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RESEARCH ARTICLE

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

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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 Y_3 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 Y_3 medium for 3 d to screen for microbial contamination. Encapsulation was carried out by suspending the EC parts in Eeuwens Y_3 medium (devoid of $CaCl_2$) 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 $CaCl_2.2H_2O$ solution prepared in Eeuwens Y_3 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 Y_3 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 Y_3 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 ($p \leq 0.05$).

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 dehydration, and osmoprotected with 0.75 M sucrose for 72 h without physical dehydration 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 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.

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 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 CCC 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	56
	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 AGT TCA AGT TCA AGA AGA CCT TTG -3'		
CNZ29	F	5'-TAA ATG GGT AAG TGT TTG TGC-3'	105–157	56
	R	5'-CTG TCC TAT TTC CCT TTC ATT-3'		
CNZ40	F	5'-CTT GAT TGC TAT CTC AAA TGG-3'	143–155	56
	R	5'-CTG AGA CCA AAT ACC ATG TGT-3'		
CNZ44	F	5'-CAT CAG TTC CAC TCT CAT TTC-3'	151–170	52
	R	5'-CAA CAA AAG ACA TAG GTG GTC-3'		
CNZ46	F	5'-TTG GTT AGT ATA GCC ATG CAT-3'	101–120	56
	R	5'-AAC CAT TTG TAG TAT ACC CCC-3'		

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.

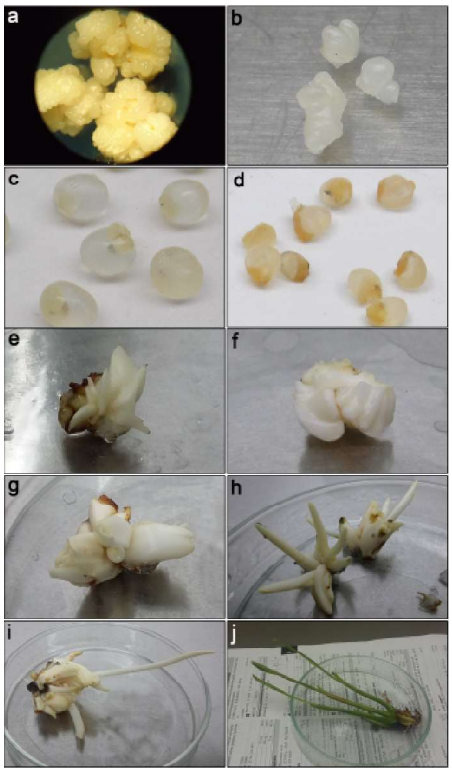

Figure 1: Cryopreservation of EC. (a) Ovary derived callus after third multiplication cycle (b) Calli parts dissected from ovary derived callus (c) encapsulated calli (d) dehydrated alginate beads (e) Somatic embryo development in cryopreserved EC. (f) Somatic embryo development from the control sample. (g) Somatic embryo germination in a non-cryopreserved sample. (h). Shoots from dehydrated, non-cryopreserved samples (e, f) Shoots from control samples.

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).

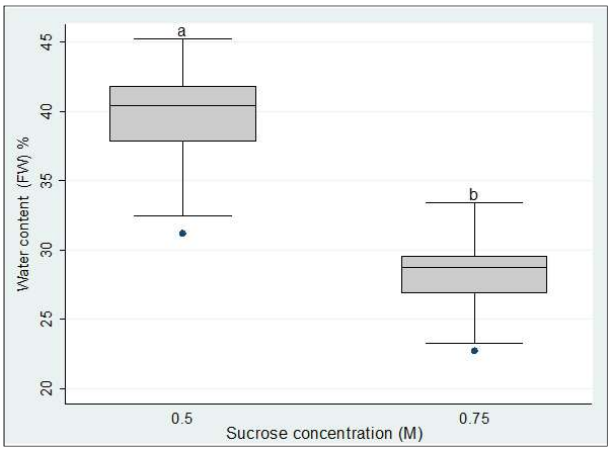

Figure 2: Effect of sucrose concentration on water content [fresh weight (FW)] of encapsulated beads. Median within boxplot having different letters is significantly different according to two-sample Wilcoxon rank-sum (Mann-Whitney) test at $p \leq 0.05$. Data represent the median of four replicates in two repeated experiments.

