CryoLetters 32 (4), 317-328 (2011)

CRYOPRESERVATION OF COCONUT (*Cocos nucifera* L.) ZYGOTIC EMBRYOS BY VITRIFICATION

K. K. Sajini[1], Anitha Karun[1*], C. H. Amarnath[2] and Florent Engelmann[3,4]

[1]Biotechnology Section, Central Plantation Crops Research Institute, Kasaragod-671124, Kerala, India.
[2]Statistics Section, Central Plantation Crops Research Institute, Kasaragod-671124, Kerala India.
[3]Institut de Recherche pour le Développement (IRD), UMR DIAPC, 911 avenue Agropolis, BP 64501, 34394 Montpellier cedex 5, France.
[4]Bioversity International, Via dei Tre Denari 472/a, 00057 Maccarese (Fiumicino), Rome, Italy.
*Corresponding author email: anithakarun2008@gmail.com

Abstract

The present study investigates the effect of preculture conditions, vitrification and unloading solutions on survival and regeneration of coconut zygotic embryos after cryopreservation. Among the seven plant vitrification solutions tested, PVS3 was found to be the most effective for regeneration of cryopreserved embryos. The optimal protocol involved preculture of embryos for 3 days on medium with 0.6 M sucrose, PVS3 treatment for 16 h, rapid cooling and rewarming and unloading in 1.2 M sucrose liquid medium for 1.5 h. Under these conditions, 70-80% survival (corresponding to size enlargement and weight gain) was observed with cryopreserved embryos and 20-25% of the plants regenerated (showing normal shoot and root growth) from cryopreserved embryos were established in pots.

Keywords: *Cocos nucifera* L., coconut, zygotic embryo, preculture, vitrification, PVS3, unloading.

INTRODUCTION

Genetic diversity, which provides the target genes available for breeding programmes, needs to be safely conserved to meet the challenges faced by changes in agricultural technology, global warming, attacks by new pests and diseases and market requirements. Coconut being a monotypic genus, the genetic diversity is mainly found in different ecotypes/landraces. The present mode of conservation of genetic diversity for coconut is through field genebanks that require large areas, high maintenance costs and are subjected to many risks including diseases, pests and natural calamities. Owing to the large size and recalcitrant behavior of the nut, long-term conservation of coconut germplasm through conventional seed storage is not feasible (3). Cryopreservation of embryos can be employed as an alternative method for long-term conservation of coconut germplasm, thereby sheltering genetic resources from biotic and abiotic threats (1). Cryopreservation is also a cost-effective method, which requires limited space and maintenance for long-term storage of frozen samples (4).

The development of an efficient cryopreservation protocol depends on a series of parameters including the type, size, water content, desiccation and freezing tolerance of explants (23). The control of explant water content is of paramount importance to achieve desiccation and freezing tolerance. Among the various cryopreservation methods available, vitrification is a very simple and effective technique for complex tissues like somatic embryos and shoots tips (28). The most commonly used vitrification solutions (VSs) are Plant Vitrification Solution 2 [PVS2 (29)] and Plant Vitrification Solution 3 [PVS3 (16, 25)]. In the case of coconut, desiccation (13) and pregrowth-dehydration (1, 27) have been employed for cryopreserving zygotic embryos and encapsulation-dehydration for cryopreserving plumules (24).

In view of the operational simplicity of the vitrification technique and of the high level of plantlet recovery obtained when it was applied to numerous species (28), it was used for cryopreserving coconut embryos in our experiments. The effect of different PVSs and of different sucrose concentrations in the preculture and unloading media on regrowth of coconut zygotic embryos after cryopreservation was compared.

MATERIALS AND METHODS

Plant material

The material used for the present study consisted of embryos (11-12 month old) collected from mature nuts of cultivar WCT (West Coast Tall) freshly harvested from the palms. The endosperm plugs containing the embryos were scooped out from the soft eye of the nuts with a corkborer and sterilized with sodium hypochlorite solution (6% active chlorine) for 20 min. Thereafter, the embryos were thoroughly rinsed four or five times in sterile water and employed in the experiments.

Cryopreservation

The cryopreservation protocol comprised a preculture step on medium with high sucrose concentration, treatment with PVS, rapid cooling and rewarming, unloading and regrowth of embryos. For initial screening of the vitrification solution, 25 embryos were used per experimental condition and experiments were repeated three times. Out of these 25 embryos, five were utilized for viability testing, 10 as PVS treatment controls and 10 were cryopreserved. To study the effect of sucrose preculture and of unloading solution, 20 embryos were utilized for each treatment combination including control and the experiments were repeated three times.

Preculture: For preculture, embryos were inoculated for 3 days on semi-solid Y3 medium containing 0.3 M sucrose for initial screening of the PVS and for 3 days on medium containing 0.5 M or 0.6 M sucrose in further experiments using PVS3.

PVS treatment: Seven PVSs of different compositions were tested (Table 1). Embryos were precultured on Y3 medium (6) with 0.3 M sucrose for 3 days, and then incubated (25 embryos in a 100 ml conical flask) in 25 ml PVS, under constant shaking (90 rpm) for 2 to 36 h. All PVSs were filter-sterilized except PVS3, which was autoclaved. In further experiments, embryos were treated with PVS3 for 16 h in view of the best results obtained with it.

