

Cryopreservation of mature coconut embryos by desiccation method

Anitha Karun¹, K.K. Sajini² and V.A. Parthasarathy³

Abstract

Mature embryos of West Coast Tall variety of coconut could be cryopreserved after desiccation pretreatments and retrieved into plantlets. The desiccation pretreatment and optimum duration for dehydration of zygotic embryos were standardized. The maximum retrieval of healthy plantlets was obtained from the embryos subjected to 18 hours silica gel or 24 hours laminar airflow desiccation pretreatment. Irreversible damage caused by desiccation to the death of the shoot meristem was noticed when the water content reduced to 20%.

Key words: Coconut (*Cocos nucifera* L.), cryopreservation, desiccation pretreatment, liquid nitrogen, mature embryos, West Coast Tall.

Abbreviations: **LF** - Laminar flow, **LN** - Liquid nitrogen, **TTC** - 2, 3, 5-triphenyl tetrazolium chloride, **WCT** - West Coast Tall.

¹ Senior Scientist, Biotechnology section CPCRI, Kasaragod-671 124, Kerala.

E-mail: karun_ani@yahoo.co.uk

² Technical officer, Biotechnology Section, CPCRI, Kasaragod-671 124, Kerala.

³ Director, Indian Institute of Spices Research, Marikunnu. P.O, Calicut, Kerala India.

Introduction

Coconut (*Cocos nucifera* L.) germplasm at present is conserved only in the form of field gene banks. Central Plantation Crops Research Institute maintains the world's largest field gene bank of coconut consisting of 189 indigenous and 132 exotic collections. The International Coconut Gene Bank for South Asia (ICG-SA) is also functioning under CPCRI. Conservation of coconut germplasm in *ex situ* requires maintenance of very large area, which is labour intensive and therefore highly expensive. Field gene bank also have the risk of loss of germplasm due to unprecedented incidence of pest/disease or other natural calamities. It is therefore necessary to conserve the coconut germplasm alternatively. The cryopreservation techniques ensure the storage of live tissues in viable state for several years (Engelmann, 2000). The standardization of protocol for retrieval of coconut zygotic embryos (Karun *et al.* 1993; Karun *et al.*, 2002) gives opportunity to conserve the germplasm *in vitro* by means of cryopreservation of zygotic embryos.

Several cryopreservation protocols have been developed for zygotic embryos of many species using various methods of freezing (Engelmann, 1992). Coconut being a recalcitrant species, the method chosen for cryopreservation of germplasm is usually that of rapid or relatively rapid dehydration of zygotic embryos prior to their introduction into liquid nitrogen. Pretreatments are necessary to avoid the lethal damage of embryos by dehydration and subsequent low temperature storage. Normah *et al.* (1986) and Assy-Bah and Engelmann (1992 a, b) reported the successful cryopreservation of coconut zygotic embryos subjected for both high sugar or dehydration techniques. Chin *et al.* (1989) obtained callusing and normal germination of one coconut embryo after 15 months freezing. Zygotic embryos of oil palm are dehydrated in air current of laminar flow cabinet without any cryoprotective treatments (Grout *et al.*, 1983). In this paper a simple method for long term conservation of coconut zygotic embryos by optimizing the condition of desiccation either

under laminar flow cabinet or silica gel before subjecting them to liquid nitrogen (-196°C) is described.

Materials and methods

Cryopreservation of coconut zygotic embryos involves the following components: Collection of embryos aseptically and sterilization; imposing a suitable pretreatment; plunging into liquid nitrogen and finally conservation. To standardize the procedures, mature (11 month after pollination) zygotic embryos (cultivar: West Coast Tall) were used. Embryos were collected as per the methods described by Karun *et al.* (1993) and the fresh weight recorded.