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).

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).

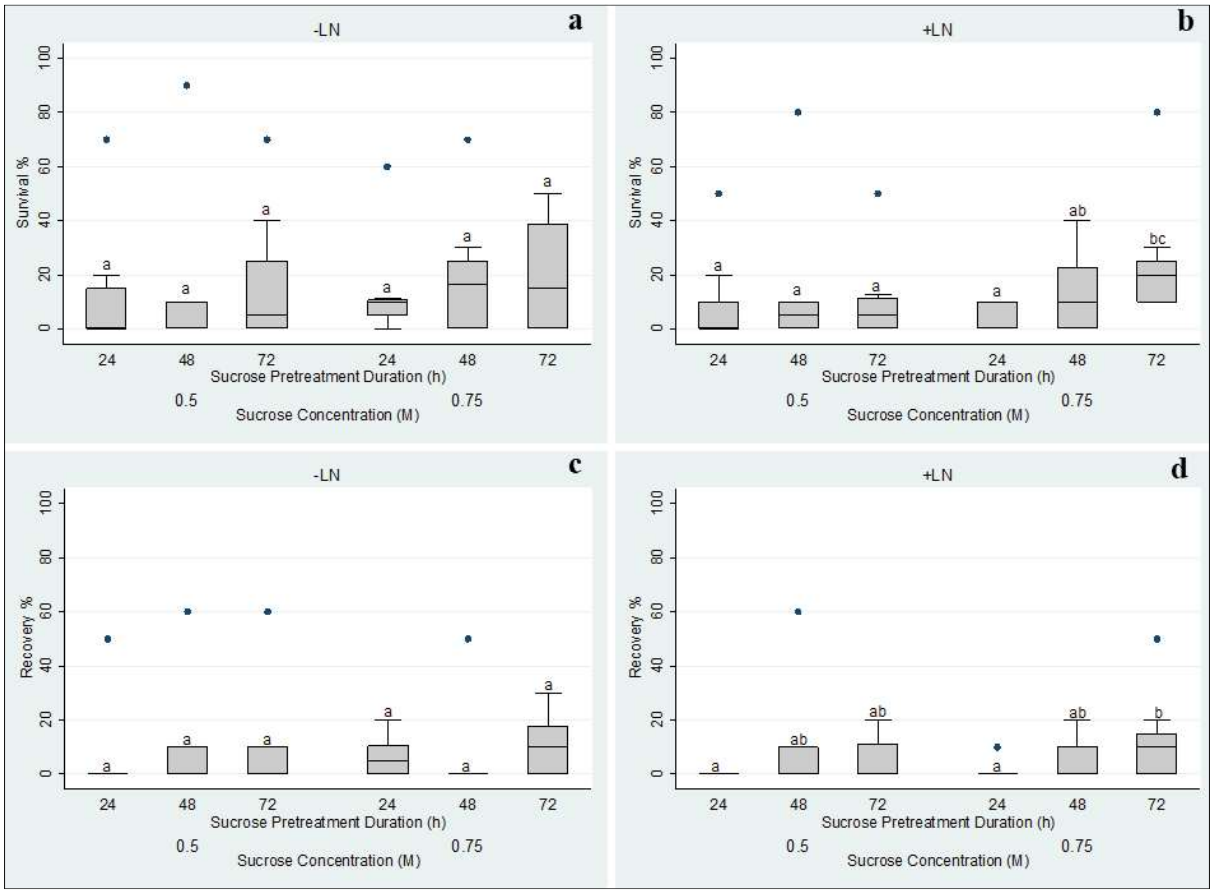

Figure 3: Effect of sucrose pretreatment duration on survival and recovery of embryogenic calli. Survival % of (a) non-cryopreserved samples (-LN); (b) cryopreserved samples (+LN). Recovery % of (c) non-cryopreserved samples (-LN); (d) cryopreserved samples (+LN). Data correspond to all the cryopreserved (+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 $p \leq 0.05$.