Cooling: A few minutes before the end of the PVS treatment, embryos were transferred to 4.5 ml cryovials (20 embryos per cryovial), which were filled with 4.5 ml fresh PVS and plunged directly in liquid nitrogen (LN). Samples were stored in LN for at least 24 h.

Rewarming: Rapid rewarming of cryopreserved samples was achieved by plunging the cryovials in a 40°C water bath for approximately 2 min, until the PVS became liquid.

Unloading: The PVS was removed and replaced with unloading solution, which consisted of liquid Y3 medium containing 0.6, 0.8 or 1.2 M sucrose. Embryos were kept in the unloading solution for 90 min. The unloading solution was replaced three times with fresh solution after 10, 30 and 50 min.

Regrowth: Embryos were transferred on semi-solid Y3 medium containing 60 g/l sucrose and incubated in the dark at 27±1°C for germination. After 2 weeks, the embryos were subcultured on semi-solid Y3 medium containing 40 g/l sucrose and cultured according to the protocol developed by Karun *et al*. (12) until transfer of whole plants in pots.

Moisture content determination

The moisture content (% MC, fresh weight basis) of embryos after different durations of PVS3 exposure was measured after oven drying at 103°C for 24 h. Three samples of five embryos per treatment were utilized for moisture content determination.

Viability testing using TTC

Viability of embryos was estimated after incubation in a 0.1% aqueous solution of 2, 3, 5-triphenyltetrazolium chloride [TTC (10)] for 24 h at 25ºC in the dark. Embryos showing red staining as a result of respiratory activity in living cells after various steps of the vitrification protocol were considered viable.

Survival and recovery assessment

Cryopreserved and non-cryopreserved embryos showing any sign of regrowth 25 days after inoculation on Y3 medium were considered to have survived. Recovery was measured as the percentage of embryos showing emergence of the plumule and of the first leaf. Thereafter, growth assessments were made with respect to embryo weight gain and shoot length after a period of 30, 90, 180, 240 and 330 days.

Sample processing for light microscopy

Embryos at different stages of the cryopreservation protocol were fixed in Carnoy's fluid (2) for 24 h. Samples were then dehydrated in alcohol-butanol series for a minimum period of 24 h in each solution. Tissues were infiltrated with paraffin wax at 60°C for 2-3 days, then embedded in paraffin wax to make sections. Sections of 10 μm thickness were cut using a microtome and stained with Periodic Acid Schiff (PAS) reagent or Toluidine blue. PAS stains starch reserves and cell walls pink and Toluidine blue stains zones containing RNA light purple and DNA bluish green.

Statistical analysis

The results were subjected to analysis of variance (ANOVA) following arcsin transformation and the means were compared using the Duncan's Multiple Range Test (DMRT) using SPSS 15.0.

RESULTS

Selection of PVS

Treatment with PVS1, PVS2, PVS4, and the Steponkus, Towill and Fahy PVSs were lethal to embryos whatever the treatment duration (2 to 36 h) (Table 1), even though the TTC test showed pink staining in all cases. Survival of cryopreserved embryos was achieved only after treatment with PVS3, reaching a maximum of 40% after 16 h treatment. Sixty percent of the embryos that withstood PVS3 treatment showed abnormal growth, such as callus formation (Fig. 1a), thus impairing shoot and root development.

Table 1. Optimal survival (%) of cryopreserved coconut embryos following treatment with the seven VSs tested. EG: ethylene glycol; DMSO: dimlethylsulfoxide; BSA: bovine serum albumin; PEG: polyethylene glycol. Preculture duration was 16 h.

Vitrification solution	Composition (%, w/v)	Reference	Survival (%)
PVS1	22.0 glycerol + 13.0 propylene glycol + 13.0 EG + 6.0 DMSO + 13.7 sucrose	(36)	0
PVS2	30.0 glycerol + 15.0 DMSO + 15.0 EG + 13.7 sucrose	(29)	0
PVS3	50.0 glycerol + 50.0 sucrose	(25)	40
PVS4	35.0 glycerol + 20.0 ethylene glycol + 20.5 sucrose	(22)	0
Steponkus	50.0 EG + 15.0 sorbitol + 6.0 BSA + 13.7 sucrose	(19)	0
Towill	35.0 EG + 6.8 DMSO + 10.0 PEG 8000 + 13.7 sucrose	(33)	0
Fahy	20.0 DMSO + 20.0 formamide + 15.0 propylene glycol	(7)	0

Optimization of PVS3 treatment

Following preculture for 3 days on medium with 0.6 M sucrose, embryos were treated with PVS3 for various durations before cryopreservation. Significant differences ($p<0.05$) were observed for survival with respect to moisture content at various treatment durations with PVS3. Embryos dehydrated very rapidly for the first hour, from an initial 78% MC to 30% MC, then more slowly, reaching 12% MC after 20 h (Fig. 2). Survival of non-cryopreserved controls remained very high, from 100% without PVS3 treatment to 75% after 20 h. After cryopreservation, no survival was observed for PVS3 exposure durations between 0 – 4 h (Fig.1b). Survival progressively increased to reach the optimum of 75% after 16 h (at 16.8% MC), then decreased, reaching 30% after 20 h (Fig. 2). More than half of the embryos surviving after 20 h PVS3 treatment displayed growth abnormalities like callus type growth, development of crevices on the embryonic axis and flaccidity.

