



Effect of cryopreservation on germination of seeds and zygotic embryos of *Calamus shendurunii*, an endemic rattan of Western Ghats

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Abstract

Calamus shendurunii is an endemic rattan of Western Ghats having restricted distribution and limited population. As a prerequisite to devise an appropriate method for *ex situ* conservation of the species, desiccation and cryopreservation of seeds and zygotic embryo has been studied. Seeds extracted from ripened fruits possessed 35 per cent moisture content and exhibited 97 per cent germination. Desiccation to 28 per cent moisture content reduced the germination to 77 per cent. Desiccation below 14 per cent moisture content caused complete loss of seed germinability. Seeds stored under ambient conditions ($28\pm2^{\circ}\text{C}/60\% \text{ RH}$) for more than seven days reduced germination to less than 40 per cent. Thus, conventional storage is not effective for their *ex situ* conservation. As an alternative method, excised zygotic embryos were subjected to desiccation and storage in liquid nitrogen. The embryos tolerated desiccation down to 5 per cent exhibiting 60 to 90 per cent germination upon culture into MS medium. Desiccated embryos subjected to liquid nitrogen exposure showed post freeze recovery and germination (80-90%) equal to that of desiccated control samples. Thus the study proved the extreme recalcitrance of *C. shendurunii* seeds and embryo cryopreservation as an alternative method of their *ex situ* conservation in gene banks.

Keywords: *Calamus shendurunii*, desiccation, embryo, storage, recalcitrant seeds

Introduction

In recent years, cryopreservation and desiccation procedures have been used for the long term storage of seeds and embryos of many species of plants to establish a gene cryobank. Cryopreservation is regarded as the most efficient tool for long term preservation of germplasm. It is generally understood as the storage of a variety of cells and tissues at a temperature between -79 and -196°C the low extreme being the temperature of the liquid nitrogen (Mycock *et al.*, 1995). The preservation regime has a major advantage that it halts the metabolic activity at the cellular level and in addition to this, it is believed that the technique also can keep the material genetically stable during the period of preservation.

The use of seeds as a major choice of material for conservation has been studied extensively due to its low cost, ease of handling and regeneration of whole plants from genetically diverse materials. However, this is limited to orthodox seeds which can tolerate desiccation and chilling and therefore are amenable to conventional long-term storage at -20°C (Normah and Makeen, 2008). Nevertheless, recalcitrant seeds are sensitive to desiccation and in such cases, isolated embryonic axes have been shown to tolerate partial desiccation and consequent cryopreservation as reported in cocoa, jack, tea, nutmeg, rubber and mango (Chandel *et al.*, 1995). In these species, the excised embryonic axes are reported as tolerant to desiccation and liquid nitrogen exposure better than whole seeds and have been preferred for long-term conservation of the

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respective germplasm in gene banks. Seeds of palms in general (Fki *et al.*, 2011), are recalcitrant in nature while their isolated embryos tolerate liquid nitrogen exposure as proved in coconut, oil palm, arecanut and *Calamus vattayila* (Engelmann *et al.*, 1995; Chabrilange *et al.*, 1997; Sajini *et al.*, 2011; Joemon and Decruse, 2015).

C. shendurunii, the plant species used in the present study is an endemic rattan palm having restricted distribution in Southern region of Western Ghats of Kerala (Anto *et al.*, 2001). Nothing is known about their seed storage behavior. Therefore, a study on desiccation and storage of their seeds as well as embryo cryopreservation has been undertaken in order to devise an effective strategy for long-term preservation of this species.

Materials and methods

Desiccation and storage of seeds

Mature fruits of *C. shendurunii* were collected from their natural habitat in Shendurunii Wild Life Sanctuary, Kollam, Kerala State, India in the month of September. Seeds were extracted through de-pulping and thorough washing in running tap water. The cleaned and blotted seeds were subjected to dehydration under charged silica gel and at ambient conditions (28 ± 2 °C/60-85% RH) for varied duration from 1-35 days. Fresh as well as desiccated seeds were stored for one week at 30, 10 and -10 °C and germination profiles were assessed by incubation in a seed germinator by rolled paper towel method at 30 ± 2 °C/ 80 per cent RH. The germination vigour was calculated as germination percentage per day of completion of germination. Desiccated seeds were exposed to 80 per cent RH for 24 hours prior to germination test to prevent imbibitional injury.

