CONSERVATION OF COCONUT (*Cocos nucifera* L.)
GERMPLASM AT SUB-ZERO TEMPERATURE

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Abstract

Protocols are proposed for the low (-20°C) and ultra-low (-80°C) temperature storage of coconut (*Cocos nucifera* L.) embryos. A tissue dehydration step prior to storage, and a rapid warming step upon recovery optimized the protocol. The thermal properties of water located within the embryos were monitored using differential scanning calorimetry (DSC). In the most efficient version of the protocol, embryos were dehydrated under a sterile air flow in a dehydration solution containing glucose (3.33 M) and glycerol (15%) for 16 hours. This protocol decreased the embryo water content from 77 to 29% FW and at the same time reduced the amount of freezable water down to 0.03%. The dehydrated embryos could be stored for up to 3 weeks at -20°C (12% producing normal plants upon recovery) or 26 weeks at -80°C (28% producing normal plants upon recovery). These results indicate that it is possible to store coconut germplasm on a medium term basis using an ultra-deep freezer unit. However for more efficient, long term storage, cryopreservation remains the preferred option.

Keywords: dehydration, cryopreservation, differential scanning calorimetry, embryo culture, medium-term conservation, recalcitrant seeds.

INTRODUCTION

Up to now, the creation of *ex situ* seed gardens has been the only technique available for the efficient conservation of coconut (*Cocos nucifera* L.) germplasm (2). Seed gardens have been created in *ca.* 30 locations covering 22 countries around the globe and presently they contain more than 1,000 accessions of coconut (5). However, such gardens are very expensive to maintain and they are under continuous threats from a number of environmental stresses
(e.g. typhoons), devastating diseases (e.g. lethal yellowing, cadang-cadang [10]) and pest (e.g. Oryctes and Brontispa [13]). Alternative ex situ conservation techniques such as seed banking has no application to coconut due to the unusually large seed size (600 to 3,000 g each; [14]) and their recalcitrant nature (12) which hampers long term storage. Therefore, an alternative method for the preservation of coconut germplasm material is needed.

A new method to preserve coconut germplasm through cryopreservation has been recently developed. This method shows that a large number of plants can be recovered (19, 21) and that no genetic or epigenetic changes were induced using this improved technology (20). Such a cryopreservation approach can be successfully implemented in well-equipped laboratories with a constant supply of liquid nitrogen. However, this would not be possible in the more remote facilities in which much of the present day coconut research and conservation activities take place.

An alternative approach for conserving coconut germplasm would be through the used of a deep (-20°C) or ultra-deep (-80°C) freezer unit. Even if this technique has been employed for many orthodox plant species (11, 22), only a few recalcitrant species have been successfully stored under such conditions.

The present paper reports on an innovative approach enabling the storage of dehydrated coconut zygotic embryos and their recovery from low (-20°C) and ultra low temperatures (-80°C). The applicability of these approaches for the preservation of coconut germplasm is discussed.

**MATERIALS AND METHODS**

**Plant materials**

Coconut (Cocos nucifera L.) zygotic embryos were of Malayan Yellow Dwarf (MYD) genetic origin and were imported into Australia from the Albay Coconut Research Centre in the Philippines. For import, 10 to 12 surface-sterilized embryos were placed into culture tubes (polycarbonate; 3 cm diameter x 10 cm height), each containing 10 mL of a solidified embryo culture medium (18), and sent by international courier. About 12 days after harvest the embryos were received at the University of Queensland and were selected for their apparent good health and uniformity in size and shape. They were then washed, re-surface-sterilized (0.5 % sodium hypochlorite solution for 3 min followed by three rinses with sterile de-ionised water) and used in the subsequent experiments.