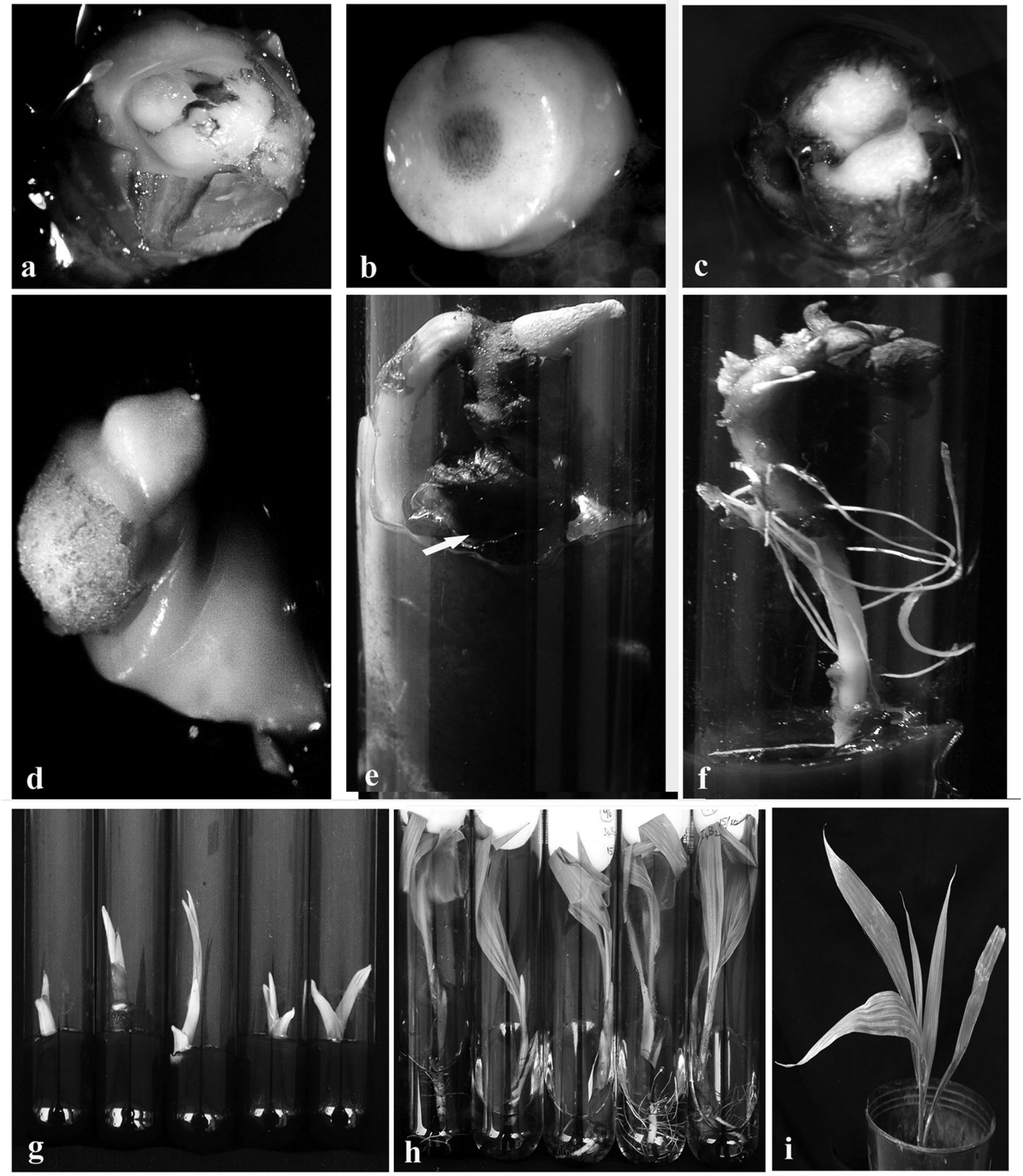

Figure 1. *In vitro* cultures of coconut embryos after various treatments. **a:** callus development on a cryopreserved embryo precultured in 0.3 M sucrose followed by PVS3 treatment for 16 h; **b:** dead embryos after preculture in 0.6 M sucrose for 3 days and PVS3 treatment for 4 h; **c:** fissure developed on the embryonic axis of a cryopreserved embryo precultured in 0.5 M sucrose and unloaded with 1.2 M sucrose; **d:** callus production on the shoot and root poles of a cryopreserved embryo after 0.5 M sucrose preculture for 16 h, PVS3 treatment and unloading in 1.2, 0.8 and 0.6 M sucrose medium; **e:** non-cryopreserved embryo showing enlarged haustorium (arrow) and rapidly growing root after preculture with 0.6 M sucrose, PVS3 treatment and unloading in 1.2 M sucrose; **f:** cryopreserved embryo showing complete deformation of the shoot and normal, rapidly growing root after the same preculture and unloading treatment as **e**; **g**: and **h**: differential growth among cryopreserved embryos cultured for same period after preculture and unloading treatments as in **e**; **i**: potted plant produced from a cryopreserved embryo following the same preculture and unloading treatments as in **e**.