Embryo cryopreservation

Seeds were extracted from partially mature fruits, surface sterilized by immersing in 30 per cent sodium hypochlorite solution for 5 to 8 minutes and washed in sterile distilled water thrice. The seeds were dipped in alcohol and flamed before isolation of embryos under aseptic conditions. The embryos were isolated by removal of opercula cap followed by lifting from base through insertion of a scalpel blade laterally. The isolated embryos were then

treated in 90 per cent alcohol for 30 seconds followed by washing in sterile distilled water. The embryos thus obtained were dehydrated in the laminar air flow chamber for 1 to 8 hours. At hourly intervals, 5 to 7 embryos were transferred to germination medium (MS basal medium) as the desiccation control and another 5 to 7 embryos transferred to 2 mL cryovials and plunged into liquid nitrogen. Desiccated seeds were also exposed to liquid nitrogen to test effect of cryopreservation on whole seeds.

Re-warming and germination assessment

On re-warming, the embryos stored in liquid nitrogen for 1 to 7 days were rapidly transferred to a water bath maintained at 40 °C and kept for 30 s. The embryos were then cultured in agar-gelled MS medium for germination and re-growth. Initially the culture was incubated in the dark for one week and subsequently under illuminated condition. Embryo germination was evaluated over 30 to 60 days and percentage re-growth was calculated.

Moisture content determination

The moisture content of both the seeds and embryos were determined through constant temperature oven method and represented on fresh weight basis (ISTA, 2008).

Results and Discussion

Effect of desiccation on seed germination

Fresh and pure seeds with 33.1 per cent moisture content registered 97 per cent germination. Germination started after 20 days and was completed by the 64th day (Fig. 1). Moisture content of the seeds kept under ambient conditions (28 ± 2 °C /80% RH) was reduced to 6.5 in 35 days. The moisture content was reduced to 28 per cent and seed viability to 77 per cent by one week (Fig. 2). The results suggest that the seeds of *C. shendurunii*



Fig. 1. Germinating seeds of *C. shendurunii* after 20-60 days [L-R]

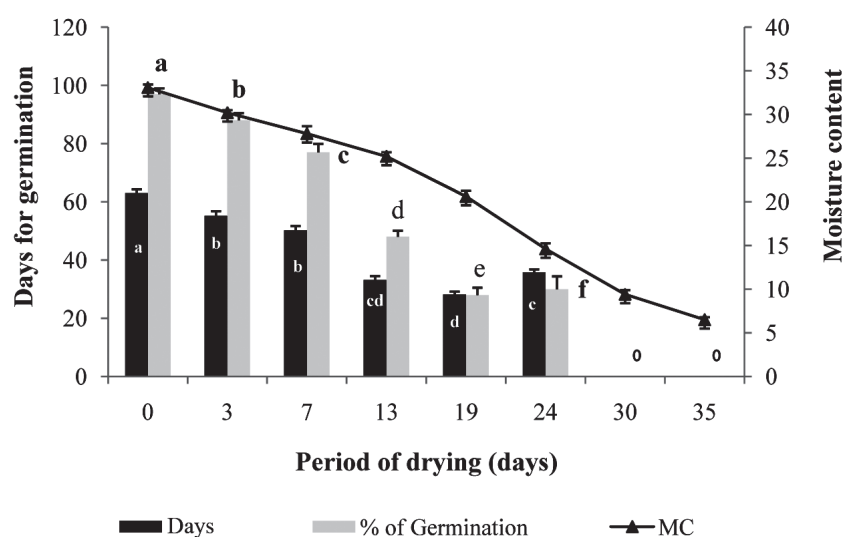


Fig. 2. Effect of desiccation at $28\pm 2^\circ\text{C}/80\%$ RH on moisture content and germination of seeds of *Calamus shendurunii*. Bars= SEM. Different letter in a data series denotes significant difference at 5% level

cannot tolerate desiccation below 27 per cent moisture content and is detrimental below 14.6 per cent (Fig. 2).

Due to forced desiccation, the moisture content was reduced very fast (Fig. 3). The moisture content was reduced drastically beyond five days and reached 9.4 per cent by 15 days when total viability

loss occurred. The desiccation sensitivity thus observed indicate that *C. shendurunii* seeds are highly recalcitrant in nature.

