field planting the plants.

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