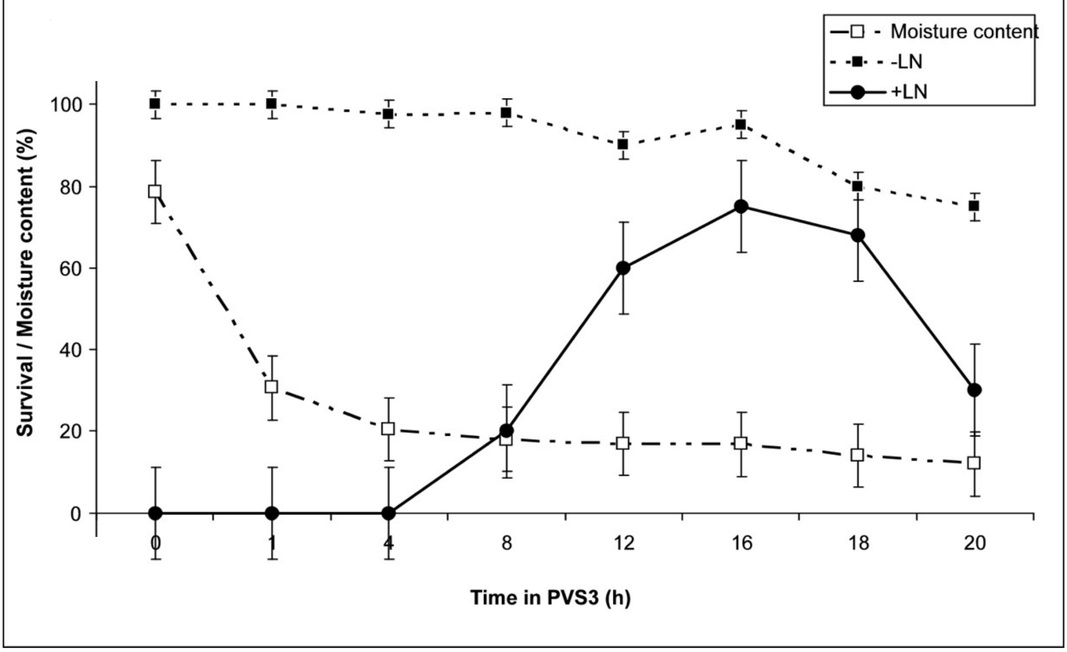

Figure 2. Effect of time exposed to PVS3 on moisture content (MC %, FW) and survival (%) of non-cryopreserved (-LN) and cryopreserved (+LN) coconut zygotic embryos. The embryos were precultured in 0.6 M sucrose medium for 3 days and unloaded in 1.2 M sucrose medium with (+LN) or without (-LN) subsequent immersion in liquid nitrogen. For moisture content, each value represents the mean of three 5-embryo replicates and for survival the mean of three 10-embryo replicates. Bars represent standard error.

Effect of sucrose concentration in preculture and unloading medium

Embryo survival and plantlet production varied depending on the sucrose concentration present in the preculture and unloading media (Table 2). Pregrowth with 0.6 M sucrose induced significantly higher survival from the control and cryopreserved embryos (cd = 7.39). Subsequent unloading in medium with 1.2 M sucrose induced significantly higher plantlet production (cd = 7.99) from control and cryopreserved embryos compared with all other treatments. Very low survival was observed after 0.5 M sucrose preculture treatment for the embryo. Among the surviving embryos callus development was observed in control embryos on the shoot and root pole regions under all experimental conditions (Fig. 1d). In cryopreserved embryos disruption of the meristematic cells on the embryonic axis resulted in the occurrence of fissures (Fig. 1c). Shoot growth of the germinating control embryos from 0.5 M sucrose pregrowth treatment was very slow, irrespective of the unloading solution employed, reaching a length of only 0.5 – 1.0 cm after 6 months in culture. But none of the embryos showed normal germination after cryopreservation.

Significant differences were observed for the three parameters studied (plantlet weight, shoot length and plantlet recovery) between the experimental conditions employed (Table 3). As regards plantlet weight, no differences were noted amongst the control explants; however, plantlets originating from cryopreserved embryos treated with unloading solution containing 1.2 M sucrose had a significantly higher weight, compared with unloading solutions containing 0.6 and 0.8 M sucrose. Shoots produced from control and cryopreserved embryos treated with 1.2 M sucrose unloading solution had a comparable length and were significantly higher than those produced from embryos treated with unloading solutions containing 0.6 and 0.8 M sucrose. There were significant differences between unloading media for plant recovery in pots. Unloading embryos in medium containing 1.2 M sucrose led to significantly higher plantlet recovery in pots, for both control and cryopreserved embryos, compared with unloading in the other two sucrose unloading solutions (0.8 and 0.6 M) (cd = 7.07).

Table 2. Effect of sucrose concentration in the preculture medium and unloading solution on survival **A** (%) and plantlet production **B** (%) of coconut embryos before (-LN) and after (+LN) cryopreservation. For Table A & B means in a row followed by different small letters imply significant differences according to Univariate ANOVA [n=20, replication 3, p<0.05)].