**Embryo dehydration and assessment of moisture content**

With the aim of optimising the tissue dehydration technique, batches of embryos (10 embryos per treatment) were placed in open Petri dishes (15 mm height x 90 mm diameter) and covered with 50 mL of a sterile liquid medium (18) containing glucose (3.33 M), glycerol (15%, v/v) and activated charcoal (1 g L⁻¹). Petri dishes were then placed into a laminar air flow hood and embryos were allowed to soak in the dehydration solution for various durations (4, 10, 12, 14, 16, 18, 20 and 24 h). After dehydration, the moisture content of the embryos was determined on both a fresh (FW) and a dry weight (DW) basis (after drying at 103°C for 24 h).

**Low temperature storage and cryopreservation**

In order to estimate the feasibility of coconut preservation at low (-20°C) and ultra-low (-80°C) temperatures, batches (n=20 each) of 16-h dehydrated embryos were individually placed into 2 mL cryo-vials (Techno Plastic Products AG, Trasadingen, Switzerland) and frozen in a deep freezer unit (VX 380 E Thermo Scientific, New South Wales, Australia) set at either -20 ± 3 °C or -80 ± 5°C. A cryopreservation treatment was used as a control.
Dehydrated embryos were placed in cryo-vials, clipped onto a cryocane, and plunged into liquid nitrogen at -196°C. After 1, 2, 3, 4, 26 or 52 weeks (for both -20°C or -80°C storage temperatures) or 24 h (at -196°C), embryos were removed from cold storage, warmed in a water bath (3 min at 40°C) and then assessed for viability. The experiment was repeated three times using embryos from different imported batches.

Recovery and viability testing

The viability of the batches of embryos was determined before and after dehydration, and after cold storage using a germination test as previously described (21). Recovered embryos were transferred into a liquid embryo culture medium, then incubated in the dark (27 ± 1°C) for 4 weeks, and then subcultured onto a solid medium (18) at 27 ± 1°C under a 14 h photoperiod for a further 8 weeks. The embryos were assessed as being viable if they produced roots or shoots, or callus, or when they were able to enlarge. Embryos were identified as being germinated if they formed shoots, roots, or both. The scoring for normal seedling development was undertaken after a further 16-week acclimatisation period in soil under glasshouse conditions.

Thermal analysis

Thermal analysis was performed (TA 2920 DSC unit with a liquid nitrogen cooling system; TA instruments, Newcastle, Delaware, USA), using a previously optimised protocol (21), on the water present in the dehydrated (16, 20 and 24 hours) and non-dehydrated embryos, and during the rewarming process. Tissues (10 - 20 mg) were held (5 min) at 25°C before cooling at 10°C per minute to -140°C, where they were held for 5 min before warming at the same rate to 40°C. The thermograms produced were processed through the DSC Universal Analysis™ 2000 version 3.9A software package, in order to determine the glass transition temperature (T_g), change in heat capacity during the glass transition (ΔC_p) and the melting temperature (T_m) of frozen water during cooling (21).

RESULTS

Dehydration process

Changes in moisture content of the embryos during dehydration (Figure 1), indicate a first phase (the first 10 h of treatment) during which water is lost rapidly from 77.8% FW (or 3.58 g g⁻¹ DW basis) to 39.7% FW (or 0.91 g g⁻¹ DW basis). During a second, slower phase, the embryo moisture content began to reach an equilibrium with the surrounding osmotic solution and thus showed only a further small decrease in moisture content. Overall, the embryo moisture content fell to 25% FW basis (or 0.33 g g⁻¹ DW) after 20 h of dehydration.

The viability of embryos was measured after each step of the dehydration process (Figure 2). The results showed that viability remained high for up to 16 h drying (down to 25.4% FW or 0.38 g g⁻¹ DW moisture content), then it began to decline rapidly. After 16 h of dehydration, the level of viability (84%) was only slightly lower than that observed for non-dehydrated embryos (93%). Not all of these viable embryos could properly undertake the germination process: indeed only 57% did successfully germinate. For dehydration treatments which were longer than 16 h, the embryo viability was found to decline rapidly and after 24 h drying it reached 27% with only 8% of embryos able to germinate. In the case of non-dehydrated embryos, the percentage of embryos which were found able to develop into normal seedlings after acclimatisation was high (67%).
Figure 1. The moisture content (fresh weight basis) of coconut embryos after various periods of dehydration. Each data point is the mean (± SE) from 10 embryos.