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

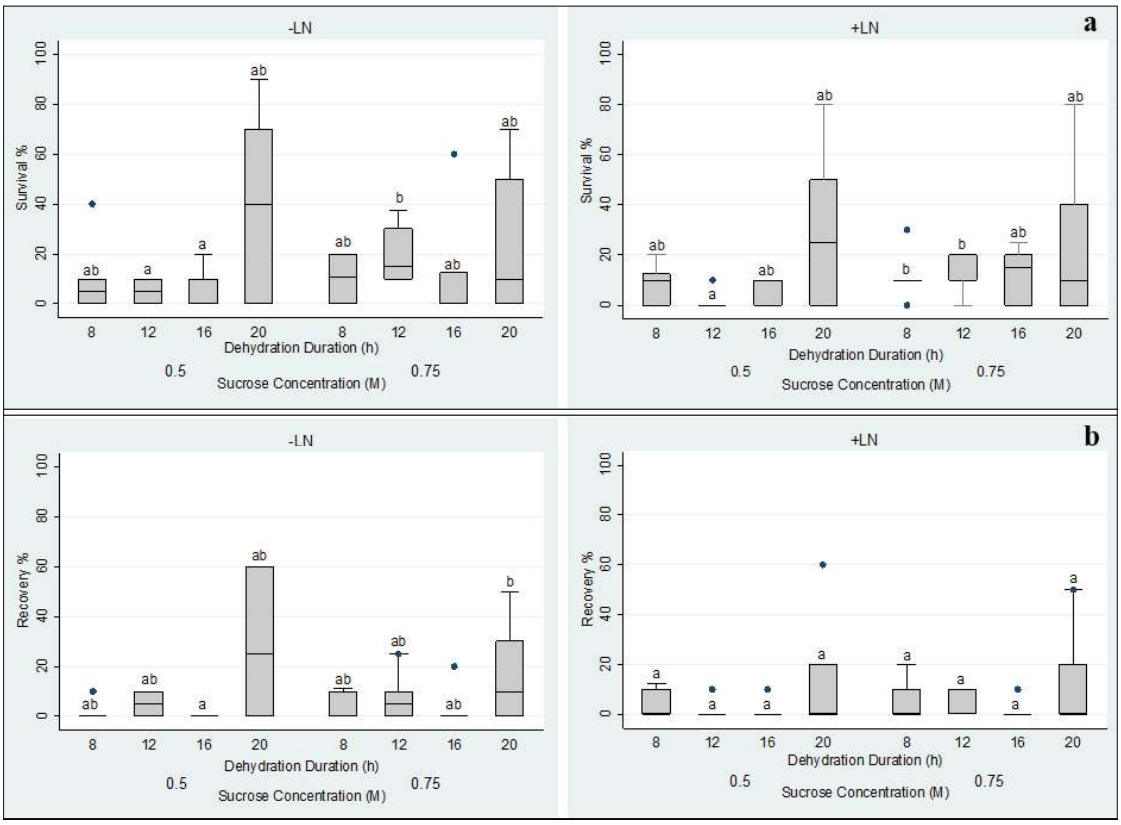

Figure 4: Effect of sucrose concentration and dehydration period on survival rates (a) and recovery rates (b) of encapsulated non-cryopreserved (+D/-LN) and cryopreserved (+D/-LN) EC. Encapsulated EC were osmoprotected with 0.5 M and 0.75 M sucrose, and dehydrated for 8, 12, 16 and 20 h durations followed 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 $p \leq 0.05$.

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).