Two types of desiccation pretreatments *viz.*, (1) desiccation using silica gel and (2) air current of laminar flow, were tried. To obtain the optimum duration, embryos after surface sterilization were subjected to pretreatments for various time intervals *viz.*, 1, 2, 3, 5, 18, 24, 30, 48, 54, 60, 72 and 96 hrs. Fifteen embryos per pretreatment were tried at room temperature (28 ±2°C and 80% RH) for each time interval. After respective duration of dehydration, moisture content on fresh weight basis was determined.

The silica gel pretreatment is imposed as follows: 50 g of completely dehydrated silica gel was filled in air-tight bottle of size 250 ml and sterilized for 20 minutes. Fifteen embryos were placed on a filter paper disc kept on the top of the silica gel and subjected to desiccation at room temperature for various time intervals.

In the case of other pretreatment, the sterilized embryos were placed on a sterilized filter paper disc kept on an open petri dish and dehydrated for different time intervals in front of air current of a laminar flow cabinet.

At the end of each time interval of the pretreatments, 13 out of 15 embryos were placed in a 5 ml sterile cryovials (screw capped) and immersed rapidly in liquid nitrogen (-196°C)

container (Dewar flask of 35 L capacity) by plunging the vials for one hour.

Viability tests

To test the viability, embryos were transferred to 1% TTC solution (pH 7.0) and incubated for 4 hrs in dark at 33° C. Viability was determined by counting number of stained embryos. The TTC test was conducted in two stages: before and after immersing the embryos in liquid nitrogen. At the first instance, out of 15 embryos subjected for every pretreatment and duration combinations, two embryos selected at random were subjected to viability test before immersing into liquid nitrogen, and three embryos subjected to TTC test after immersing into liquid nitrogen.

To study the germination and growth of cryopreserved embryos, after taking from the liquid nitrogen, the vials were thawed rapidly by immersing the vial into 40°C water bath for 2 minutes and immediately transferred to retrieval medium and cultures were maintained as per the CPCRI embryo culture protocol (Karun *et al.*, 1993, 1999). Control treatment included with and without desiccation and exposure to LN. The cultures were incubated in dark condition without sub culturing (27±2°C; 70-80% RH). On germination the cultures were transferred to illuminated room for further plantlet development.

Results and discussions

Moisture content on fresh weight basis of embryos varied from 72.46 to 81.98%. High moisture content is a limiting factor of viability when embryos are cryopreserved. The importance of moisture content at the time of LN treatment of embryos is depicted in Fig. 6. It can be seen that cryo-stored embryos having moisture content above 40% did not germinate while retrieving *in vitro*. Similarly moisture content below 20% was also found to be not ideal for cryopreservation of coconut embryos. Among

pretreatments tried, initial dehydration was more in silica gel than laminar flow. Embryos desiccated for short periods (1, 2, 3 and 5 hrs) as well as for more than 48 hrs failed to germinate while retrieving following place marked in LN. However embryos that are not subjected to LN treatment but having moisture content above 20% at the end of pretreatment showed cent percent germination; when the moisture content was below 20%, only few embryos germinated.

Following desiccation during the pretreatment, embryos were shrunken to smaller in size. On inoculating in retrieval medium, embryos that are viable regained their initial size (that is the size prior to desiccation). Slight browning was noticed with viable embryos, which may be due to the external injury when they were subjected to very low temperature (-96°C).

As stated earlier, embryos subjected to pretreatment for 18 to 48 hours and cryopreserved only germinated in the retrieval medium. The pretreatment methods showed differences in percent germination of embryos (Table 1). In the silica gel method, maximum germination and normal plantlet development was obtained for 18 hours duration and with laminar-air-flow method it was for 24 hours duration. The moisture content in the embryos at the end of these two pretreatments was 19.8% and 23.7%, respectively. Irreversible damage caused by desiccation to the death of the shoot meristem was noticed when the water content reduced below 19.7% (Fig.1). From the foregoing it was concluded that the optimum relative water content in coconut zygotic embryos prior to incubation in LN for cryopreservation is around 20%. Similar conditions are observed in cryopreservation of apple shoot tips with sucrose pre culture (Niino, 1993).

