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Cryopreservation of 'Laguna Tall' Coconut (*Cocos nucifera* Linn.) Mature Zygotic Embryos

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'Laguna Tall' coconut (*Cocos nucifera* L.) zygotic embryos were successfully cryopreserved through cryoprotection using pretreatment-desiccation or vitrification, rapid freezing in liquid nitrogen, rapid thawing at 40°C for 2 min, and recovery and growth of frozen embryos in coconut germination medium. Pretreatment in 0.5 M sucrose for 1 d followed by 24 h desiccation in silica gel gave the highest recovery and complete germination (47%) of cryopreserved embryos. This was followed by 24 h desiccation alone (43%), 3 d sucrose pretreatment + 24 h desiccation (33%), and the least recovery from vitrification using PVS4 solution (13%). Embryo reinvigoration as a post thawing treatment and addition of 0.5 mg L^{-1} benzyl amino purine + 1.0 mg L^{-1} naphthalene acetic acid onto Y3 germination medium hastened recovery and significantly improved complete germination of cryopreserved embryos. The study demonstrated the feasibility of cryopreservation for long-term conservation of coconut genetic resources and for the first time reported on the success in cryopreservation of 'Laguna Tall', an endemic coconut variety grown in the Philippines.

Key Words: coconut, cryopreservation, embryo culture, germplasm conservation, pre-growth, pretreatment-desiccation, vitrification

Abbreviations: BAP – benzyl amino purine, EC – electrical conductivity, LN – liquid nitrogen, MC – moisture content, NAA – naphthalene acetic acid, TTC – triphenyl tetrazolium chloride

INTRODUCTION

Coconut (*Cocos nucifera* L.) is a tropical species with large recalcitrant seeds. It is very slow growing, with a single shoot meristem at the apex of the plant, and no asexual production of shoots or suckers from the primary stem. It is traditionally grown from seed and thus gives rise to plants which are quite variable in terms of productivity. The number of seeds produced per plant also varies depending on the type of coconut.

Primary collections of coconut genetic resources are commonly held in field genebanks. The trees in the field allow confirmation of their morphological identity. In addition, the performance of the genotypes can be evaluated for resistance to pest and diseases and response to environmental conditions as well. Unfortunately, trees in field genebanks are vulnerable to environmental catastrophes such as typhoons, flood, drought, pests, disease epidemics, and other calamities (Withers and Engels 1990; Monette 1995). The labor costs and the requirement for technical personnel are likewise very high. In addition, the distribution and exchange from field genebank is difficult because of the condition of the material and the greater risk of disease transfer (Engelmann 1998).

A promising method of germplasm conservation for recalcitrant seeds is storage in liquid nitrogen (LN), which is also called cryopreservation (Chin and Roberts 1980). For recalcitrant and asexually propagated crop species, it is considered as the most stable method of germplasm conservation, as all metabolic activities stopped at –196°C. The plant material can thus be stored without alteration or modification for a theoretically unlimited period of time. Moreover, cultures are stored in a small volume, protected from contamination, and require very limited maintenance (Engelmann 2000).

Earlier studies on the cryopreservation of coconut embryos revealed that the embryo could be regenerated

into single plant 15 mo after freezing (Chin et al. 1989). Bajaj (1984) reported coconut embryos showed swelling and elongation 4 mo after cryopreservation. The survival rates for cryopreserved immature embryos ranged from 8% to 43%, however, limited number of control embryo and cryopreserved embryos developed into normal plantlets (Assy-Bah and Engelmann 1992a). Using mature embryos, however, survival rate after cryopreservation was observed to be comparable to that of normal embryos, ranging from 30% to 93% (Assy-Bah and Engelmann 1992b). Accordingly, cryopreserved embryos germinated and developed into whole plants after 3 mo of culture.

Sisunandar et al. (2010) successfully regenerated cryopreserved embryos of Malayan Yellow Dwarf and other Indonesian cultivars. Bandupriya et al. (2010) went further by encapsulating plumules excised from coconut embryos in sodium alginate then the beads were pretreated with 0.75 M or 1.0 M sucrose and finally subjected to further desiccation by exposure to silica gel for 16 h prior to freezing in liquid nitrogen; they achieved a 40% recovery rate. Sajini et al. (2011) observed 70% to 80% survival from cryopreserved coconut zygotic embryos using the vitrification solution PVS3 wherein 20% to 25% regenerated into normal palms. Kim et al. (2019) also reported 90% germination and 35% regeneration in cryopreserved 'Malayan Yellow Dwarf' embryos using a preculture-desiccation procedure. The embryos were progressively precultured with sucrose 17.5% (1 d), -35% (1 d), and -50% (1 d) followed by silica gel desiccation for 17 h. However, the cryopreservation protocols so far developed for coconut are cultivar specific and the success of plant recovery after cryopreservation has been very low and found to be highly variable.

