



Cryopreservation of coconut (*Cocos nucifera* L.) zygotic embryos after pre-growth desiccation

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Abstract

Coconut, being a recalcitrant species with large nuts, cryopreservation is the only option for long-term conservation of coconut genetic resources, which can provide a viable backup to field gene banks. A simple cryopreservation technique involving pre-growth desiccation using sucrose was standardized for coconut zygotic embryos. Zygotic embryos extracted from WCT variety of coconut were pre-grown in 1M, 2M and 3M concentrations of sucrose and thereafter subjected to liquid nitrogen exposure for 24 hours. The results showed that 1M sucrose pre-treatment was not sufficient for dehydration as it resulted in death of the embryos after cryogenic treatment. At 2M and 3M sucrose concentrations, the moisture content of the embryos was reduced to 30% and 27% with the corresponding final recovery of plantlets after cryopreservation of 20.8% and 29%, respectively.

Key words: Coconut zygotic embryos, sucrose, pre-growth desiccation, cryogenic storage

Introduction

A complementary conservation strategy involving both *in situ* and *ex situ* conservation of coconut germplasm is indispensable for its utilization in future breeding programmes. At present coconut germplasm is conserved mainly in field gene banks which are exposed to climatic and biological hazards and are costly to maintain over a large area. Coconut being a recalcitrant species with large nuts, cryopreservation of embryos is the only safe option for long-term conservation of coconut genetic resources to provide a viable backup to field gene banks. This emphasizes the development of a cost effective and easy method of cryopreservation with high plantlet recovery for the long-term conservation of coconut germplasm. Of the various cryopreservation protocols developed for different species, pre-growth desiccation technique is mainly applicable in the case of zygotic (Assy-Bah and Engelman, 1992a,b) and somatic embryos (Dumet *et al.*, 1993). Preliminary studies conducted by Bajaj (1984) and Chin *et al.* (1989) have shown that coconut zygotic embryos are amenable to cryopreservation. Pre-growth, the key step in cryopreservation process allows the intracellular accumulation of sucrose during sucrose incubation which helps in preventing freezing injury. The

present study highlights the effect of sucrose pre-growth for the successful recovery of plantlets after cryopreservation of coconut zygotic embryos.

Materials and Methods

Plant material: Zygotic embryos from 11 month-old West Coast Tall nuts were utilized for the present study. Embryo collection and its surface sterilization was done as per CPCRI embryo culture protocol (Karun *et al.*, 1993)

Pre growth: The pre growth medium consisted of autoclaved sucrose solution at 1M, 2M and 3M concentrations. The embryos were incubated in the sucrose solution for a period of twenty-four hours and thereafter dried in silica gel for seven hours (20 embryos/50g. sterile silica gel in a 250 ml wide mouthed bottle). Each treatment contained 20 embryos and the experiment was replicated thrice. The data was analyzed using SPSS package. The best pre-growth treatments consisting of 2M and 3M sucrose were repeated with 34 embryos/treatment.

Moisture content: Moisture content of the fresh and sucrose-treated embryos (expressed in % of their fresh

weight) was calculated after the pre-treatment period in the respective sucrose solutions.

Cryopreservation: The embryos, after incubation in sucrose solution and subsequent drying in silica gel, were placed in 5 ml screw-capped sterile cryo-vials and directly immersed in liquid nitrogen (-196°C). After 24 hours of cryopreservation in liquid nitrogen, the cryovials were thawed rapidly in a water bath at 40°C for two minutes and the embryos were incubated in Y3 medium for germination. The control treatment consisted of pre-grown embryos without liquid nitrogen treatment and absolute control, with direct inoculation in Y3 medium.

Culture conditions: The cultures were initially kept in dark for germination. After the emergence of shoots of 3-4 cm length the embryos were transferred to illuminated culture room maintained at 27±2°C and RH 70-80 %.

Viability test: Viability of embryos before and after cryopreservation were checked with 1% TTC (Triphenyl tetrazolium chloride) solution at 25 °C for 24 hours. The presence of red colour indicated viability of embryos.

Histology: In order to study the cellular changes during low temperature exposure to liquid nitrogen, the tissues were processed for light microscopic studies by paraffin method. The embryos were fixed in Carnoy's fluid for 24 hours, dehydrated in ethyl alcohol-butanol series, infiltrated with paraffin wax at 60°C in an oven and blocks prepared for microtomy. Sections were cut at 10mm thickness and stained with Toulidine blue.