Figure 2. The embryo re-growth characteristics following chemical dehydration on a HEC medium supplemented with glucose (3.33 M) and glycerol (15%) for specific periods of time (0 to 24 h). These characteristics were recorded after 12 weeks of recovery. The data show the percentage of viable embryos (A), dead embryos (B), embryos germinating (C) and embryos producing normal seedlings (D). In each bar chart, treatments that are ascribed with different letters are significantly different at p-value ≤ 0.05. The bars are means (± SE) of three replications of 20 embryos.
Performance assessment of low temperature storage

Embryos that had not been dehydrated could not survive after more than 1 week of storage under both the low and the ultralow temperatures. However, 70 to 80% of the embryos which had been previously dehydrated for 16 h and stored under the same conditions remained viable for the first week of storage. The percentage of viable embryos then dropped down to 43% after 3 weeks of storage and 0% after 4 weeks of low temperature (-20°C) storage (Figure 3). The results also demonstrated that storage of dehydrated embryos at -80°C proved more efficient. The percentage of viable embryos was still high (63%) after 26 weeks, but it dropped down to 30% after 52 weeks of storage.

In both treatments, not all of the viable embryos were found able to germinate. For the embryos stored at -20°C for 3 weeks, only 15% of them germinated properly, with a further 28% enlarging and / or producing callus. For the embryos stored at -80°C for 26 weeks, germination rates were high (52%) although not all the germinated embryos did produce normal seedlings after acclimatisation. Indeed, only 12% of the embryos stored at -20°C for 3 weeks produced normal seedlings, while 28% of the embryos stored at -80°C for 26 weeks produced viable plantlets (Figure 4).

Figure 3. The embryos re-growth characteristics following chemical dehydration (16 h) and storage at -20°C (■) or -80°C (□) for 0 to 52 weeks. These characteristics were recorded after 12 weeks of recovery. Data show the percentage of viable embryos (A), dead embryos (B), embryos germinating (C) and embryos producing normal seedlings (D). In each bar chart, treatments that are ascribed with different letters are significantly different at p-value ≤ 0.05. The bars are means (± SE) from three replicates of 20 embryos.
Figure 4. Recovery process for embryos that were chemically dehydrated for 16 h then stored at -80°C for 26 weeks before being recovered. (A) Three normal seedlings and one abnormal seedling (white endosperm with stunted shoot) at 8 weeks after recovery; (B) after 20 weeks the normal seedlings were ready for acclimatisation; (C) the first step of the acclimatisation process; (D) the seedlings ready to be planted into the field after 16 weeks of acclimatisation. In (D) the first three plants from the left were grown from embryos that were recovered from -80°C storage, while the three plants on the right were grown from non-stored embryos; (E) abnormal germination of embryos stored at -80°C for 52 weeks, as seen after 8 weeks recovery; (F) abnormal germination after 16 weeks in the recovery medium.
Suitability of cryopreservation

Using the previously optimised protocol (20, 21) the percentage of viable embryos that could be recovered after cryopreservation was high (60%). This figure included both viable but non-germinating embryos (29%) and germinating embryos (31%). However, only 19% of the cryopreserved embryos produced normal plants that withstood the acclimatisation step (Figure 5).

Figure 5. Various steps in the recovery process of cryopreserved coconut embryos. (A) Three normal and one abnormal seedling following cryopreservation and after 8 weeks recovery; (B) embryos of normal seedlings following 12 weeks of recovery; (C) the normal plants were ready for acclimatisation after 24 weeks of recovery.