In this present investigation, three different cryogenic protocols: pretreatment-desiccation, pre-growth in a cryoprotectant, and vitrification were compared to determine the best and effective method for cryopreserving zygotic embryos of 'Laguna Tall' coconut.

MATERIALS AND METHODS

Plant Material

The plant material consisted of mature embryos (12 mo after pollination) from seed nuts of cultivar 'Laguna Tall'. The dehusked nuts were split open and solid endosperm cylinders containing the zygotic embryos were extracted using a stainless steel cork borer (size no. 10). The endosperm cylinders were collected in plastic beaker containing sterile distilled water, transferred to sterile plastic bag, and brought to the laboratory for disinfection. Initial sterilization of the endosperm cylinders was done by washing in tap water three times, followed by quick soaking in 95% (v/v) ethanol for 30–60 s and rinsed three times with sterile tap water. Decontamination of the endosperm cylinders followed using pure commercial bleach (5.25% sodium hypochlorite) for 20 min and rinsed five times with sterile tap water (Del Rosario 1998). The embryos excised from endosperm cylinders were disinfected again in 70% ethanol for 1 min, rinsed three times with sterile distilled water, soaked in 10% commercial bleach (5.25% sodium hypochlorite) for 10 min, and finally rinsed five times with sterile distilled water. Sterile embryos were then transferred to sterile petri dishes before assignment to various treatments.

Cryopreservation of Coconut Embryos

Four procedures namely: desiccation, pretreatment desiccation, pre-growth and vitrification were studied to determine the best treatment for cryopreservation of coconut embryos. For the desiccation method, the sterile embryos were placed in an open petri dish and dehydrated for 24 h in an air-tight glass jar (120 mm x 100 mm x 95 mm (H x W x D); 800 mL) containing 100 g activated silica gel (LabChem 5–10 mesh). For the pretreatment-desiccation, the embryos were initially pre-cultured on 0.5 M sucrose solution for 1 and 3 d and then subjected to 24 h desiccation in airtight jars containing 100 g silica gel. For the pre-growth, embryos were pre-cultured on 0.5 M sucrose solution for 1, 3 and 7 d. While for the vitrification, the embryos were either transferred directly in 2 mL sterile cryovial containing 1.5 mL PVS4 solution which contained 35% glycerol and 20% ethylene glycol in basal medium containing 0.6 M sucrose (Sakai 2000) or pre-cultured initially in 0.5 M sucrose for 3 and 7 d prior to transfer to PVS4 solution. All embryos were kept in PVS4 solution for 30 min.

After cryoprotection treatments, five embryos were then placed in 2 mL sterile cryovial and immersed rapidly in LN (+LN). After 24 h at -196°C, the cryopreserved embryos were thawed rapidly by immersing the cryovials in 40°C water bath for 2 min. Except for those subjected to vitrification treatment, thawed cryopreserved embryos were transferred on standard coconut medium for recovery and germination. For the vitrification treatment, embryos were transferred from the PVS4 solution to a washing solution which contained 1.2 M sucrose for 30 min and then transferred onto standard Y3 medium. A total of 45 embryos (three replications with 15 embryos per replication) and were used per treatment. Control treatments involved recovery on medium with and without cryoprotection and with (+LN) and without exposure to LN (–LN). Other embryos were used for destructive sampling for the determination of the various parameters; 10 embryos for the moisture content (MC)

determination, 10 embryos for the viability test using triphenyl tetrazolium chloride (TTC) and 5 embryos for the electrical conductivity (EC) test. All treatments were arranged in completely randomized design with three replications.

Post Thawing Recovery

To increase the recovery and growth of cryopreserved embryos, the effects of embryo reinvigoration and addition of plant growth regulators 0.5 mg L^{-1} benzyl amino purine (BAP) and 1.0 mg L^{-1} naphthalene acetic acid (NAA) onto the coconut germination medium were studied. Reinvigoration was carried out by placing embryos on petri dishes lined with a thin layer of cotton pad wet with 20 mL sterilized distilled water. Embryos were reinvigorated for 24 h after and then subsequently transferred to germination medium. These post thawing treatments were used in embryos subjected to three different methods of cryopreservation used in this study. The treatments were arranged in completely randomized design with three replications with 30 embryos per replicate.

In Vitro Culture

All embryos were cultured on coconut embryo culture medium developed at the University of the Philippines Los Baños (del Rosario 1998). The embryos were initially cultured in 20 x 150 mm test tubes containing 10 mL of the medium and after germination sub-cultured at monthly intervals to bigger vessel containing 50 mL of medium. Culture tubes were kept in the dark at 25 + 2°C and when the plumule emerged transferred to growth room with 16 h photoperiod.