Seeds having 30-33 per cent moisture content stored under 30 and 10 °C showed 60- 70 per cent germination after one week (Table 1). Further reduction in moisture content reduced germination

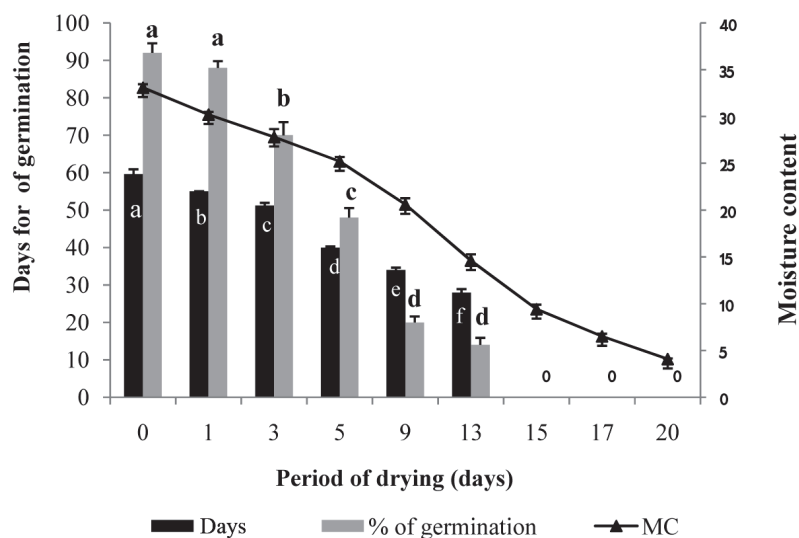


Fig. 3. Effect of fast desiccation under charged silica gel on germination of seeds. Bars = SEM. Different letter in a data series denotes significant difference at 5% level based on LSD multiple 't' test

Table 1. Effect of moisture content and storage temperature on germination after one week storage of seeds

Moisture content (%) ± SE	Germination after one- week storage at different storage temperatures ± SE	
	30 °C	10 °C
33.1 ± 0.37	70 ± 2.9 a	65 ± 1.2 a
30.2 ± 0.30	60 ± 1.6 b	60 ± 1.0 a
27.8 ± 0.86	32 ± 1.2 c	40 ± 1.6 b
25.2 ± 0.48	32 ± 1.2 c	31 ± 1.8 c
20.6 ± 0.67	-	-

Means followed by the same letter in a column do not differ significantly at 5 per cent level based on LSD multiple 't' test

to 31 to 40 per cent on storage at both temperatures. None of the seeds stored at -10 °C germinated showing their chilling sensitive nature.

Desiccation and cryopreservation of excised embryos

The excised embryonic axes possessed 77.8 per cent moisture content and upon desiccation to 2 hours, it was reduced to 11.1 per cent. Desiccation beyond 2 hours did not make any appreciable change in moisture content (Fig. 4). Desiccation to 4.9 to 5.6 per cent moisture content attained after 4-5 hours was observed as the optimum to obtain maximum (90%) survival of liquid nitrogen exposed embryos (Fig. 4 and 5).

Rattan seeds are known to be desiccation sensitive as in the case of *Calamus merrillii* and *C. manillensis*, where desiccation below 40 per cent moisture content was detrimental (Lapis *et al.*, 2004). Therefore, storage under wet conditions was preferred in those cases to keep them viable for a maximum of two months. Similarly, the rattan species from Western Ghats viz., *C. thwaitesii*, *C. pseudotenius*, *C. rotang* and *C. hookerianus* have been observed to lose complete seed viability below 10 per cent moisture content (Renuka, 2003). Nevertheless, seeds of those species packed in air tight bags have been shown to be viable after three months of storage at 5 °C in a refrigerator. The results obtained in *C. shenduruni* suggest that it is more sensitive to moisture loss and chilling. This is due to the large seed size, probably the largest of Western Ghats's rattans. Similar results have been obtained in *C. vattayila*, another endemic rattan palm of Western Ghats, where none of the seeds desiccated below 12 per cent moisture content germinated (Joemon and Decruse, 2015).