Thermal analysis

The DSC thermograms (Figure 6) obtained from embryos which were dehydrated and stored at low temperature (-140°C) and then heated to 40°C show two thermal transitions, the glass transition point ($T_g$ value) and the melting ($T_m$) point of freezable water. The $T_g$ value increased sharply (from -123 to -92°C) when the embryos had been dehydrated for 16 h (with the internal moisture content reduced to 29% FW; see Table 1). The endothermic shift indicated by the $\Delta C_p$ (heat capacity change) value increased 20-fold from the value obtained for non-dried embryos (0.03 J g$^{-1}$ °C$^{-1}$) compared to 16-h dehydrated embryos (0.60 J g$^{-1}$ °C$^{-1}$). $T_m$ values were found to be affected by the dehydration process, as they decreased from -1°C in non-dehydrated embryos to -8°C in 16-h dehydrated embryos. Similarly, the enthalpy of melting was also impacted by the dehydration process. The non-dehydrated embryos (77% FW) showed a highest enthalpy (ca. 275 J g$^{-1}$) which decreased to a fraction of this value (0.3 J g$^{-1}$) after 16 h of dehydration (29% FW). More than 63% of the total water present in the non-dehydrated embryos was estimated to be freezable water, while this amount was decreased to almost zero (0.03%) following dehydration for 16 h. It is worth noticing that the $T_g$ and $\Delta C_p$ values did not increase further when the embryos were dehydrated for longer periods of time (i.e. for 20 h or 24 h treatments). Indeed, these longer dehydration times did not reduce the melted and frozen water component any further.
Figure 6. A detailed section of the DSC thermograms for coconut embryos, showing the glass transition ($T_g$; insert) and the melting of ice crystals ($T_m$) formed in the embryos during thawing process. Embryos were dehydrated using a HEC medium supplemented with 3.33 M glucose and 15 % glycerol for 16 h (-b-), 20 h (-c-) and 24 h (-d-), and were compared to a non-dehydrated embryo (-a-). The curves were shifted slightly downwards to avoid overlap.

Table 1. Physical characteristics of MYD embryos obtained from differential scanning calorimetry analysis during the rewarming process after freezing of embryos following dehydration for different periods of time (0 to 24 h). Each data set is the mean of three embryos ± SE. For each characteristic, the duration of chemical dehydration treatment that was significantly different from others was denoted by an asterisk (*) at $p$-value ≤ 0.05.

<table>
<thead>
<tr>
<th>Physical characteristics</th>
<th>Duration of dehydration (hours)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
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<tr>
<td>Water content (% FW)</td>
<td>77.13 ± 0.55*</td>
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<tr>
<td><strong>Melting</strong></td>
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<tr>
<td>Onset, $T_{m, \text{onset}}$ (°C)</td>
<td>-9.94 ± 0.39*</td>
</tr>
<tr>
<td>Peak, $T_{m, \text{peak}}$ (°C)</td>
<td>-0.84 ± 0.43*</td>
</tr>
<tr>
<td>Melt water (J g$^{-1}$)</td>
<td>275.31 ± 8.11*</td>
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<tr>
<td>Frozen water (% of total)</td>
<td>63.52 ± 2.23*</td>
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<td><strong>Glass transition</strong></td>
<td></td>
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<tr>
<td>Onset, $T_{g, \text{onset}}$ (°C)</td>
<td>-124.08 ± 0.65*</td>
</tr>
<tr>
<td>Glass transition, $T_g$ (°C)</td>
<td>-123.82 ± 0.67*</td>
</tr>
<tr>
<td>End, $T_{g, \text{end}}$ (°C)</td>
<td>-122.77 ± 0.63*</td>
</tr>
<tr>
<td>Change in heat capacity, $\Delta C_p$ (J g$^{-1}$ °C$^{-1}$)</td>
<td>0.03 ± 0.01*</td>
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DISCUSSION

