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Cryopreservation of Arecanut (*Areca catechu* L.) Embryogenic Calli by V Cryo-plate Method

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AK, MKR, KSM and KP conceived the research idea and designed the experiments. Authors KSM, KKS, PK, KP, AAS, MKR and AK executed the experimental works and data analysis, authors KSM, KKS and KP wrote the initial draft of the manuscript. MKR and AK reviewed and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Arecanut, a perennial palm species of Arecaceae family, has huge commercial value, and is grown mainly for its masticatory nuts. The ever-increasing demand for uniform quality plantlets from growers necessitates putting in place *In vitro* mass multiplication and other crop improvement programmes. The present study was carried out to standardize the procedure for cryopreservation of embryogenic calli of arecanut, derived from immature inflorescence cultures, by vitrification based cryo-plate technique.
Study Design: Completely randomized design (CRD) with three replications.
Place and Duration of Study: ICAR-Central Plantation Crops Research Institute, Kerala, India during 2019.
Methodology: The embryogenic calli were precultured in Eeuwen's Y3 basal medium supplemented with sucrose (0.2, 0.3 and 0.4 M) for three days. Explants were affixed on cryo-plates and later dehydrated using plant vitrification solution 3 (PVS3) for 30 min. Cryoplates were

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inserted in cryovials and cryopreserved. Explants with no cryostorage served as control. Explants were rewarmed quickly in a water bath (40ºC) for 2 min and treated with unloading solution and cultured on recovery medium.

Results: The results showed 8-10 % recovery of embryogenic calli that resulted in normal plantlet production. The clonal fidelity studies, using Start Codon Targeted (SCoT) marker, showed no variation of cryopreserved calli in comparison to the original calli.

Conclusion: This preliminary study demonstrated the successful use of vitrification (V) cryo-plate technique in cryopreservation of embryogenic calli of arecanut. With better recovery percentage, the optimal concentration of sucrose in the preculture medium was found to be 0.3 M. Desiccation in PVS3 solution for 30 min had no adverse effect.

Keywords: Arecanut; cryopreservation; embryogenic calli; PVS3; V cryo-plate.

ABBREVIATIONS

BAP : Benzyl amino purine
IAA : Indole acetic acid
NAA : α-Naphthalene Acetic Acid
PVS3 : Plant Vitrification Solution 3
SCoT : Start Codon Targeted
V cryo-plate : Vitrification using cryo-plate
Y3 medium : Eeuwen's Y3 Basal Medium

1. INTRODUCTION

Arecanut (*Areca catechu* L.; Arecaceae) is commonly referred to as betel nut and grows in the tropical Pacific, Asia, and parts of East Africa. The genus *Areca* consists of 76 species of which *Areca catechu* is the only commercially cultivated species [1]. In India, arecanut is mainly cultivated in the states of Assam, Karnataka, Kerala, Maharashtra, Tamil Nadu, Goa, West Bengal and Tripura. Being a high valued commercial crop, its contribution in terms of livelihood, employment and income to the national economy is significant [2]. Genetic diversity in arecanut is generally maintained as live collections in field gene banks [3]. In India, arecanut germplasm is mainly maintained by ICAR- Central Plantation Crops Research Institute, Regional Station, at Vittal, Karnataka State [3]. This research station maintains the largest collection of arecanut (176 accessions) in the world [4].

As a perennial tree crop, arecanut is normally propagated through seeds. The natural population resulting from cross-pollination is highly heterozygous, leading to many variations in yield characteristics of the palm. Through the intervention of tissue culture, it is possible to multiply arecanut palms with desirable characteristics like high yield and disease resistance [5-7]. Maintenance of embryogenic potential of the tissue is usually achieved by passage of cultures in medium with high to a low concentration of auxins. However, handling a large *In vitro* collection of callus lines is cumbersome as it requires regular subculturing into the same medium; besides, contamination risks [8]. As a result, the embryogenic potential may get reduced with time.

To overcome these difficulties, cryopreservation technology offers long term storage of tissues and maintaining them in their original forms without any genetic modification or variation [9]. Cryopreservation involves storing of tissues in liquid nitrogen (-196ºC), to stop all metabolic activities in explants that were passed through preculturing, loading and desiccation treatments [10]. Cryopreservation technology emerged during 1980's, and its advent has resulted in the development of cryogene banks for several plant species especially vegetatively propagated plants or plants possessing recalcitrant seeds [11-15].