Results and Discussion

The moisture content of the freshly excised coconut zygotic embryo was found to be 81.98 to 92.46%. In the present study, the embryos incubated in varying concentrations of sucrose (1M, 2M and 3M) with silica gel desiccation (7 hours), showed decreased moisture content with increasing concentration of sucrose incubation (Fig.2). After 24 hours of 3M sucrose incubation, the water content of the embryo was reduced to 27% whereas after 2M and 1M sucrose incubation it was 30% and 37%, respectively. In our previous studies, by simple desiccation of coconut zygotic embryos either in the laminar air flow cabinet (24 hours) or in silica gel (18 hours), the optimum relative water content prior to incubation in liquid nitrogen was around 20% below which the death of shoot meristem occurred (Karun *et al.*, 2005). In oil palm, somatic embryos pre-grown on a medium containing 0.75M sucrose and desiccated in silica gel for 16 hours showed 13 to 53% survival rates with corresponding moisture contents of 28 ±1 and 19 ±2% respectively, after freezing in liquid nitrogen (Dumet *et*

al.,1993). In oil palm zygotic embryos, reduction of moisture content to 10.4% by desiccation inside the laminar air flow and cooling in liquid nitrogen caused no loss of viability (Grout *et al.*, 1983). Sucrose is widely used as a cryoprotective compound for the non-injurious freezing of plant material by cryopreservation (Wang *et al.*, 1998). Sugars, when added before dehydration and low temperature stresses, are known to stabilize membranes and proteins during desiccation (Crow *et al.*, 1990), which would improve recovery following freezing.

The viability of embryos immediately before and after exposure to liquid nitrogen was tested with TTC solution kept at 25°C for 24 hours. The red stained embryos were scored as viable. The viable embryos in culture swelled and protuberance was seen at the plumular region. Normally, the shoot emerges first from this region. In absolute control (without any treatment or exposure to liquid nitrogen), it took 25-35 days for the emergence of plumule whereas in cryopreserved embryos it was further delayed for another 7 to 10 days. After one month of culture in the retrieval medium, browning was intense (64%) in 1M sucrose pre-treated embryos and only very few embryos showed early signs of growth, like size enlargement and weight increment. Initially, 22% of the cultures exhibited plumule emergence and this was taken as germination. There was high incidence of abnormalities (Fig1.) in the case of 1M sucrose pre-treated embryos. In these cultures, the shoot pole either degenerated completely by callus like growth (Fig. 1 A&C) or suppressed inside the embryo (Fig1.D&E). On subsequent subcultures (3-4 months duration) these embryos turned brown and died. This may be the result of partial removal of the freezable water as indicated by the high moisture content (37%) leading to extracellular ice formation and death of tissues (Farrant, 1980).

Browning was less intense in 2M and 3M treated embryos but it did not affect germination. Germination of 41.7% and 25% was observed in 2M and 3M sucrose treated embryos exposed to liquid nitrogen, whereas it was 27.8% each in control embryos for 2M and 3M sucrose treatments without liquid nitrogen exposure, after one month in culture medium. The corresponding moisture content of these embryos (2M- 30% and 3M- 27%, respectively) were relatively high compared to simple desiccation of embryos in laminar air flow chamber or silica gel (Karun *et al.*, 2005). This may be attributed to the intracellular sugar uptake replacing water molecule in the tissues. Sugars may act by substituting for the water in the membrane surface (Crowe and Crowe, 1986). Studies in cabbage petioles and asparagus shoot apex