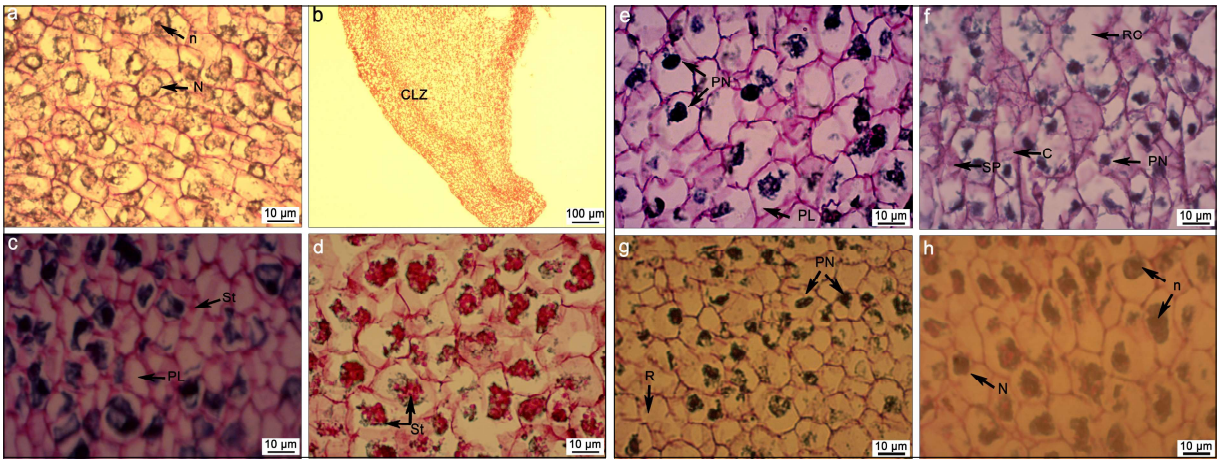

Figure 5: Histological observations. untreated EC (a, b); osmoprotected, non dehydrated EC (c); osmoprotected in 0.75 M sucrose (d, e), 0.5 M sucrose (f), dehydrated and non-cryopreserved EC; osmoprotected in 0.75 M sucrose for 2 days (g), 3 days (h), dehydrated and cryopreserved EC. Dehydration duration 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 pretreatement 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 6 b 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 6 a 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

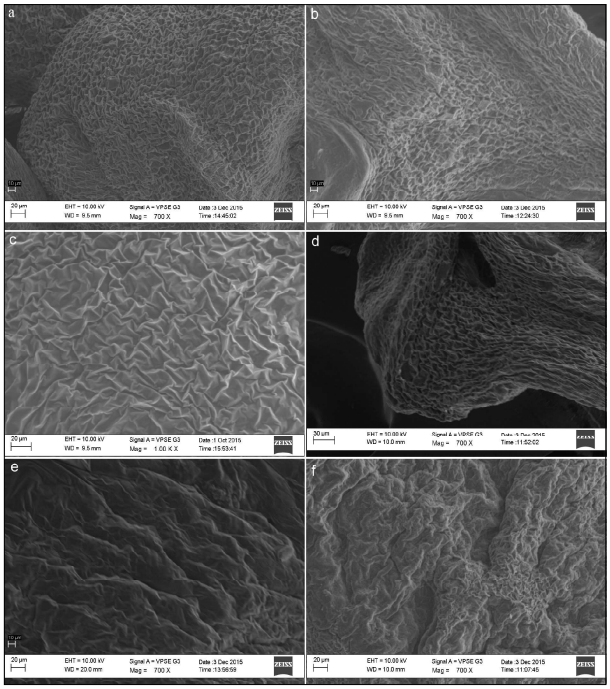

Figure 6: Scanning electron microscopic observations of cryopreserved and non-cryopreserved EC: (a, c) surface of the untreated EC. Note the apparent epidermal layers. (a,c bar = 20 µm); (b) surface of the cryopreserved (+LN) and (d, f) non-cryopreserved (-LN) EC after sucrose pretretment and 20 h dehydration. 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)

changes like aneuploidy could also occur during somatic embryogenesis and they cannot be detected using SSR markers.