Moisture content in the embryos above 20% may lead to freezing injury and death of embryos as observed with shorter desiccation periods (less than 18 hours). Desiccation beyond 24 hrs may cause dehydration injury and death of embryos. However, in the latter case many embryos

showed germination by producing only roots (Fig. 2).

When the embryos were subjected to desiccation beyond 18 hours on silica gel and 24 hours on laminar-air-flow, some of the germinated embryos exhibited damaged shoot meristem (Fig. 2). This abnormality was observed only after 50 days of culture initiation. Similar type of abnormality was reported in cryopreservation trials of other species (Pritchard and Prendergast, 1986 and Pence, 1995). This probably explains the complex nature of the coconut embryo, more specifically, the haustorium which consists of more of parenchymatous tissue with shoot and root meristem. It exhibits differential sensitivity to desiccation on subsequent freezing. In the present study also the shoot meristem damage was observed when the moisture content was reduced below 20% causing desiccation injury. However, reduction in water content is necessary to increase the resistance to cryopreservation. Germination was effected by emergence of plumule and radicle (Fig. 3). *In vitro* retrieved plantlets were transferred to the same basal medium supplemented with NAA (1 mg^{-1}) IBA (5 mg^{-1}) for proper root development (Fig. 4).

Ex vitro establishment

Acclimatization of *in vitro* retrieved plantlets is a critical step, wherein individual plantlet's root portion were treated with carbendazim (1 gm/l) subsequently they were transferred to IBA 1000 ppm solution for one hour each before transferring to pots. Potting mixture consists of sterile sand, soil, and coir dust in equal parts. Humidity control and application of nutrient solution was given to plants as per CPCRI protocol (Karun *et al.*, 1999). The well-established plantlets in pots are shown in Fig. 5.

TTC test

The TTC test conducted prior to LN treatment showed the viability of embryos irrespective of the pre-treatment that they were subjected to. On the other hand the TTC test that followed the LN treatment showed no viability of embryos subjected to pre-treatment for a period up to 5 hours, but cent per cent viability thereafter. Moreover, no difference in viability was noticed among the desiccation methods. Nevertheless, the germination of embryos *in vitro* showed differences as mentioned earlier, especially with pre-treatment duration more than 48 hours. The TTC incubation showed darkly stained root pole and unstained shoot pole. One could not differentiate the root and shoot pole in embryo stage. Therefore, TTC based viability test is unsuitable for rapid determination of coconut embryo viability. Staining nature of embryo in the present study indicates that the root meristem can efficiently withstand the desiccation and freezing compared to shoot meristem.

Replacement of water in silica gel pretreatment method was very quick than laminar airflow dehydration. Similar types of result were reported in various species (Vertucci *et al.*, 1991). But in somatic embryos of oil palm, it was reported that the dehydration with silica gel was slower than that achieved under the laminar air flow (Dumet *et al.*, 1993). An increase in resistance of embryos to desiccation probably attributes to level of ABA in embryo at the time of desiccation (Grey *et al.*, 1987; Kim and Janick, 1989).

From the present study it was observed that silica gel desiccation ensured more reproducible dehydration conditions than those of a laminar flow cabinet. Laminar airflow based dehydration is largely controlled by external factors like speed of the airflow, room humidity etc. Thus reproducibility through this method cannot be assured.

Table 1. Plantlets retrieved after 75 days in silica gel and LF pretreatments

Parameters recorded	Pretreatment duration (hrs)							
	Laminar flow				Silica gel			
	18	24	30	48	18	24	30	48
No. of embryos inoculated	10	10	10	10	10	10	10	10
No. of embryos germinated	6	9	6	6	8	6	6	6
Shoot developed	6	7	5	1	7	3	2	0
Shoot damaged	0	0	2	5	0	3	4	5
Root developed	4	6	5	5	6	5	5	1
Abnormalities*	0	0	2	6	0	0	4	5