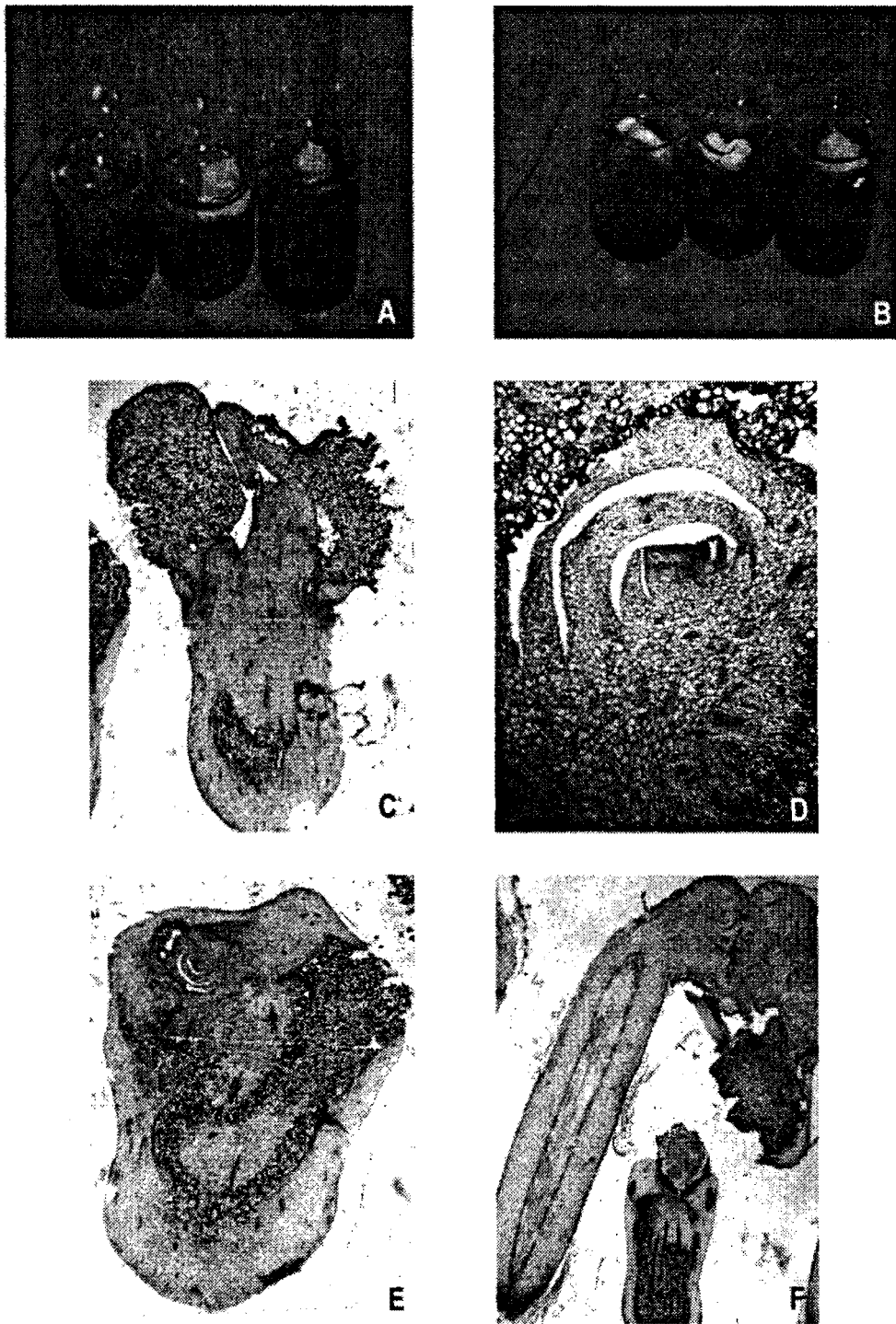


Fig: A to F - Abnormalities in cryopreservation retrieved embryos

A & C : Flacid embryos and callus formation - 1 M Sucrose pre-treatment

D & E : Supressed shoot pole inside the embryo - 1 M Sucrose pre-treatment

B & F : Abnormal root growth supressing the shoot pole - 3 M Sucrose pre-treatment

cells have shown that during incubation in 0.8M sugar solution, sugars are taken up intracellularly by osmotically induced fluid phase endocytotic vesicles or by sugar transporters. The sugars taken up by sugar transporters are mainly responsible for the increased freezing tolerance in cabbage petioles and asparagus shoot apices (Jitsuyama *et al.*, 2001).

After four months, the germination percentage increased to 68.8% and 47.9% in 2M and 3M pre-grown embryos, respectively, whereas it was 61.1% and 55.6% in controls without liquid nitrogen exposure and 100% in absolute control. The Fig. 2 shows the relationship between moisture content and germination percentage of embryos after 4 months in culture. There was not much difference between shoot length in 2M and 3M sucrose treated embryos. Whereas in 3M sucrose treatment, root growth was higher than that of 2M sucrose pre-treatment. The simultaneous development of root along with shoot helped in the higher percentage of pot establishment of plantlets retrieved from 3M sucrose pre-treated embryos after cryopreservation.