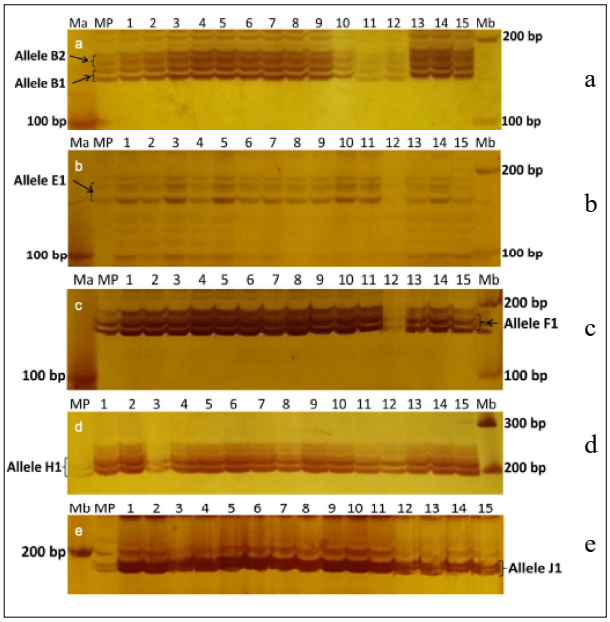

Figure 7: Amplification of DNA extracted from *in vitro* shoots and somatic embryos developed from non-treated EC as control, cryopreserved and non-cryopreserved EC with respective mother palm for eleven SSR primers. Lanes: Ma - 10 bp DNA ladder; Mb - 100 bp DNA ladder; MP - DT-1680 mother palm; 1–5 - non-treated; 6–10 - non-cryopreserved and 11–15 - cryopreserved. (a) CNZ 10; (b) CNZ 40; (c) CNZ 44; (d) CAC 08; (e) CAC 23

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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REFERENCES

Ai P.F., Lu L.P. & Song J.J. (2012). Cryopreservation of *in vitro*-grown shoot-tips of *Rabdosia rubescens* by encapsulation-dehydration and evaluation of their genetic stability. *Plant Cell Tissue and Organ Culture* **108**: 381–387. DOI: https://doi.org/10.1007/s11240-011-0049-x

Alla-N'Nan O., Tiécoura K., Bi S.G., Verdeil J-L. & Malaurie B. (2014). Ultrastructural changes during cryopreservation of plumules and embryos of coconut (*Cocos nucifera* L.). *International Journal of Agronomy and Agricultural Research* **5**(6): 103–115.

Assy-Bah B. & Engelmann F. (1992a). Cryopreservation of mature embryos of coconut (*Cocos nucifera* L.) and subsequent regeneration of plantlets. *CryoLetters* **13**: 117–126.

Assy-Bah B. & Engelmann F. (1992b). Cryopreservation of immature embryos of coconut (*Cocos nucifera* L.). *CryoLetters* **13**: 67–74.

Bachiri Y., Bajon C., Sauvanet A., Gazeau C. & Morisset C. (2000). Effect of osmotic stress on tolerance of air-drying and cryopreservation of *Arabidopsis thaliana* suspension cells. *Protoplasma* **214**: 227–243. DOI: https://doi.org/10.1007/BF01279067

Bagniol S. & Engelmann F. (1992). Histo-cytological study of apices from *in vitro* plantlets of date palm (*Phoenix dactylifera* L.) during a cryopreservation process. *CryoLetters* **13**: 405–412.

Bandupriya H.D.D., Fernando S.C., Verdeil J-L. & Malaurie B. (2007). Effect of abscisic acid on survival and recovery of cryopreserved plumule explants of coconut (*Cocos nucifera* L.). *Cocos* **18**: 45–51. DOI: http://doi.org/10.4038/cocos.v18i0.989

Bandupriya H.D.D., Iroshini W.W.M.A., Perera S.A.C.N., Vidhanaarachchi V.R.M., Fernando S.C., Santha E.S. & Gunathilake T.R. (2017). Genetic fidelity testing using SSR marker assay confirms trueness to type of micropropagated coconut (*Cocos nucifera* L.) plantlets derived from unfertilized ovaries. *The Open Plant Science Journal* **10**(1): 46–54. DOI: http://dx.doi.org/10.2174/1874294701710010046

Barraco G., Sylvestre I., Collin M., Escoute J., Lartaud M., Verdeil J.L. & Engelmann F. (2014). Histocytological analysis of yam (*Dioscorea alata*) shoot tips cryopreserved by encapsulation-dehydration. *Protoplasma* **251**(1): 177–189. DOI: https://doi.org/10.1007/s00709-013-0536-5