Seeds of palms in general, are sensitive to desiccation as known in *Livistona chinensis* (Wen, 2009), *Cocos nucifera* (King and Roberts, 1980), arecanut (Raja *et al.*, 2004), oil palm (Engelman *et al.*, 1995) and *Calamus vattayila* (Joemon and Decruse, 2015). In those cases, zygotic

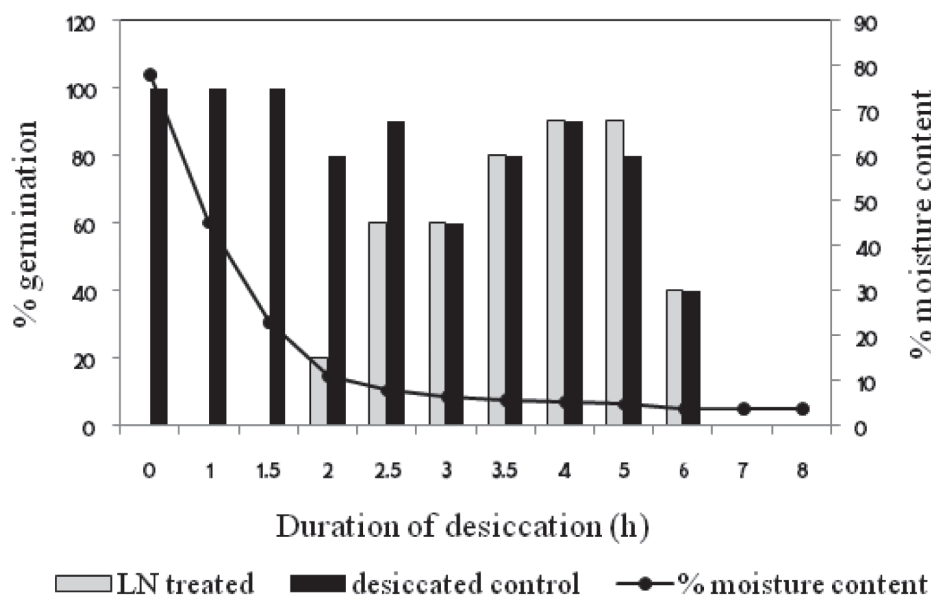


Fig. 4. Effect of desiccation and liquid nitrogen exposure on germination of embryos

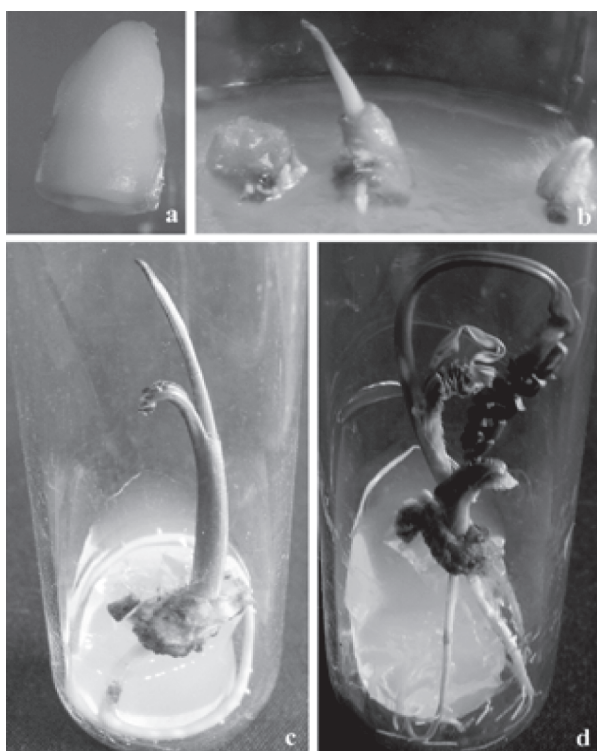


Fig. 5. Plant regeneration from cryopreserved embryos of *C. shendurunii*. a) Isolated zygotic embryo; b) Cryopreserved embryos showing recovery; c) & d) Plantlets developed from cryopreserved embryos

embryo has been proved as an alternative strategy for gene bank storage. However, zygotic embryo at different developmental stages is known to behave differently to desiccation and liquid nitrogen exposure (Shao *et al.*, 2009; Joemon and Decruse, 2015). The fruits of *C. shendurunii*, harvested in July, were far behind the ripening stage (which usually happens in September). Therefore, these fruits were considered partially mature. The excised embryos possessing 77.8 per cent moisture content and undergoing desiccation very fast to reach 11.1 per cent by 2 hours indicated that they were not at mature stage. Moisture content of 4.9 to 5.6 per cent attained after 4 to 5 hours supporting optimum recovery (90%) after liquid nitrogen exposure suggests that in contrast to seed, the embryos are highly tolerant to desiccation. However, why their embryos isolated from ripened seeds failed to tolerate desiccation as that of partially mature embryos (data not provided) is not clear. It is reported in other palm species *Archontophoenix alexandrae*, desiccation tolerance

and liquid nitrogen tolerance increased with maturity of embryos from 146 to 174 days after flowering followed by a decline (Shao *et al.*, 2009). They assumed that the embryos become physiologically mature by 174 days. Dry matter accumulation is shown to be responsible for desiccation tolerance of oil palm immature embryos. However, whether such factors are involved in *C. shendurunii* is yet to be investigated.