Assessment of Recovery

Survival of the embryos was recorded after 1 mo of culture. Embryos were considered alive when enlargement was observed. Germination was deemed completed when the plumule emerged from within the cotyledonary sheath, and roots produced. The emergence of plumule only is considered partial or incomplete germination. After 3 mo in culture, the development of the surviving embryo was assessed by the number of embryos having developed both plumule and roots.

Determination of Viability

The TTC method devised by Steponkus and Lanphear (1967) was used to determine the viability of coconut embryo after every step in the treatments including the control. Ten embryos were used before and after all treatments. The embryos were sliced in half and soaked in 0.1% TTC solution for 12 h in the dark and washed with distilled water before observation. The chemical was imbibed by the embryos as a colorless solution and is reduced by dehydrogenase enzymes to a red color. In the absence of active enzymes, tissues remain unstained. Those embryos that turned red were considered viable while those that did not turn red or remained white were considered non-viable. Embryos stained in the apical area containing plumule and radicle (Sugimura and Murakami 1990) were counted as viable but if staining was limited to the haustorial end, then these were considered non-viable.

Moisture Content (MC) Determination

The MC of the embryos (expressed in % of their initial fresh weight) was monitored during treatment. Fresh weight measurements were taken from batches of 10 embryos before and after treatment for different periods of time. Dry weight was determined after 48 h drying in an oven set at 102°C.

Determination of Temperature

The temperature of the embryos immersed in LN was monitored during treatment using an analog model BAT-12 Sensortek thermometer (USA) which was capable of measuring within the range of –100°C to 200°C. Thermocouple sensors were imbedded within the embryos. After immersion of the embryos in LN, the temperature was read every 10 s until it reached the limit of detection which is -100°C. This procedure was conducted to detect any exotherm (ice formation) and determine the time duration before the temperature within the embryos reached -100°C.

Electrical Conductivity Measurements

Electrical conductivity (EC) was measured following the procedure of Chandel et al. (1995) with some modifications. Fresh (control) and treated embryos were soaked in 25 mL deionized water. Five embryos were used for each treatment. Conductivity of the water was measured 4 h later at room temperature using a conductivity meter (Type CDM 2e) with a conductivity cell type CDC 104. HPLC grade water (JT Baker™) was used so that the background electroconductivity was kept to a minimum. Five embryos were used for each treatment. Conductivity was measured as μSiemens/cm (μS cm^{-1}). This is a unit of measure which estimates the quantity of electrolytes leached from the embryos, which indicates membrane quality in general.

Experimental Design and Statistical Analysis

All experiments were set up in completely randomized design with three replications per treatment and 15 to 30 embyos per replication. Percentage data were transformed using the Square-Root Transformation and encoded in Microsoft Excel and analyzed using Statistical Analysis

Software (SAS) version 6.12 system. Analysis of variance (ANOVA) was done to test for significant differences among the treatments. Comparison of treatment means was done using the Least Significant Difference test.

RESULTS

Pretreatment-Desiccation

The MC of coconut embryos after desiccation was significantly reduced from 80.3% (control or non-desiccated embryos) to less than 35.2% in all desiccation treatments (Table 1). The greatest reduction in MC was obtained with 1 d sucrose pretreatment followed by 24 h desiccation in silica gel (9.8% MC) although this was not significantly different from the 24 h desiccation treatment alone (11.85% MC). Prolonging the sucrose pretreatment for 3 d, however, did not result in further reduction in MC (22.5%). On the other hand, desiccation followed by sucrose pretreatment showed the least reduction in MC (35.2%).

Table 1. Mean moisture content of coconut embryos subjected to pretreatment-desiccation method.

Treatment	Moisture Content (%)
A Control	80.29
B Desiccation 24 h	11.85
C Desiccation 24 h + sucrose 0.5 M 1 d	35.15
D Sucrose 0.5 M 1 day + desiccation 24 h	9.80
E Sucrose 0.5 M 3 day + desiccation 24 h	22.50
$LSD_{.05}$	2.48

Moisture content expressed in % of their initial fresh weight; dry weight was determined after 48 h drying in an oven set at 102°C.

Electrical conductivity reading increased significantly as the embryos became dehydrated and increased further in all treatments after immersion in LN (Fig. 1a). The highest EC reading was obtained in embryos desiccated for 24 h for both –LN (2266 µS cm^{-1}) and +LN (2341 µS cm^{-1}), respectively. On the other hand, the lowest reading was observed in embryos subjected to 24 h desiccation + 1 d sucrose pretreatment both in –LN (583 µS cm^{-1}) and +LN (1216 µS cm^{-1}), respectively.