The present study is the first report on the conservation of coconut germplasm (zygotic embryos) at a -80°C temperature and on the recovery of normal plants at reasonable rates (Figure 4). By dehydrating for 16 h and storing the embryos at -80°C for 26 weeks, a high percentage of viable embryos (63%) and a good number of plants, ready for field-planting (28%; Figure 3), could be produced. These results suggest that for medium-term conservation (up to 26 weeks) a standard ultra-deep freezer unit set at -80°C could be used to conserve coconut germplasm. The protocol is simple, offers less risk of contamination and no risk of embryo germination during storage compared to earlier short- to medium-term storage techniques (1, 9, 16, 17). This technique would be useful for remote laboratories that have a constant electrical supply but do not have easy access to liquid nitrogen and therefore cannot undertake cryopreservation. For periods longer than 26 weeks the low temperature technique does not seem to be reliable enough, with a significant loss of embryo viability occurring after this time of storage (Figure 3). One possible explanation for this failure over the longer term might be because the temperature of -80°C is warmer than the Tg observed for the embryos (ca. -93°C, see Table 1 and Figure 6). As a result, the low storage treatment (-80°C) was probably not cold enough to enable the formation of the glassy state in the cytoplasm of the zygotic embryo cells. Hence, the molecular mobility in the cytoplasm of these cells would not have been fully impeded during treatment. As a result, the physical and chemical reactions that promote cell aging would still have been undertaken at this temperature. Consequently, the longevity of the embryos would have been progressively reduced (6, 7, 23), becoming more apparent after 26 weeks of storage.

Storage of embryos at -20°C was much less successful than storage at -80°C. After storage at -20°C no viable embryos could be recovered after more than 3 weeks of storage (Figure 3). The rapid loss of viability was probably caused by the high molecular mobility that was possible in coconut embryo cells at this temperature. The physical and chemical reactions that promote cell aging would still be going on at -20°C (6, 7).

In the case of cryopreservation, and as previously reported (21), the rate of recovery of viable embryos is high (60%) and these embryos have a high capacity to produce normal plants ready for field-planting (19%; Figure 5). It is thought that at the very low temperature utilized by the cryopreservation process all of the physical and chemical reactions that promote cell aging are stopped and viability is retained for long periods of time (6, 7; 23).

The DSC analysis (Figure 6) showed that high amounts of water (up to 64%) melted from ice crystals during the warming process of the non-dehydrated embryos. With the Tg (-123.8 to -87.4°C) of the embryos being far lower than their Tm (-8.5 to -0.8°C) there is a high risk of ice formation and devitrification when applying cryopreservation to non-dehydrated embryos. The reduction of coconut embryo moisture content to 25% FW (0.38 g g⁻¹ DW) by chemical dehydration for 16 hours seems to be the point to which they can be dried before they start dying due to desiccation stress (Figure 1 and 2). Research in the past decades has shown that tissues of most recalcitrant species lose their viability upon dehydration (when dried into the range of 50 to 23% moisture content; depending upon the species) and cannot be stored easily (3). Most reports have classified coconut seeds as recalcitrant (15) and our present study supports this view.

Two points coming from the present study will be important for future research work; viz. 1) not all of the viable embryos after recovery could be made to germinate, and 2) not all of the embryos that germinated could develop into normal seedlings (Figure 3). We observed that 24% of coconut embryos, which were stored at -80°C for 26 weeks, produced abnormal seedlings with either roots only, or shoots only, or with poorly developed shoot and roots (Figure 6). Abnormal seedling production is a common side effect of low temperature
conservation of embryos in many recalcitrant species (4), such as European chestnut (*Castanea sativa* Mill.), for which 40% of the recovered embryos produced seedlings with roots only (8). The best explanation for the tendency of roots to develop normally but for shoot growth to be stunted is that, structurally, the root meristem is more protected by other compact cells, while the shoot meristem is not so protected. Thus, the shoot apical meristems are probably more damaged during the low temperature treatments (4). These observations suggest that research work should be undertaken in the aim of to improving the rate of seedling development and acclimatization.

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**REFERENCES**


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