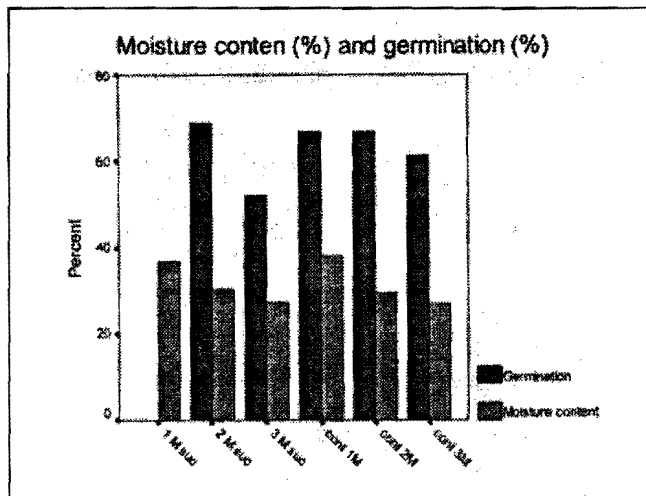


Fig.2. Moisture content and germination percentage of cryopreservation treated and control plantlets

After four months, abnormality in 3M treated embryos was found to be 8.33% and in 2M it was 6.25%. In 3M treated embryos, some cultures showed fast root growth suppressing the initially emerged shoot primordial development (Fig. 1.G). In our previous coconut embryo culture studies also, early development of the roots suppressed shoot growth (Karun *et al.*, 2002). Considering the emergence of plumule as indicative of germination, the initial survival percentage of the embryos was high. But due to slow growth, abnormalities, variability of embryo size and improper shoot: root ratio, complete recovery of plantlets after 10 months in culture reduced

considerably. The slow growth of frozen coconut embryos, which affects the rate of development, was reported by Assy-Bah and Engelmann (1992a). The *ex vitro* development and pot establishment of the cryopreservation retrieved plantlets were similar to those of normal embryo cultured plantlets (Fig.3). Table 1 shows the *in vitro* and *ex vitro* recovery of cryopreservation retrieved plantlets and normal control plantlets. Repetition of the best treatments gave similar results.

The importance of sucrose incubation for acquiring freezing tolerance during cryopreservation has been widely reported (Dumet *et al.*, 1994; Suzuki *et al.*, 1997 and Hitmi *et al.*, 1999). Incubation in high concentration of osmotica such as sucrose induces intracellular sucrose accumulation and changes in cell structure such as plasmolysis in asparagus embryogenic cells (Jitsuyama *et al.*, 2002). It has been reported in wheat suspension culture that plasmolysis of cells incubated in a highly concentrated sucrose solution increased freezing tolerance, reducing protoplast deformation during freezing and preventing structural changes in plasma membrane (Sopina and Samygin, 1993). Loading of sugars promotes plasma membrane stability by reducing close apposition of plasma membrane with intracellular organelle membranes under freezing in asparagus meristematic cells (Fujikawa *et al.*, 1999).

Assy-Bah and Engelmann (1992b) could achieve recovery of plantlets after pretreatment of mature coconut embryos for 4 hour desiccation in the laminar air flow current followed by culture on a medium containing 600g/l glucose and 15% glycerol. In the present study, pre-growth was carried out with high osmoticum such as sucrose solution, which had a protective effect on freezing of tissues. Even though the sugar incubation helps in the acquisition of resistance to desiccation and freezing tolerance in liquid nitrogen, tissues of different plant species have different tolerance thresholds of intracellular sucrose. In banana, Panis *et al.*, (1996) reported that media containing more than 0.3M sucrose retarded growth of the proliferating meristems and caused intense browning of tissues. Sucrose at high concentrations induced an osmotic shock, which caused browning and reduced growth. So sugar concentration in the pre culture medium must be carefully determined. Coconut embryos are comparatively large and it has got a different structural integrity with meristematic pole and haustorium containing vacuolated cells. The concentrations of sucrose used in this study have not shown such deleterious effect on coconut embryos.