The success of this technique depends on the recovery percentage of cryostored tissues [10], and research has been conducted to develop such protocols for several crop species [16]. Efficient use of V cryo-plates, with very high regrowth after cryopreservation, has been reported for various plants [17-22]. However, there is dearth of knowledge on the potential application of this technique in arecanut. Simplicity and user-friendliness associated with this technique make it more preferable. Also, a very high cooling and rewarming rates of treated samples in V cryo-plate technique alleviate freezing damage to a large extent [16].

The present study was carried out to develop a protocol for cryopreservation of embryogenic calli of arecanut using the V cryo-plate method for practical use in long term maintenance of its embryogenic potential.

2. MATERIALS AND METHODS

2.1 Establishment of *In vitro* Cultures

Embryogenic callus, originating from immature inflorescence cultures, were used as initial explant for the cryopreservation (Fig. 1A). Callus from immature inflorescence of arecanut (Hirehalli Dwarf, *Areca catechu* L.) were produced through inoculation of rachille bits on Y3 medium supplemented with picloram (200 µM), following the standardized protocol [5]. Whitish or transparent and granular type calli were considered to be embryogenic. Explants were precultured in three different semi-solid Y3 medium (agar 6.5 g l^{-1}) supplemented with 0.2, 0.3 or 0.4 M sucrose. The preculture medium was also supplemented with activated charcoal (1 g l^{-1}). Explants were incubated for three days in the preculture medium at room temperature in dark condition.

2.2 Pre-growth, Vitrification and Cryopreservation of Embryogenic Callus

Equal sized embryogenic calli, precultured on 0.2, 0.3 and 0.4 M sucrose medium, were placed in each groove of cryo-plate (Fig. 1B, C). Explants were encapsulated by pouring sodium alginate solution (3%) into each groove of the cryo-plate carefully, and flooding the cryo-plate with 0.01 M calcium chloride solution at room temperature (Fig. 1D). After 15 minutes, the cryo-plates, along with encapsulated explants, were treated with loading solution (Y3 medium with 2M glycerine and 1M sucrose) for 30 min at room temperature. The cryo-plates with encapsulated explants were then treated in PVS3 solution (Y3 + 50% glycerine+ 50% sucrose) in test tubes and incubated for 30 min at room temperature (Fig. 1E). The cryo-plates were then placed in cryovials filled with fresh PVS3 solution and immersed in liquid nitrogen container (+LN) (Fig. 1F) for a minimum period of 24 h. Explants with no cryostorage served as control.

2.3 Rewarming and Post-cryopreservation Recovery

Samples stored in liquid nitrogen containers (+LN), were taken out and then rewarmed quickly in a water bath (40ºC) for 2 min at room temperature. The cryo-plates with explants (+LN/-LN) were placed in the unloading solution (Y3 medium with 1.2 M sucrose) for 45 min by changing the unloading solution every 15 min at room temperature. Sodium alginate gel was removed carefully from the explants and inoculated onto recovery medium (Y3 + sucrose 60 g l^{-1} + activated charcoal 1g l^{-1} + agar 6.5g l^{-1}). After incubation for two weeks, explants were transferred either to callus multiplication medium (Y3 + picloram 2.5 µM + sucrose 30 g l^{-1} + activated charcoal 1g l^{-1} + agar 6.5 g l^{-1}) or to the medium devoid of picloram. Observations were taken on the rate of survival (any sign of growth), recovery growth (multiplication and regeneration) and mortality after cryopreservation are compared with the control. After the formation of somatic embryos, germination and plantlet development was carried out using a standardized protocol [5]. Genetic fidelity test in the cryopreserved sample was carried out using SCoT 13 marker (5'- ACGACATGGCGACCATCG -3') following the method of Rajesh et al. [23] compared with control.

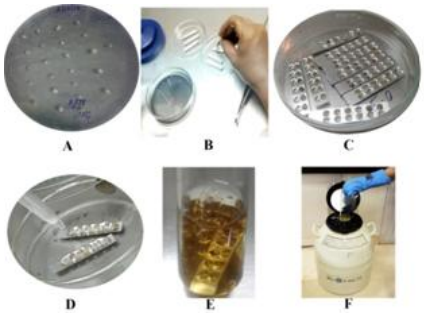

Fig. 1. Preparation of embryogenic callus explants. A) Arecanut embryogenic calli generated from immature inflorescence culture. B) Fixing the embryogenic calli explants on cryo-plate. C) Cryo-plates accommodated completely with the explants. D) Encapsulation using sodium alginate (3%) and calcium chloride (0.01 M) solutions. E) Dehydration in PVS3 solution. F) Plunging the cryovials containing cryo-plates in liquid nitrogen

A completely randomized design was followed with three replicates per treatment. Significant differences in the treatment means were picked up by Duncan's multiple range test [24] (SPSS v 16) at P ≤ 0.05 level.