Significant differences in the percent viability of embryos were observed among desiccation treatments both in –LN and +LN, respectively (Fig. 1b). Embryos dehydrated for 24 h showed the highest percent viability in both –LN (83.3%) and +LN treatments (73.3%), respectively. While for other desiccation treatments, no significant differences were observed in –LN and +LN, respectively.

None of the control embryos survived LN treatment. The percent survival of embryos taken after 1 mo in culture was highest in embryos subjected to sucrose pretreatment for 1 or 3 d + 24 h desiccation for both –LN (81–85%) and + LN (82–86%), respectively (Fig. 1c). On the other hand, the least survival of embryos was obtained with 24 h desiccation + 1 d sucrose pretreatment; percent survival was reduced significantly from 55% in –LN to only 5% in + LN.

The control embryos showed complete germination (both plumule and root emergence) after 6 wk in culture while for other treatments, delayed germination was noted and some embryos started to germinate after 7 wk in culture. After 3 mo in culture, control embryos had 73% complete germination while those subjected to desiccation treatments had less than 60% germination even in –LN (Fig 1d). For those immersed in LN, the complete germination was 0–46%. The highest was observed in embryos subjected to 1 d sucrose pre-growth + 24 h desiccation (47%) although this was not significantly different from the 24 h desiccation only (43%). Furthermore, for these treatments, the difference in germination between –LN and +LN was relatively small. This result shows that these desiccation treatments provided sufficient dehydration (9.8% and 11.8% MC) to enable the embryos to survive storage in LN. On the other hand, prolonging sucrose pretreatment for 3 d prior to silica desiccation did not offer additional cryoprotection to the embryos; percent germination was 33% compared to 43% for 1 d sucrose pretreatment. Incomplete or partial germination (plumule only) of embryos was likewise observed in all treatments for both –LN and +LN; the highest (27%) was observed in 1 d sucrose pretreatment + 24 h desiccation after LN treatment.

Pregrowth in Cryoprotectant

Pre-growth in cryoptotectant involves initial culture of the embryos in 0.5 M sucrose as cryoptotectant for 1, 3 and 7 d followed by freezing rapidly by direct immersion in LN. The MC of embryos subjected to sucrose pre-growth was still high even after 7 d in sucrose (Table 2). The lowest MC attained was only 57.1%, which was still very much higher compared with those obtained with desiccation treatments (Table 2 vs. Table 1). Such high MC will not offer any cryoprotection to embryos. The EC

Table 2. Mean moisture content of coconut embryos subjected to pre-growth in cryoprotectant method.

Treatment	Moisture Content (%)
Control	80.29
Sucrose 0.5 M 1 d	72.11
Sucrose 0.5 M 3 d	65.87
Sucrose 0.5 M 7 d	57.61
$LSD_{.05}$	2.9

MC expressed in % of their initial fresh weight; dry weight was determined after 48 h drying in an oven set at 102°C.

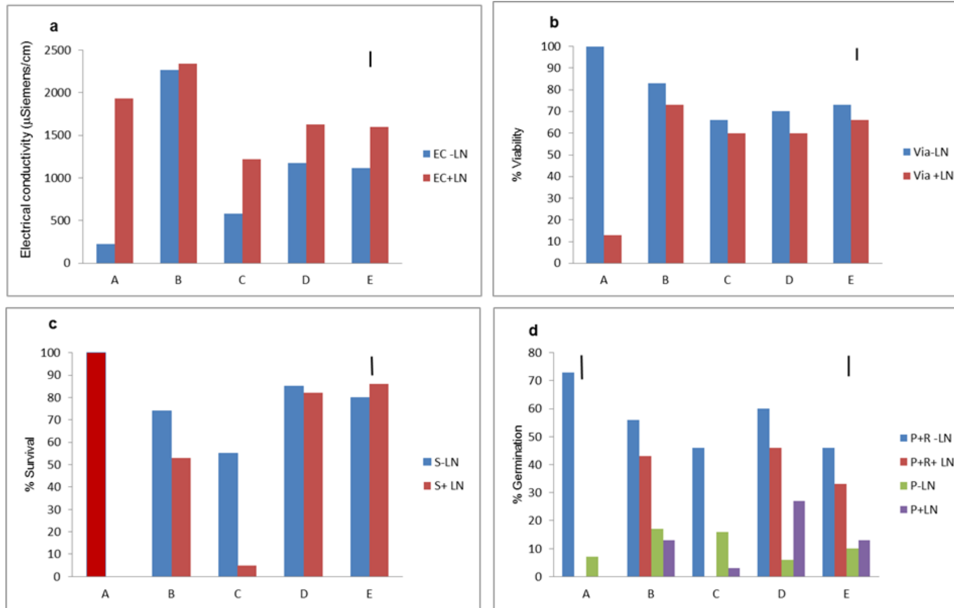

Fig. 1. (a) Electrical conductivity reading, (b) % viability based on TTC test, (c) % survival based on enlarging embryos taken after 1 mo of culture, and (d) % germination taken after 3 mo in culture before (-LN) and after (+LN) immersion in LN of embryos subjected to pretreatment-desiccation method. Treatments: A - Control, B - Desiccation 24 h, C - Desiccation 24 h + sucrose 0.5 M 1 d, D - Sucrose 0.5 M 1 d + desiccation 24 h, E - Desiccation 24 h + sucrose 0.5 M 3 d. Bars indicate LSD at *α* = 0.01.