In conclusion, embryos excised from partially mature seeds tolerate desiccation and liquid nitrogen exposure, and displayed embryo germination and seedling development. Thus embryos isolated from fruits, 30 to 45 days before ripening is ideal for gene bank storage as seeds are sensitive to desiccation and storage.

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References

- Anto, P.V., Renuka, C. and Sreekumar, V.B. 2001. *Calamus shendurunii* a new species of Arecaceae from Kerala, India. *Rheedea* II (1): 37-39.
- Chabrillange, N., Aberlenc-Bertossi, F., Engelman, F. and Duval, Y. 1997. Effect of oligosaccharide content during maturation on tolerance to desiccation and cryopreservation of oil palm zygotic embryos. *Cryo-Letters* **18**: 68.
- Chandel, K.P.S., Chaudhury, R., Radhamani, J. and Malik, S.K. 1995. Desiccation and freezing sensitivity in recalcitrant seeds of tea, cocoa, and jackfruit. *Annals of Botany* **76**: 443-450.
- Engelmann, F., Chabrillange, N., Dussert, S. and Duval, Y. 1995. Cryopreservation of zygotic embryos and kernels of oil palm (*Elaeis guineensis*). *Seed Science Research* **5**: 81-86.
- Fki, L., Bouaziz, N., Sahnoun, N., Swennen, R., Drira, N. and Panis, B. 2011. Palm cryobanking. *Cryo Letters* **32**: 451-462.
- International Seed Testing Association (ISTA). 2008. International rules for seed testing. Published by International Rules for Seed Testing Association, Zurich, Switzerland.
- Joemon, J. and Decruse, S.W. 2015. Zygotic embryo cryopreservation of *Calamus vattayila* Renuka, an endangered rattan palm of India, as influenced by seed maturity. *CryoLetters* **36**: 299-307.

- King, M.W. and Roberts, E.H. 1980. Maintenance of recalcitrant crop seeds in storage In: *Recalcitrant Crop Seeds*. (Eds.) Chin, H.F. and Roberts, E.H. Tropical Press SDN. BDH. Kuala Lumpur, Malaysia. pp. 53-89.
- Lapis, A.B., Decipulo, M.S. and Salazar, A.M. 2003. Rattan Nursery and Planting Stock Production. IITO-Philippines., 18p.
- Mycock, D.J, Smith, J.W and Berjack, P. 1995. Cryopreservation of somatic embryos of four species with and without cryoprotectant pre-treatment. *Annals of Botany* **75**: 331-336.
- Normah, M.N. and Makeem, A.M. 2008. Cryopreservation of excised embryos and embryonic axes. In: *Plant Cryopreservation- A Practical Guide*. (Ed.) Reed, R.M. Springer, New York. pp. 211-215.
- Raja, K., Selvaraju, P. and Palanisamy, V. 2004. Desiccation and cryopreservation of recalcitrant arecanut (*Areca catechu* L.) embryos. *FAO/IPGRI Plant Genetic Resources Newsletter* **133**: 16-18.
- Renuka, C. (2003). Maintenance of seed stands and species trial plots of rattans. *KFRI Research Report*, No. 254. pp. 29-30.
- Sajini, K.K, Anitha Karun, Amarnath, C.H and Engelmann, F. 2011. Cryopreservation of coconut (*Cocos nucifera* L.) zygotic embryos by vitrification. *CryoLetters* **32**: 317-328.
- Shao, Y.T., Yin, S.H. and Wang, Z.J. 2009. Desiccation tolerance and cryopreservation of *Archontophoenix alexandrae* excised embryos at different developmental stages. *Seed Science and Technology* **37**: 147-160.
- Wen, B. 2009. Storage of recalcitrant seeds: A case study of the Chinese fan palm, *Livistona chinensis*. *Seed Science and Technology* **37**: 167-179.