3. RESULTS AND DISCUSSION

Cryopreservation offers a solution to long-term maintenance of embryogenic potential of callus, which otherwise would deteriorate over a period of time in culture medium. User friendly handling methods makes a cryopreservation protocol efficient and involves the optimization of preculture conditions and vitrification procedures. In the present study, embryogenic callus obtained from immature inflorescence explants of arecanut, were precultured for a duration of three days in Y3 medium with 0.2, 0.3 and 0.4 M sucrose. The precultured explants were then subjected to PVS3 solution for 30 min after affixing in aluminum cryo-plates and exposing to loading solution. Post cryopreservation growth (a lag period of 30 to 45 days) was observed in the cryopreserved samples (+LN) as compared to control (-LN) for any visible sign of growth. Rate of survival (%) was found to be highest in samples precultured in 0.2 M sucrose medium as compared to 0.3 or 0.4 M. On the other hand mortality (%) was highest in tissues precultured in 0.4 M sucrose medium. Initially, friable white masses were seen on the cryopreserved tissues, which later multiplied in the medium containing a low concentration of picloram (2.5 µM). Even though survival of tissues was found to be highest in 0.2 M sucrose treatment, the growth recovery was found to be highest in 0.3 M sucrose treatment (Figs. 2 and 3). Preculturing of the tissues on medium with high sucrose is of great significance as it prepares the tissues for cryopreservation and leads to better post cryopreservation recovery [17]. Optimization of pre-conditioning of explants would provide tolerance to the cryoprotectant solution which has been considered as crucial in enhancing recovery percentage [25]. However, sucrose concentration above the optimal level causes adverse effects in tissues [26]. Similarly, we have observed a higher mortality rate in 0.4 M sucrose concentration, probably due to higher desiccation and membrane damage. Vitrification procedure generally involves treatment of the explants in vitrification solution for a duration of 30 to 90 min [27]. In the present work, it is apparent that 30 minutes of exposure to PVS3 solution could protect the explants during vitrification and devitrification processes and assisted in their recovery and regeneration. A cryoprotectant solution, which comprises of a mixture of chemicals, has the capacity to vitrify rapidly in liquid nitrogen in comparison to a cryoprotectant solution comprising of a single chemical [28]. In the present study, PVS3 (a mixture of sucrose and glycerine in equal parts) was found to aid survivability of the explants during cryopreservation. Vitrification using PVS3 solution has been earlier reported to enhance survival and regeneration in coconut zygotic embryos after cryopreservation [14].

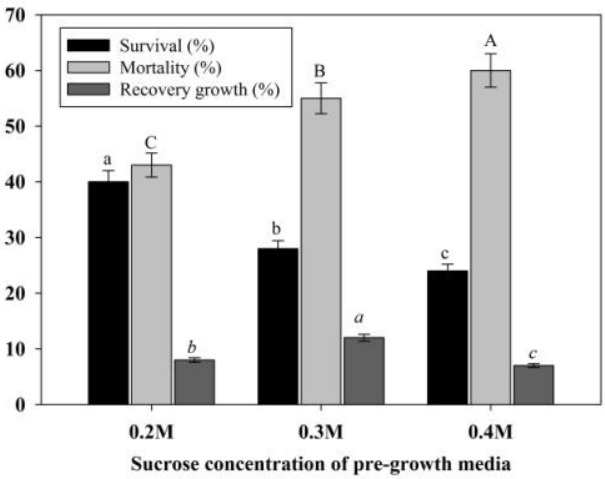

Fig. 2. Effect of sucrose concentrations (0.2, 0.3 or 0.4 M) in preculture medium on survival rate (%), mortality (%) and recovery growth (%) in cryopreserved (+LN) embryogenic calli using V cryo-plate method in *Areca catechu* L. Different alphabets for a parameter indicate significant difference at *P*=0.05 level