reading after pre-growth in sucrose increased significantly from 225 µS cm^{-1} for the control embryos to 1016 µS cm^{-1} after 7 d sucrose pre-growth (Fig. 2a). However, except for the control embryos, there was no significant increase in EC reading in all treated embryos after immersion in LN.

Without LN, viability of the embryos was almost 100% in all treatments (Fig. 2b). However, in +LN, viability of sucrose treated embryos was significantly reduced to 50–63% (Fig. 2b). The survival after 4 wk of culture was likewise almost 100% in all treatments if without LN (Fig. 2c). In contrast, in + LN, survival was significantly reduced to 11–54% and with the highest survival noted in embryos subjected to 3 d pre-growth in sucrose.

Germination either complete or partial was observed only for embryos not subjected to LN (Fig. 2d). Of the 100% surviving embryos, only 20–63% showed complete germination, the rest showed plumule emergence only (3 –40%). Prolonged pre-growth in sucrose solution, however, was detrimental to the germination of the embryos; it was reduced from 73% (control) to 63% (1 d pre-growth), 43% (3 d pre-growth) and 20% (7 d pre-growth), respectively. All surviving embryos in + LN treatment did not show any signs of germination even after more than 3 mo in culture.

Vitrification

Vitrification involves treatment ('loading') of samples with cryoprotective substances, dehydration with highly

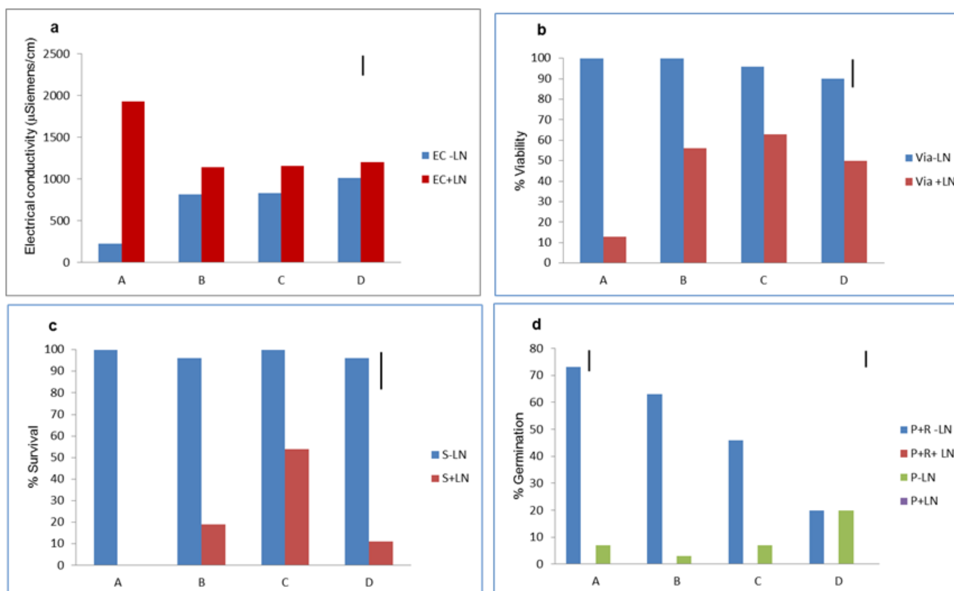

Fig. 2. (a) Electrical conductivity reading, (b) % viability based on TTC test, (c) % survival based on enlarging embryos taken after 1 mo of culture, and (d) % germination taken after 3 mo in culture before (-LN) and after (+LN) immersion in LN of embryos subjected to sucrose pre-growth method. Treatments: A - control, B - Sucrose 0.5 M 1 d, C - Sucrose 0.5 M 3 d, D - Sucrose 0.5 M 7 d. Bars indicate LSD at *α* = 0.01.

concentrated vitrification solutions, rapid freezing and thawing, removal of cryoprotectant (‘unloading’) and recovery (Engelmann 1997).

Dehydration of embryos in PVS4 solution showed significant reduction in MC from 80.1% in the control embryos to about 40.4–47.3% in treated embryos (Table 3). Additional sucrose treatment prior to PVS4 treatment, however, resulted in only a minimal decrease in MC (from 47.3% with PVS4 treatment to 40.4% with 3 d sucrose treatment + PVS4). The MC of embryos after vitrification treatments was still relatively high compared with the desiccation treatments (Table 3 vs. Table 1).