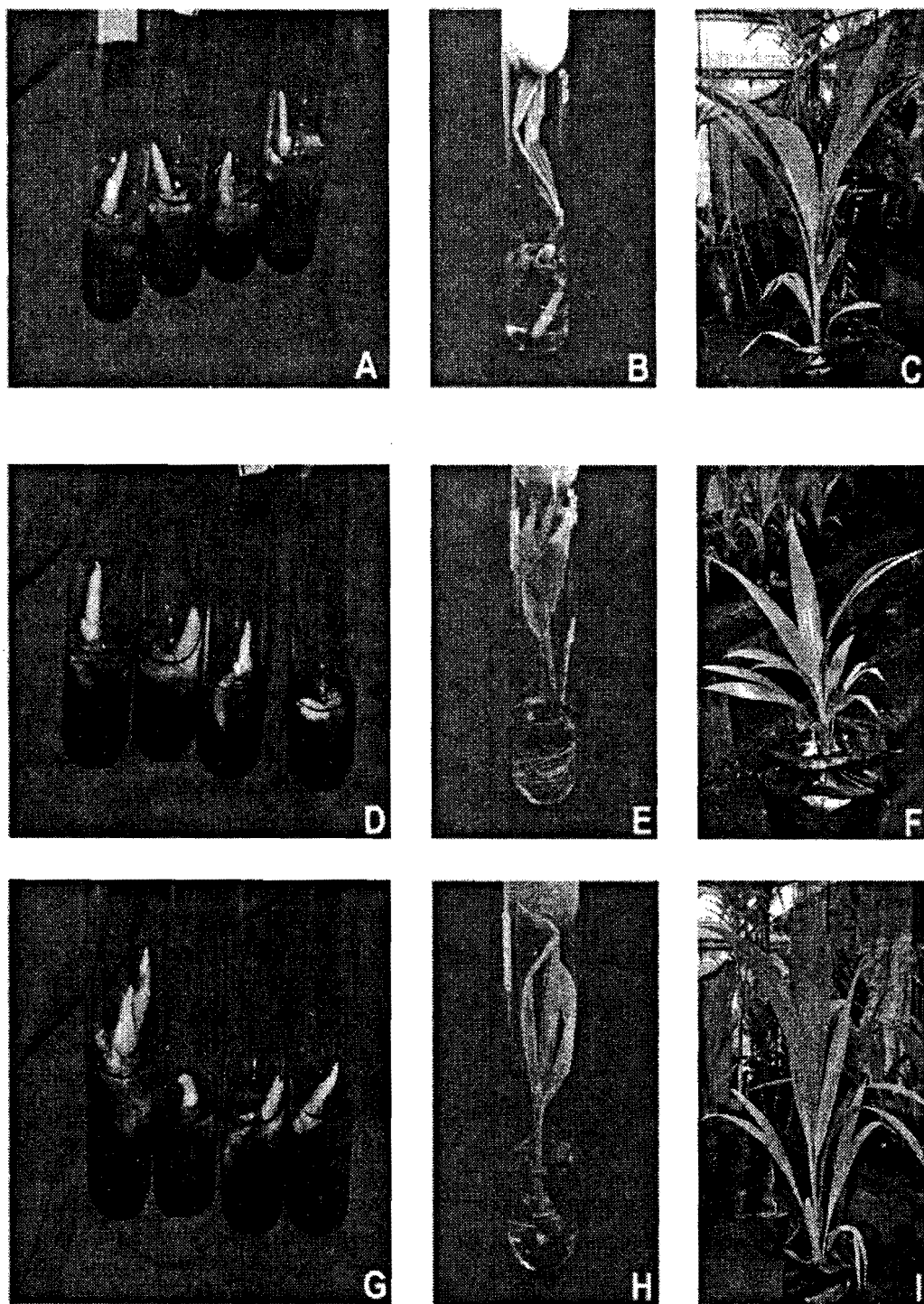


Fig.3. *In vitro* and *ex vitro* establishment of cryopreservation retrieved plantlets

A, B and C: 2 M Sucrose treated cryopreservation retrieved plantlets

D, E and F: 3 M Sucrose treated cryopreservation retrieved plantlets

G, H and I: Absolute control plantlets

Conclusion

The results of the present study shows that, sucrose at 3M concentration can ideally be used for dehydrating coconut zygotic embryos to an optimum moisture content of 27% which resulted in 29% of plantlet recovery in pot culture after cryogenic storage. The highest final recovery of plantlets in the case of 3M treated embryos shows the beneficial effect of sucrose for the pre-treatment of coconut zygotic embryos for cryopreservation without losing its structural integrity and viability. The method employed here is very simple and easy to handle and it can be utilized for the long-term conservation of coconut germplasm *ex situ*.

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