*Hyperhydricity, swelling of the embryos

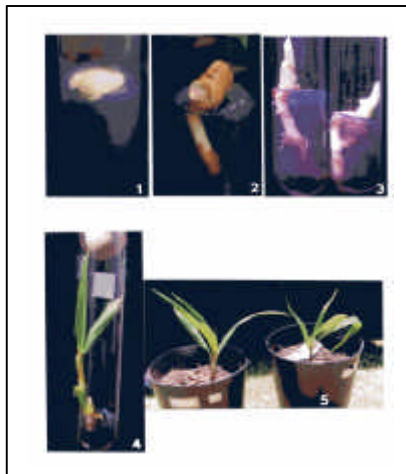


Fig. 1-5 In vitro retrieval of cryopreserved embryos, subjected to desiccation pretreatments

Fig. 1. Germinating embryo showing hyperhydricity due to over desiccation (45 days after inoculation into retrieval medium).

Fig. 2. Damaged shoot meristem of 60 hours desiccation treatment. Note the normal root development. (75 days after inoculation in retrieval medium).

Fig. 3. Normal germination of embryos observed after 18 hours of silica gel desiccation pretreatment. (75 days after inoculation in retrieval medium).

Fig. 4. Plantlet developed from an embryo subjected to 24 hours of laminar flow desiccation (120 days after inoculation into retrieval medium).

Fig. 5. Pot established plantlets from embryos desiccated 18 hours of silica gel and 24 hours LF pretreatment (7 months after inoculation into retrieval medium).

Relationship between germination and moisture content

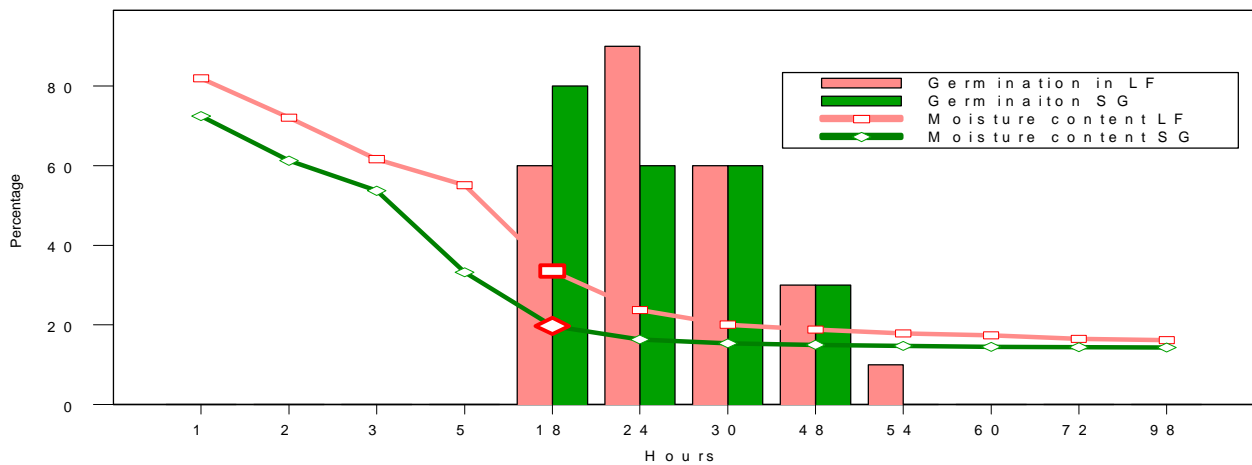


Fig. 6. Relationship between moisture content and germination

The silica gel desiccation technique is simple and less expensive. The recovery rate of embryos of different coconut accessions in the field gene bank maintained by the Central Plantation Crops Research Institute (CPCRI) at the International Coconut Gene Bank for South Asia (ICG-SA) need to be studied before recommending this technique for long term storage of coconut germplasm.

Acknowledgement

Authors wish to thank Director, Central Plantation Crops Research Institute, Kasargod.