Table 3. Mean moisture content of coconut embryos subjected to vitrification method.

Treatment	Moisture Content (%)
Control	80.29
PVS4	47.26
Sucrose 0.5 M 1 d + PVS4	42.68
Sucrose 0.5 M 3 d + PVS4	40.45
$LSD_{.05}$	2.9

Moisture content expressed in % of their initial fresh weight; dry weight was determined after 48 h drying in an oven set at 102°C.

Contrary to other cryoprotective treatments such as desiccation and sucrose pre-growth, the EC reading of the embryos subjected to vitrification treatments was almost similar in –LN and +LN (Fig. 3a). Treatment with PVS4 alone showed the least EC reading in both –LN (316.67 µS cm^{-1}) and +LN (350.33 µS cm^{-1}), respectively.

Without LN treatment, the viability of the embryos was similar for all vitrification treatments (96–100%) including the control embryos (Fig. 3b). However, with+ LN, the viability was dropped to 60–63% and no significant differences were observed among vitrification treatments. In terms of embryo survival, only 15.4% of embryos treated with PVS4 survived LN treatment (Fig. 3c). The percent survival was even lower (7%) with pre-growth in sucrose for 1–3 d + PVS4. The germination of embryos subjected to vitrification treatments was likewise significantly reduced in both –LN and +LN, respectively (Fig 3d). After 3 mo in culture, only those embryos treated with PVS4 solution showed complete germination (13%) after immersion in LN. Even in –LN, treatment with PVS4 alone or with sucrose pre-growth seemed to be detrimental to the survival and subsequently the germination of embryos.

Post Thawing Recovery

Post thawing treatments such as reinvigoration and addition of plant growth regulators (BAP and NAA) to the germination medium significantly improved survival and germination of cryopreserved embryos (Table 4). With these treatments, percent germination of cryopreserved embryos was about 3 to 5 times higher (20–30%) than the non-treated (no post thawing) cryopreserved embryos (5–8%). Moreover, cryopreserved embryos started to germinate as early as 4 wk after incubation in culture medium, about 2 wk earlier than the control embryos (data not shown). Generally, control and cryopreserved embryos attained complete germination in about 3 mo (Fig. 1d, 2d, and 3d). However, with these post thawing treatments, about 20–30% of the cryopreserved embryos already showed complete germination in 2 mo, which is a significant improvement in recovery of cryopreserved embryos.

DISCUSSION

The study was done to test several cryopreservation techniques for ‘Laguna Tall’ coconut embryos with the

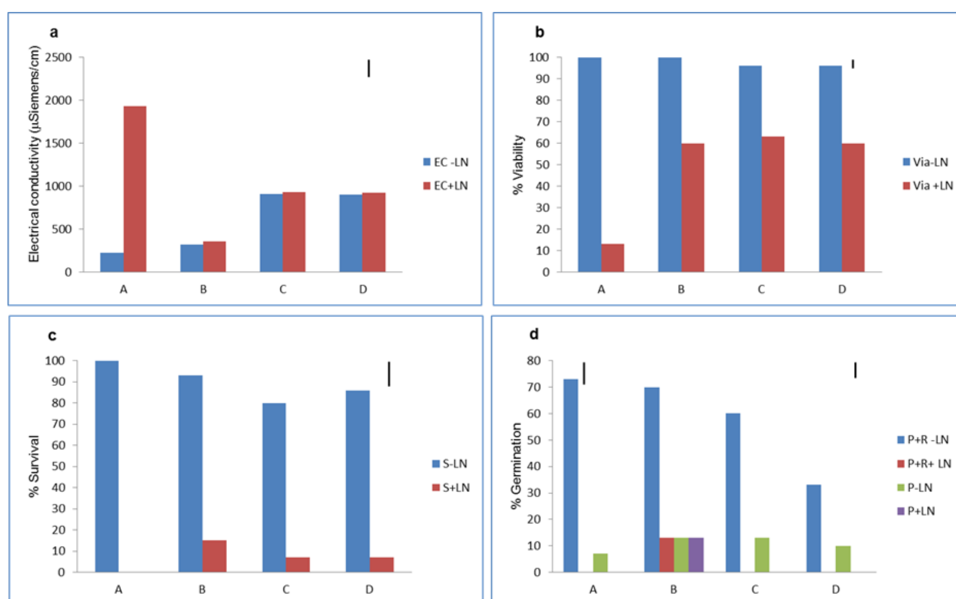

Fig. 3. (a) Electrical conductivity reading, (b) % viability based on TTC test, (c) % survival based on enlarging embryos taken after 1 mo of culture, and (d) % germination taken after 3 mo in culture before (-LN) and after (+LN) immersion in LN of embryos subjected to vitrification method. Treatments: A - Control, B - PVS4, C - Sucrose 0.5 M 1 d + PVS4, D - Sucrose 0.5 M 3 d + PVS4. Bars indicate LSD at α = 0.01.