A

Unloading medium (M)	Preculture medium -LN			Preculture medium +LN		
	0.5 M	0.6M	Mean	0.5M	0.6M	Mean
0.6	15	65	40	0	50	25
0.8	35	77	56	5	70	37.5
1.2	35	**95**	65	5	**75**	40
Mean	28.33	79	53.66[a]	3.33	65	34.16[b]

SD- Pregrowth medium & (-LN&+LN) = 2.2, Unloading medium =2.70

B

Unloading medium (M)	Preculture medium -LN			Preculture medium +LN		
	0.5M	0.6M	Mean	0.5M	0.6M	Mean
0.6	4	13	8.5	0	10	5
0.8	5	30	17.5	0	11.6	5.8
1.2	10	**56**	33.3	0	**55**	27.5
Mean	6.33	33.2	19.76[d]	0	25.53	12.76[e]

SD- Pregrowth medium & (-LN&+LN) = 1.5, Unloading medium =1.83

Table 3. Effect of sucrose concentration in the unloading medium on weight (g), shoot length (cm), and pot survival (%) of plantlets originating from control (–LN) and cryopreserved (+LN) embryos after preculture in 0.6 M sucrose for 3 days. Means in a row followed by different letters imply significant differences according to DMRT (*p<0.05*), with n=20 and three replicates. For embryo weight and shoot length data were recorded after 240 days of culture initiation and after 330 days for plantlet recovery in pot.

Character	Unloading solution					
	0.6 M		0.8 M		1.2 M	
	-LN	+LN	-LN	+LN	-LN	+LN
Plantlet weight (g)	1.178 a	0.513 b	1.471 a	0.578 b	1.589 a	1.357 a
Shoot length (cm)	1.305 b	0.448 b	3.073 a	0.805 b	3.533 a	3.15 a
Plantlet recovery in pot (%)	10	0	20	5	30	22.5

(SE. for plantlet weight 0.20, shoot length 0.61 and plantlet recovery in pot 4.08)

At the time of potting, the height of plantlets originating from control and cryopreserved embryos treated with 1.2 M sucrose unloading solution varied between 8.5 cm and 19.0 cm; they had two to four leaves and two to three actively growing roots. In both control and cryopreserved embryos, some plantlets showed a normal growth rate whereas others grew slowly. Due to the high variability in their growth rate (Fig. 1 g, h, i), all plantlets produced from cryopreserved embryos (precultured in 0.6 M sucrose for 3days & unloading in 1.2 M sucrose) could not be transferred to pots simultaneously. When compared to plants produced from control embryos, the rate of shoot growth was slower in plantlets coming from cryopreserved embryos, even though all plantlets were morphologically similar. In non-cryopreserved samples, the development of an enlarged haustorium and of a rapidly growing root, which impeded normal shoot development (Fig. 1e), was the abnormality observed in approximately 5% of the embryos. In cryopreserved embryos, complete shoot deformation along with a normal rapidly growing root (Fig. 1f) was the abnormality observed for about 5% of the explants.

The effect of preculture and unloading medium on cell structure of coconut embryo

The cellular structure of cryopreserved and non-cryopreserved embryos was observed to understand the effect of sucrose (0.6M) preculture and unloading medium (1.2 M, 0.8 M and 0.6 M) on survival and germination of the embryo. In untreated embryos, cells of the shoot meristematic dome and surrounding primordial leaf sheaths showed the presence of a round nucleus, a dense cytoplasm, high nucleocytoplasmic ratio and intact cell walls (Fig. 3A, B) Osmotic dehydration after preculture of embryos in 0.6 M sucrose medium for 3 days resulted in the retraction of the cytoplasm in meristematic cells (Fig. 3 C). Cellular integrity of control and cryopreserved embryos was retained after unloading treatment with 1.2 M sucrose (Fig. 3 D, E, F, G). When embryos were transferred to unloading medium with a lower sucrose concentration, the cell wall structure of cryopreserved explants was disturbed. Undulating cell walls as a result of cell lysis were observed in the outer cell layers of the embryo treated with 0.8 M sucrose unloading medium (Fig. 3 I), whereas such deformations were not observed in non-cryopreserved embryos (Fig. 3H). After treatment with 0.6 M sucrose unloading medium, complete cell wall destruction was observed after cryopreservation. Damage was severe in primordial leaf sheaths surrounding the meristematic dome (Fig. 3K, L). Damage was not much pronounced in non-cryopreserved samples (Fig. 3J).

DISCUSSION

The present study clearly demonstrated the effectiveness of PVS3 solution, which contains glycerol and sucrose, for cryopreservation of coconut embryos. The other vitrification solutions tested, which include other chemicals like DMSO, ethylene glycol and propylene glycol became cytotoxic even after short treatment durations, resulting in the absence of embryo recovery. Glycerol has been considered a very efficient cryoprotectant due to its small size and stereochemically oriented OH groups along one side of the molecule that result in better hydrogen bonding qualities with membrane phospholipids, thereby imparting better membrane stabilization (34). In accordance with the results of Kim *et al.* (18) with garlic shoot tips, PVS3 was the most adapted vitrification solution for coconut embryos, which have a heterogenous cell structure, large size, are sensitive to biochemical toxicity and relatively tolerant to osmotic stress.