References

- Assy-Bah, B. and Engelmann, F. 1992a. Cryopreservation of mature embryos of coconut (*Cocos nucifera* L.) and subsequent regeneration of planlets. *Cryo-Letters* **13**:117-126.
- Assy-Bah, B. and Engelmann, F. 1992b. Cryopreservation of immature embryos of coconut (*Cocos nucifera* L.) *Cryo-Letters* **13**:67-74.
- Chin, H.F., Krishnapillay, B. and Hor, Y.L. 1989. A note on the cryopreservation of embryos from young coconut (*Coconut nucifera* L.) var.*Mawa*. *Pertanika* **12**: 183-186.
- CPCRI, 2000. Annual Report - 2000. Central Plantation Crops Research Institute, Kasaragod. Kerala, India, pp.30-31.
- Dumet, D., Engelmann, F., Chabrillange and Duval, Y. 1993. Cryopreservation of oil palm (*Elaeis guineensis* Jacq) somatic embryos involving a desiccation step. *Plant Cell Reports*: **12**:352-355.
- Engelmann, F. 1992. Cryopreservation of embryos, pp.281-290. In: *Reproductive Biology and Plant Breeding*, V. Dattee, C. Dumas and A. Gallais (Eds.) Springer Verlag Berlin.
- Engelmann, F. 2000. Importance of cryopreservation for conservation of plant genetic resources. In: *Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application*. F. Engelmann and H. Takagi (Eds). JIRCAS, Japan/ IPGRI, Italy.
- Gray, D.J., Conger, B.V, and Songstad, 1987. Desiccated quiescent somatic embryos of orchardgrass for use as synthetic seeds. *In Vitro* **23**:29-33.
- Grout, B.W.W , Shelton, K. and Pritchard, H.W. 1983. Orthodox behaviour of oil palm seed and cryopreservation of the excised embryo for genetic conservation *Ann. Bot.* **52**:381-384.
- Kim, Y.H and Janick, J. 1989 ABA and polyox-encapsulation of high humidity increases survival of desiccated somatic embryos of celery. *Hort. Sci.* **24**:674-676.
- Karun, A., Shivashankar, S., Sajini, K.K. and Saji, K.V. 1993 Field collection and *in vitro* germination of coconut embryos. *J. Plant. Crops* **21** (Suppl.): 291-294.
- Karun, A., Sajini K.K. and Shivashankar, S. 1999. Embryo culture - A CPCRI protocol. *Indian J. Horti.* **56**(4): 348-353.
- Karun, A., Parthasarathy V.A., Kumaran P.M., Iyer, R.D. and Sajini, K.K. 2002. Coconut embryo culture - A protocol for germplasm collection *Tech. Bull.* 45 CPCRI, Kasaragod, India.
- Niino, T. 1993. Cryopreservation of deciduous fruits and mulberry trees. In: *Cryopreservation of Plant Genetic Resources* A. Sakai (Ed) Ref No. 6 FARDA, Tokyo, pp.71-76.
- Normah, M.N., Chin, H.F and Hor, Y.L. 1986. Desiccation and cryopreservation of embryonic axes of *Hevea brasiliensis* Mucl. Arg. *Pertanika* **9**:299-303.

Cord 2004, **20** (2)

Pritchard, H.W. and Prendergast, F.G. 1986. Effect of desiccation on the *in vitro* viability of embryos of the recalcitrant seed species. *Araucaria hunstenii* K. Schum. *J. of Plant Physiol.* **37**:1388-1397.

Pence, V.C. 1995. Cryopreservation of recalcitrant seeds, pp. 29-52. *Biochemistry in agriculture and forestry Vol 32* Cryopreservation of Plant Germplasm I. Bajaj Y.P.S (Ed). Springer Verlag, Berlin.

Vertucci, C.W., Berjack .P., Pammentee N.W. and Cram, T. 1991. Cryopreservation of embryonic axes of an homeohydrous (recalcitrant) seed in relation to calorimetric properties of tissue water. *Cryo-Letters* **12**:339-350.