Table 4. Mean percent survival and percent germination of cryopreserved embryos after reinvigoration and treatment with plant growth regulators.

Post Thawing/Treated with Cryopreservation Treatment[1]	% Survival[1]	% Germination[2] (Plumule + Roots)[3]
Without post thawing treatment (Non treated)		
Control		
Desiccation 24 h	53.8	5.6
Sucrose 0.5 M 1 d Desiccation 24 h	82.1	8.6
+Reinvigoration in moist cotton pad for 24 h		
Desiccation 24 h	83.3	30.0
Sucrose 0.5 M 1 d Desiccation 24 h	86.7	23.3
+Growth regulators BAP (0.5 mg L^{-1}) and NAA (1.0 mg L^{-1}) on germination medium		
Desiccation 24 h	80.0	30.0
Sucrose 0.5 M 1 d Desiccation 24 h	96.7	20.0
$LSD_{0.01}$	7.1	4.8

[1]Cryopreservation of embryos using the best treatments from previous study
[2]Based on enlarging embryos taken after 1 mo of culture
[3]Complete germination with both plumule and roots emergence taken after 2 mo of culture

eventual goal of storing coconut germplasm in LN for long-term preservation. Among the cryoprotection techniques tested, 24 h pretreatment in 0.5 M sucrose + 24 h desiccation in silica gel gave the highest recovery and complete germination (47%) of cryopreserved embryos. This was followed by 24 h desiccation alone (43%), 3 d sucrose pretreatment + 24 h desiccation (33%), and the least was vitrification using PVS4 solution (13%). On the other hand, pre-growth of embryos on 0.5 M sucrose for 3–7 d did not offer any cryoprotection to frozen embryos.

The recovery and germination of cryopreserved embryos using the pretreatment-desiccation method obtained in this study is comparable to earlier findings of Assy-Bah and Engelmann (1992b) for Indian Tall (48% germination) and Rennell Tall (33% germination) using the desiccation-pre-growth technique. Similarly, using 8 h rapid dehydration technique, Sisunandar et al. (2010) obtained 20–40% complete germination and normal seedling growth for cryopreserved embryos of Malayan Yellow Dwarf. Moreover, this 8 h rapid dehydration technique was found applicable to a range of Indonesian coconut cultivars; 16 out of the 20 cultivars tested showed 10–40% normal seedling recovery and the remaining four cultivars had 0–10% normal seedling recovery from cryopreserved embryos (Sisunandar et al. 2010).

The pre-growth desiccation (embryos desiccated in laminar air flow for 4 h and pre-grown in MS medium containing 600 g L^{-1} glucose + 15% glycerol) of Assy-Bah and Engelmann (1992b), 8 h rapid physical dehydration (embryos dehydrated for 8 h in fan-operated drying chamber containing silica gel) of Sisunandar et al. (2010), and the pretreatment–desiccation used in the present study are basically similar and all employ physical dehydration of the cells to a critical low MC required to survive LN storage. The three techniques, however, differ in the way physical dehydration is done, ease or complexity of the procedure, and lowest attainable critical MC. For many plant species, one of the most important variables contributing to success in cryopreservation is the MC of the tissues prior to freezing; the optimal rates for survival being about 10–20% fresh weight basis (Assy-Bah and Engelmann 1992a). In the present study, only those treated embryos which attained MC of about 10–20% (Table 1 and Fig. 1d) survived and germinated after cryopreservation. Complete germination was highest (43–46%) at 9.8 to 11.8% MC, and decreased with increasing MC. With the pre-growth desiccation, 5–7% MC gave germination which ranged from 33% to 93% (Assy-Bah and Engelmann 1992b) while rapid dehydration to about 19% MC resulted in 10–40% normal germination and seedling development in a range of coconut cultivars (Sisunandar et al. 2010).

On the other hand, in most tissues, the ability to survive desiccation is not a guarantee that cryo damage will be avoided. Even when the loss of non-freezable water can be tolerated, there is only a relatively very narrow range of moisture at which LN exposure is tolerated, as with the intermediate *Coffee arabica* seeds, or LN may not be tolerated, even though there is survival at –20°C, as in the case of wild rice seeds (Pence 1995). In most cases though, the critical and threshold moisture levels of the tissue will determine whether or not desiccation can be used as a protection against LN damage.