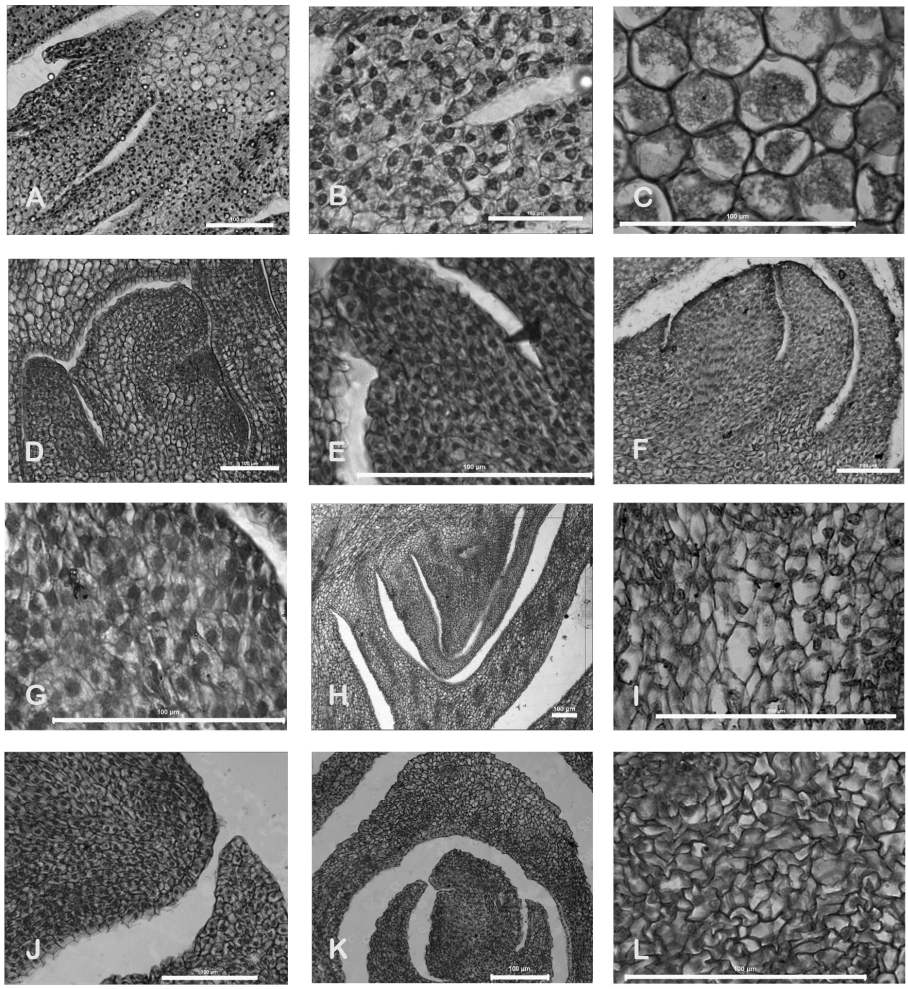

Figure 3. Histological analysis of coconut zygotic embryos at various stages of the experiment. Su (Sucrose), Un (Unloading).

A, B : Untreated control embryos with intact cell wall and nucleus
C : Retracted cytoplasm after 0.6M Su preculture for 3days
D, E : After1.2M Su Un Note the retention of cellular integrity –LN
F, G : +LN, H: 0.8M Su Un Partial cell wall damage –LN
I : Undulating cell wall after cell lysis +LN ;
J : 0.6 M Su Un Cell wall damage –LN
K : Cell wall breakage in the meristematic dome +LN
L : Complete cell wall collapse in the cells of primordial leaf +LN.
(Scale bar = 100µm)

Even though the TTC test has been used for immediate assessment of viability after cryopreservation in some plants (38), our results showed that this was not feasible for coconut. This is in agreement with the fact that the presence of living cells in the specimens is not predictive of the ability of explants to undergo further development (26). In studies on cryopreservation of yam shoot tips, Quain *et al*. (26) cautioned the interpretation of TTC results as they showed higher success than the actual survival observed in tissues after cryopreservation. It was also observed in azalea *in vitro* cultures that the brown colour of explants caused by oxidation as a result of freezing damage sometimes looked similar to red coloured reduced TTC (38). Hence, the evaluation of viability using TTC staining is subjective and may lead to an overestimation of viability. It should thus be used only as a rough indication of success/failure of experiments during screening for new cryopreservation protocols.

The duration of PVS3 treatment is critical for regrowth of embryos after cryopreservation. The fast initial influx of sugar and glycerol into the embryos resulted in the sharp decline in MC from an initial 78.9% to 30.6% after 1 h dehydration with PVS3 solution. Thereafter, the lower penetration rate resulted in the slower embryo MC decrease. A similar pattern was observed by Kim *et al.* (17) with garlic shoot tips. As observed with other materials (14, 20), the percentage of plantlet recovery was affected by PVS3 treatment duration. Optimal survival (75%) was achieved after 16 h treatment (embryo MC of 16.8%) but longer treatment durations were lethal for coconut embryos, due to the cytotoxicity of cryoprotectants and/or osmotic stress induced by extended PVS3 exposure. This effect was manifested as damage of meristematic tissues of the embryo.