Bian H.W., Wang J.H., Lin W.Q., Han N. & Zhu M.Y. (2002). Accumulation of soluble sugars, heat-stable proteins & dehydrins in cryopreservation of protocorm-like bodies of *Dendrobium candidum* by the air-drying method. *Journal of Plant Physiology* **159**: 1139–1145. DOI: https://doi.org/10.1078/0176-1617-00824

Crowe J.H., Crowe L.M., Carpenter J.F., Rudolph A.S., Wistrom C.A., Spargo B.J. & Anchordoguy T.J. (1988). Interactions of sugars with membranes. *Biochimicaet Biophysica Acta* **947**: 367–84. DOI: https://doi.org/10.1016/0304-4157(88)90015-9

Cueto C., Rivera R.L., Kim H.H., Kong H.J., Baek H.J., Sebastian L. & Park H.J. (2013). Development of cryopreservation protocols for cryobanks of coconut zygotic embryos. In: II International Symposium on Plant Cryopreservation **1039**: 297–302. DOI: https://doi.org/10.17660/ActaHortic.2014.1039.37

Engelmann F. (1997). *In vitro* conservation methods. In: *Biotechnology & plant genetic resources: conservation & use* (eds. B.V. Ford-Lloyd, J.H. Newbury & Callow J.A.), pp 119–162. CABI, Wallingford.

Eeuwens C.J. (1976). Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palm (*Cocos nucifera* L.) cultured *in vitro*. *Physiologia Plantarum* **36**: 23–28.

Fang J.Y., Wetten A. & Hadley P. (2004). Cryopreservation of cocoa (*Theobroma cacao* L.) somatic embryos for long-term germplasm storage. *Plant Science* **166**(3): 669–675. DOI: https://doi.org/10.1016/j.plantsci.2003.11.002

Feng C.H., Cui Z.H., Li B.Q., Chen L., Ma Y.L., Zhao Y.H. & Wang Q.H. (2013). Duration of sucrose preculture is critical for shoot regrowth of *in vitro*-grown apple shoot-tips cryopreserved by encapsulation-dehydration. *In vitro Cellular and Developmental Biology* **112**: 369–378. DOI: https://doi.org/10.1007/s11240-012-0245-3

Fisher D.B. (1968). Protein staining of ribbonedepon sections for light microscopy. *Histochemie* **19**: 92–96.

Frison E.A., Putter C.A.J. & Diekmann M. (1993). *FAO/IBPGR Technical Guidelines for the Safe Movement of Coconut Germplasm*. FAO/IBPGR, Rome, Italy.

Harding K. (2004). Genetic integrity of cryopreserved plant cells: a review. C*ryoLetters* **25**: 3–22.

Malaurie B., Bandupriya H.D.D., Fernando S.C. & Verdeil J-L., (2006). Optimisation du procédé de cryoconservation de la plumule de cocotier. Les Actes du BRG **6**: 449–468.

Martín C. & González-Benito M.E. (2005). Survival and genetic stability of *Dendranthema grandiflora* Tzvelev shoot apices after cryopreservation by vitrification and encapsulation-dehydration. *Cryobiology* **51**: 281–289. DOI: https://doi.org/10.1016/j.cryobiol.2005.08.001

Nguyen Q.T., Bandupriya H.D.D., Lo´pez-Villalobos A., Sisunandar S., Foale M. & Adkins S.W. (2015). Tissue culture and associated biotechnological interventions for the improvement of coconut (*Cocos nucifera* L.): a review. *Planta* **242**(5): 1059–1076. DOI: https://doi.org/10.1007/s00425-015-2362-9

N'Nan O., Hocher V., Verdeil J-L., Konan J-L.K., Ballo K., Mondeil F. & Malaurie B. (2008). Cryopreservation by encapsulation-dehydration of plumules of coconut (*Cocos nucifera* L.). *CryoLetters* **29**(4): 339–350.