Vitrification is another effective mechanism against freezing, wherein highly concentrated cryoprotective solution supercools to a very low temperature and solidifies into a metastable glass without undergoing crystallization (Fahy et al. 1984). The vitrification procedure using PVS4 solution used in the study, however, did not offer enough cryoprotection to the embryos since the highest germination obtained was only 13% (Fig. 3d). The relatively high moisture content of embryos after PVS4 treatment (Table 3) and membrane damage as shown by EC reading (Fig. 3a) could have contributed to very low survival and germination of cryopreserved embryos. Sajini et al. (2011) likewise used vitrification technique for cryopreservation of coconut zygotic embryos, and found that of the seven plant vitrification solutions that were tested, only PVS3 was the most effective for regeneration of cryopreserved embryos. With this protocol, about 20–25 % of the plants showing

normal shoot and root growth were regenerated from cryopreserved embryos (Sajini et al. 2011). According to Sakai et al. (2000), the vitrification technique worked best for cryopreservation of shoot apices of species like wasabi and chrysanthemum and in more than 140 plant species or cultivars in both temperate and tropical origins (Sakai 1997; 2000). Accordingly, because vitrification uses highly concentrated toxic solutions, the duration of treatment has to be very precisely timed and optimized for a particular explant. Due to some inherent difficulties and limitations in the use of embryos for cryopreservation, coconut plumules have been used for cryopreservation by encapsulation dehydration using different sucrose concentrations and dehydration periods; the technique, however, still needs further refinements (Bandupriya et al. 2010).

All cryoprotection treatments (i.e., desiccation, pre-growth and vitrification) showed a significantly increased membrane leakage as indicated in the increased EC reading even before LN treatment and a much higher reading after immersion in LN (Fig. 1a, 2a and 3a). These results suggest that cryoprotection treatments and freezing cause irreversible damage to the membrane. Several studies have also used membrane leakage as a measure of desiccation and freezing damage. The integrity of the membrane is needed to perform functions such as regulating the flow of material between subcellular compartments, supplying a dynamic framework for numerous enzyme activities, and maintaining separations between mutually incompatible cellular compartments (Priestly 1986).

In the present study, delay in germination of treated and cryopreserved embryos was also evident. Observations indicated that embryos started to germinate after 6 wk for control and 7 wk for treated embryos. Bajaj (1984) obtained transient callusing on frozen embryos after 4 mo while Chin et al. (1989) could regenerate only one coconut plant 15 mo after cryopreservation of the embryo. Assy-Bah and Engelmann (1992a, 1992b) also noticed similar delays in the germination of frozen embryos compared to the pretreated control. The delay in germination may be due to reduced capability of embryos to cope with stress brought about by desiccation and freezing injury.

Reinvigoration during post thawing recovery and the addition of plant growth regulators BAP and NAA onto the germination medium significantly improved rate and percent germination of frozen coconut embryos (Table 4). With these treatments, frozen coconut embryos started to germinate in 4 wk, which is about 2 wk earlier than the non-treated embryos, and likewise increased the number of completely germinated embryos. Reinvigoration promoted the water availability after dehydration and addition of plant growth regulators could have triggered cell division and differentiation.

The drawbacks of field gene banking of coconut genetic resources can be overcome by cryopreservation and this is more attractive because of the potential reduction in maintenance cost and reduced exposure of germplasm to pest and diseases and natural disasters. The present study shows that cryopreservation of ‘Laguna Tall’, an endemic coconut cultivar grown in the Philippines, is feasible and that the success and recovery is comparable, or in some instances, even higher than those obtained with other coconut cultivars (Chin et al. 1989; Assy-Bah and Engelmann 1992b; Sisunandar et al. 2010; Sajini et al. 2011). Cryopreservation using pretreatment desiccation is a simple method and could be applied to other cultivars and used for long-term conservation of Philippine coconut genetic resources.

CONCLUSION

The present study demonstrated the feasibility of cryopreservation for long-term conservation of coconut genetic resources and for the first time reported on the success in cryopreservation of ‘Laguna Tall’, an endemic coconut variety grown in the Philippines. The technique involves cryoprotection of embryos by pretreatment desiccation either by silica gel desiccation for 24 h or sucrose pre-growth + desiccation for 24 h, followed by rapid freezing in LN, rapid thawing at 40°C for 2 min, and post thawing and recovery onto coconut embryo culture medium. The embryos cryopreserved using the developed procedure showed 33–47% normal (both shoot and root present) germination after 3 mo in culture. Recovery and germination of cryopreserved embryos was improved further by embryo reinvigoration during post thawing and culture onto coconut embryo culture medium supplemented with 0.5 mg L^{-1} BAP + 1.0 mg L^{-1} NAA. The cryopreservation using pretreatment desiccation is a simple method and could be applied to other cultivars for the long-term conservation of Philippine coconut genetic resources. ‘Laguna Tall’ cultivar would now be included in the list of coconut genotypes responsive to cryopreservation.

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