Preculture of plant material in sucrose enriched medium is essential for the mitigation of the subsequent injurious effect of vitrification solution and osmotic adjustments (32). The concentration of sucrose in the preculture medium had a significant influence on survival of coconut embryos after cryopreservation. In the present study, preculture with 0.6 M sucrose was found to be optimal for survival of cryopreserved embryos. With a lower sucrose concentration (0.5 M) in the preculture medium, none of the embryos showed normal regrowth. Similar results were observed by Nan *et al.* (24) with coconut plumules after cryopreservation. The induction of cryopreservation tolerance by incubation in sugar rich medium is described as a complex mechanism involving accumulation of sugar, dehydrins and a plasmolytic effect (11). As suggested by Malik and Chaudhury (21), the beneficial effect of sucrose preculture is attributed to the equalization of MC within the tissues of a given embryos axis and between axes of different embryos, which are highly heterogenous with regards to axis MC, so that tissues could be optimally and homogeneously desiccated. However this effect of sucrose preculture was only partially exemplified in the present study since the plantlets developed from embryos subjected to cryopreservation did not exhibit uniform growth rate. Preculture with high sucrose concentration (0.75 – 1.0 M) has been shown to increase the viability of cryopreserved cells of *Medicago sativa* (30) and *Vitis vinifera* (39). Sucrose is considered as a non-penetrating cryoprotectant, which aids dehydration of explants by means of its osmotic effect. When present above a threshold level, its breakdown products can enter the cell and accumulate in the form of starch, as evidenced by histological observation (15) or by calorimetric methods (5). Sugars can also maintain the liquid crystalline state of membrane bilayers and stabilize proteins under cryopreserved conditions (30). As sugars are known to protect plant cells during desiccation (8), it was proposed by Hoekstra *et al.,* (9) that at water levels below 20% FW, sugars can replace the hydration shell and form hydrogen bonds with macromolecules, thereby preventing protein denaturation and phase transition in membranes. This could explain the high recovery of embryos pregrown in 0.6 M sucrose and dehydrated to a low MC (16.8%) after PVS3 treatment. Uemura and Steponkus (35) reported that exogenous sucrose at low concentration serves as a metabolic substrate for low-temperature induced metabolic alterations, while at higher concentration it has a direct cryoprotective effect on cellular membranes.

Initial survival and germination were significantly higher after unloading in medium with 1.2 M sucrose. The low growth rate of cryopreserved embryos at the later stages of recovery resulted in significant differences in the percentage of plantlets available for potting originating from non-cryopreserved and cryopreserved material. The low growth rate of the plantlets observed in the present study may be due to the deleterious effect exerted by the damaged primordial tissues of leaf sheaths surrounding the shoot apical dome of embryos as has also been suggested by Varghese *et al.* (37). This was evident from the histological studies performed, which showed more prevalent damage in tissues of the primordial leaf sheaths compared to those of the embryo meristematic dome.

Unloading samples in hypotonic medium allows the reincorporation of a sufficient amount of material in the plasma membrane to accommodate further osmotic expansion, which will take place upon their transfer onto standard medium (31). It also reduces the cytotoxicity of the remaining cryoprotectants (17). In the present study, unloading in medium with 1.2 M sucrose was found to be most effective for plantlet recovery from zygotic embryos after cryopreservation. Cellular damage, as evidenced by histological observations, occurred as a result of severe osmotic shock when embryos were transferred from PVS3 solution, which contains a high sucrose concentration, to unloading medium, which contains a lower sucrose concentration. This was illustrated by the results achieved after unloading with medium containing 0.6 M sucrose, where very low plantlet recovery (10%) was achieved with non-frozen embryos and no recovery was obtained with cryopreserved embryos.

The utilization of the vitrification technique is reported for the first time for cryopreservation of coconut embryos. Our studies led to the conclusion that PVS3 was the most effective vitrification solution for coconut embryo cryopreservation. The relative simplicity of the vitrification protocol developed in the present study makes it easily applicable for the long-term storage of coconut germplasm. It was observed that sucrose concentration in the preculture and unloading media is critical for survival and growth of coconut embryos after cryopreservation. With this vitrification protocol, considering the percentage of weanable plantlets produced from cryopreserved embryos, we need to cryopreserve five embryos to get at least one plant. Further improvements to the protocol may be achieved by modifying the original PVS3 solution (18) to increase survival after cryopreservation and by adding growth regulators such as gibberellins in the recovery medium to increase the growth rate of the embryos. Before this technique is adopted for germplasm banking, it needs to be tested with genetically diverse genotypes to assess the genotypic dependent responses for cyopreservation and to ascertain the genetic stability of plants regenerated from cryopreserved embryos.

Acknowledgements: We gratefully acknowledges Dr. George V. Thomas, Director, Dr. R. V. Nair, Head, Crop Improvement Division, CPCRI, Kasaragod, for providing facilities and guidance. Our thanks are due to Dr. M. K. Rajesh, Sr. Scientist and Mr. Jiji George, SRF, CPCRI. We thank Mrs. Sugatha Padmanabhan, Technical Officer for help in biochemical analysis and Mr. K. Shyama Prasad, Technical Officer, CPCRI, for photography.