N'Nan O., Borges M., Konan J-L.K., Hocher V., Verdeil J-L.,

Tregear J., N'guetta A.S.P., Engelmann F. & Malaurie B. (2012). A simple protocol for cryopreservation of zygotic embryos of ten accessions of coconut (*Cocos nucifera* L.). *In vitro Cellular and Developmental Biology-Plant* **48**: 160–166.
DOI: https://doi.org/10.1007/s11627-012-9425-4

Perera P.I.P., Hocher V., Verdeil J., Doulbeau S., Yakandawala D.M.D. & Weerakoon L.K. (2007). Unfertilized ovary: a novel explant for coconut (*Cocos nucifera* L.) somatic embryogenesis. *Plant Cell Reports* **26**: 1–8.
DOI: https://doi.org/10.1007/s00299-006-0216-4

Poobathy R., Sinniah U.R., Rathinam X. & Subramaniam S. (2013). Histology and scanning electron microscopy observations of cryopreserved protocorm-like bodies of *Dendrobium sonia*-28. *Turkish Journal of Biology* **37**(2): 191–198.

Sajini K.K., Karun A., Amarnath C.H. & Engelmann F. (2011). Cryopreservation of coconut (*Cocos nucifera* L.) zygotic embryos by vitrification. *CryoLetters* **32**(4): 317–328.

Sisunandar, Peter A., Sopade P.A., Samosir Y.M.S., Rival A. & Adkins S.W. (2010). Dehydration improves cryopreservation of coconut (*Cocos nucifera* L.). *Cryobiology* **61**: 289–296.
DOI: https://doi.org/10.1016/j.cryobiol.2010.09.007

Subaih W.S., Shatnawi M.A. & Shibli R.A. (2007). Cryopreservation of date palm (*Phoenix dactylifera*) embryogenic callus by encapsulation-dehydration, vitification and encapsulation-vitrification. *Jordan Journal of Agricultural Sciences* **3**(2): 156–171.

Suranthran P., Gantait S., Sinniah U.R., Subramaniam S., Alwee S.S.R.S. & Roowi S.H. (2012). Effect of loading and vitrification solutions on survival of cryopreserved oil palm polyembryoids. *Plant Growth Regulation* **66**: 101–109.
DOI: https://doi.org/10.1007/s10725-011-9633-7

Suzuki M., Ishikawa M., Okuda H., Noda K., Kishimoto T., Nakamura T., Ogiwara I., Shimura I. & Akihama T. (2006). Physiological changes in gentian axillary buds during two-step preculturing with sucrose that conferred high levels of tolerance to desiccation and cryopreservation. *Annals of Botany* **97**(6): 1073–1081.
DOI: https://doi.org/10.1093/aob/mcl054

Rivera R., Edwards K.J., Barker J.H., Arnold G.M., Ayad G., Hodgkin T. & Karp A. (1999). Isolation and characterization of polymorphic microsatellites in *Cocos nucifera* L. *Genome* **42**(4): 668–675.
DOI: https://doi.org/10.1139/g98-170

Vickers A.J. (2005). Parametric *versus* non-parametric statistics in the analysis of randomized trials with non-normally distributed data. *BMC Medical Research Methodology* **5**(1): 35.

Wang Q., Laamanen J., Uosukainen M. & Valkonen J.P. (2005). Cryopreservation of *in vitro*-grown shoot tips of raspberry (*Rubusidaeus* L.) by encapsulation-vitrification and encapsulation–dehydration. *Plant Cell Reports* **24**(5): 280–288.
DOI: https://doi.org/10.1007/s00299-005-0936-x

Welewanni I., Jayasekera A. & Bandupriya D. (2017). Coconut cryopreservation: present status and future prospects. CORD **33**(1): 41–61.

Zhou Q.N., Sun A.H., Li Z., Hua Y.W., Jiang Z.H., Huang T.D., Dai X.M. & Huang H.S. (2012). Cryopreservation and plant regeneration of anther callus in *Hevea* by vitrification. *African Journal of Biotechnology* **11**(28): 7212–7217.
DOI: https://doi.org/10.5897/AJB12.258