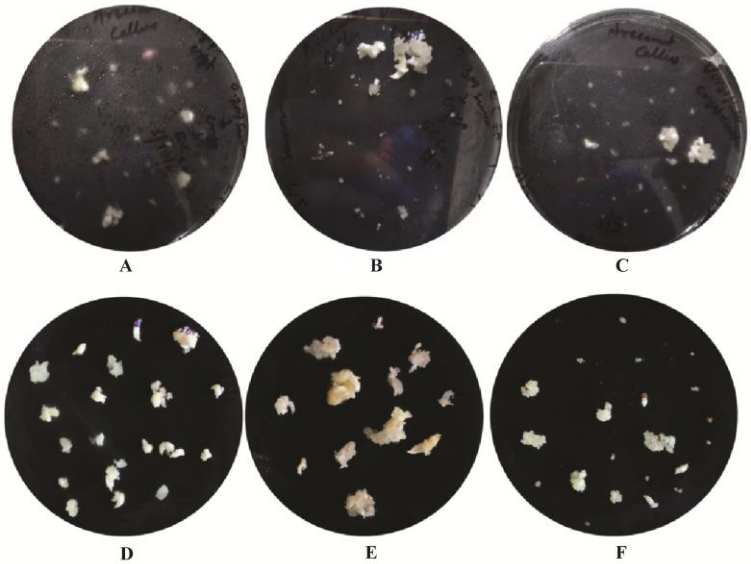

Fig. 3. Recovery and multiplication of the cryopreserved embryogenic calli of arecanut using V cryo-plate method. Recovery and multiplication of cryopreserved embryogenic calli which were precultured in Y3 medium supplemented with 0.2 M (A and D), 0.3 M (B and E) and 0.4 M (C and F) sucrose for three days

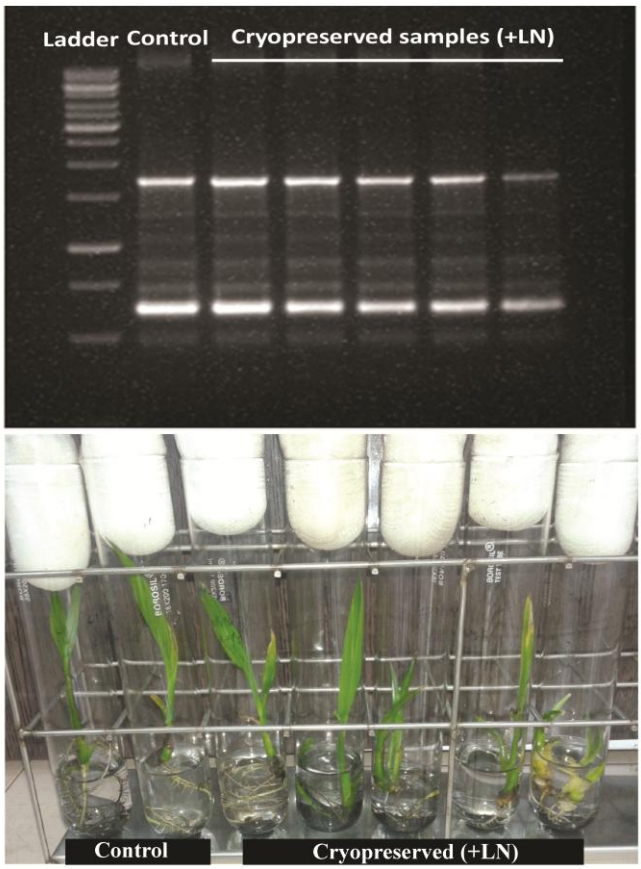

Fig. 4. Comparison of genetic fidelity in cryopreserved embryogenic calli (+LN) with non-cryopreserved counterparts (control) using SCoT 13 (ACGACATGGCGACCATCG) marker (top) and *in vitro* regeneration of arecanut plantlets from cryopreserved embryogenic calli (bottom)

Genetic fidelity studies of cryopreserved (+LN) and non-cryopreserved (control) embryogenic calli using SCoT 13 marker revealed uniformity among these cultures (Fig. 4). Some of the calli, which were cultured in the regeneration medium, resulted in the formation of somatic embryos and meristemoids. Normal development of plantlets was observed from somatic embryo and meristemoids when they were transferred in medium containing BAP (4 mg l^{-1}). Normal root development was observed in the medium supplemented with NAA (2 mg l^{-1}), IAA (2 mg l^{-1}) and BAP (2 mg l^{-1}) (Fig. 4). The results obtained in arecanut corroborate with that of strawberry [20], mulberry [21] and sugarcane [17].

The V cryo-plate method seems to be a promising technique to cryopreserve tissues as it is easy to handle, and its unique structure offers speedy cooling and rewarming of the explants. In the case of arecanut, the embryogenic calli masses could be cryopreserved conveniently using this technique which otherwise would be difficult to handle. The V cryo-plate technique, thus, ensures the retention of the embryogenic potential of the callus for a prolonged period and also need-based supply on the requirement. This technique can also be utilized to cryopreserve somatic embryos of arecanut.

4. CONCLUSION

In the current study development of a robust protocol for cryopreservation of embryogenic calli of arecanut using the V cryo-plate method aids the long-term maintenance of embryogenic potential of arecanut calli that could be exploited for conservation. Future studies should incorporate more numbers of re-growth treatments, its duration and vitrification periods for further refinement of the protocol.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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