REFERENCES

1. Assy-Bah B & Engelmann F (1992) *CryoLetters* **13,** 117-126.
2. Anna B & Bozena P (2003) *Actabiologia Cracoviensia Series Botanica* **45/2**, 79-86.
3. Chin HF & Roberts EH (1980) *Recalcitrant Crop Seeds*. Tropical Press SDN, BDH., Kuala Lumpur .
4. De-Zhu Li & Pritchard HW (2009) *Trends in Plant Sci.* **14**, 614-621.
5. Dubois M**,** Gills KA**,** Hamilton JK**,** RebersPA & Smith F (1956) *Anal. Chem.***26,** 350.
6. Eeuwens CJ (1976) *Physiol. Plant.* **36**, 23-28.
7. Fahy GM, MacFarlane DF, Angell CA & Meryman HT (1984) *Cryobiology* **21**, 407-426.

8. Halperin SJ & Koster KL (2006) *J. Exp. Bot*. **57,** 2303-2311.
9. Hoekstra FA, Golovina EA, Tetteroo FAA & Wolkers WF (2001) *Cryobiology* **43,** 140-150.
10. ISTA (1999) *Seed Sci. Technol.* **27**, Suppl.
11. Jitsuyama Y, Suzuki T, Harada T & Fujikawa S (2002) *CryoLetters* **23,** 103-112.
12. Karun A, Parthasarathy VA, Kumaran PM, Iyer RD, & Sajini KK (2002) *Coconut Embryo Culture Protocol for Germplasm Collection*, Technical Bulletin No 45, CPCRI, Kasaragod, India.
13. Karun A, Sajini KK & Parthasarathy VA (2005) *Cord.* **21**, 13-19.
14. Kartha KK & Engelmann F (1994) in *Plant Cell Tissue Culture*, (eds) Vasil IK & Thrope TA, Kluwer, Dordrecht, pp. 195-230.
15. Kendall EJ, Kartha KK, Qureshi JA & Chermak P (1993). *Plant Cell Rep.* **2,** 89-94.
16. Kim HH, Cho EG, Baek HJ, Kim CY, Keller ERJ & Engelmann F (2004) *CryoLetters* **25,** 59-70.
17. Kim JB, Kim HH, Beak HJ, Cho EG, Kim YH & Engelmann F (2005) *CryoLetters* **26,** 103-112.
18. Kim HH, Lee YG, Shin DJ, Ko HC, Gwag JG, Cho EG & Engelmann F (2009) *CryoLetters* **30**, 320-334.
19. Langis R, Schnabel-Preikstas B, Earle ED & Steponkus PL (1989) *CryoLetters* **10**, 421-428.
20. Lagis R & Steponkus PL (1990)*Plant Physiol.* **92,** 666-671.
21. Malik SK & Chaudhury R (2006) *Plant Genet. Res. Charac. Util.* **4**, 204-209.
22. Matsumoto T (1999) Doctoral Thesis, Bull. Shimane Agric. Exp. Station, Japan.
23. Mycock DJ, Smith JW & Berjak P (1995) *Ann. Bot.* **75**,331-336.
24. Nan ON, Hocher V, Verdeil JL, Konan JL, Ballo K, Mondeil F & Malaurie B (2008) *CryoLetters* **29,** 339-350.
25. Nishizawa S, Sakai A, Amano AY & Matsuzawa T (1993) *Plant Sci.* **91**, 67-73.
26. Quain MD, Berjak P, Acheampong E & Kioko JI (2009) *CryoLetters* **30**, 212-223
27. Sajini KK, Anitha Karun & Kumaran PM (2006). *J. Plant.Crops* **34,** 576-581.
28. Sakai A & Engelmann F (2007) *CryoLetters* **28,** 151-172.
29. Sakai A, Kobayashi S & Oiyama I (1990) *Plant Cell Rep.* **9**,30-33
30. Sibli RA, Haagenson DM, Cunningham SM, Berg WK & Volence JJ (2001) *Plant Cell Rep*. **20,** 445-450.
31. Steponkus PL**,** Langis R & Fujikawa S (1992) in *Advances in Low Temperature Biology***.**Vol 1**,** (ed) Steponkus PL**,** JAI Press Ltd**,** Hampton Mill**,** UK**,** pp 1-61.
32. Thinh NT, Takagi H & Yashima S (1999) *CryoLetters* **20**, 163-174.
33. Towill LE (1990) *Plant Cell Rep.* **9**, 178-180.
34. Turner S, Senaratna T, Touchell D, Bunn E, Dixon K & Tan B (2001) *Plant Sci***.160**, 489-497.
35. Uemura M & Steponkus PL (2003) *Plant Cell Environ*. **26**, 1083-1096.
36. Uragami A, Sakai A, Nagai M & Takahashi T (1989) *Plant Cell Rep.* **8**, 418-421.
37. Varghese D, Berjak P & Pammenter (2009) *CryoLetters* **30**, 280-290.
38. Verleysen H, Samyn G, Van Bockstaele E & Debergh P (2004) *Plant Cell Tissue & Organ culture* **77**, 11-21.
39. Wang QC, Gafny R, Sahar N, Sela I, Mawassi M, Tanne E & Perl A (2002) *Plant Sci.* **162,** 551-558.

Accepted for publication 11/05/2011