International Journal of Innovative Horticulture. 6(1):79-83, 2017 **Original Article**

Immature embryo culture in wild *Areca* spp.

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ABSTRACT

Areca triandra and *Areca concinna,* wild relatives of cultivated arecanut, *Areca catechu* L., are possible candidates to develop disease resistant inter-specific hybrids of arecanut, particularly against *Phytophthora meadii,* which causes fruit rot of arecanut. Inter-specific hybrids often results in premature embryo abortion and fruit fall. A protocol to extract and culture of immature embryos, thus, is of immense significance. Maturation stage of the zygotic embryo and composition of culture media can influence the efficiency of protocol for raising plantlets *in vitro*. In this study, immature embryos (four months old) were extracted from nuts of *A. triandra* and *A. concinna* under sterile conditions were cultured onto four different basal media *viz*., *Y3*, MS, B5 and White. All basal media were supplemented with 3 % sucrose and 0.1 % charcoal. Germination of the zygotic embryos was found to be initiated after three weeks of culturing. Germination percentage was more in *A. triandra* as compared to *A. concinna*. *Y3 medium was found to be the best basal medium as indicated by higher germination both in A. triandra* and *A. concinna.*

Keywords: *Areca catechu*, *Areca concinna, Areca triandra,* challenge inoculation, embryo culture.

INTRODUCTION

Arecanut (*Areca catechu* L.; 2n = 32), commonly referred as betel nut, is an extremely popular masticatory in India and other Asian countries. It is the only cultivated species of the genus *Areca* which comprises of 76 species (Murthy and Bavappa, 1960). India ranks first in the world for production of arecanut; cultivation is mainly confined to the states of Assam, Karnataka, Kerala, Maharashtra, Tamilnadu, Goa, West Bengal and Tripura (FAO, 2014). No fossil remains of the genus *Areca* is known to exist and there are no definitive records of the origin of arecanut palm (Prabhakaran Nair, 2010). The maximum diversity of species, in addition to various additional indicators, implies that the original habitat is in the contiguous regions of Malaya Celebes and Borneo (Bavappa, 1963; Raghavan, 1957). *Areca triandra* Roxb. and *Areca concinna* Thwaites are considered as wild relatives of cultivated arecanut (*Areca catechu* L.) (Fig. 1). Both *A. triandra* and *A. concinna* are also used as masticatory similar to *Areca catechu* (Murthy and Pillai, 1982), though to a limited extent. India and Sri Lanka are the main regions of geographical distribution of *Areca triandra* and *Areca concinna*, respectively (Prabhakaran Nair, 2010). In contrast to *A. catechu*, both these species have suckering properties. Bavappa (1974) had carried out extensive research work on *Areca triandra* and recorded four different ecotypes.

The damage caused by *Phytophthora meadii* on *Areca catechu* remains an important concern. The pathogen causes crown rot and fruit rot (*Mahali*) in arecanut and results in significant yield losses. Farmers take up prophylactic measures to control the disease through spraying Bordeaux mixture. However the disease could become severe during South West monsoon, if timely spraying is not undertaken, leading to heavy yield losses. Developing an arecanut variety having resistance to *P. meadii* would be, therefore, of great significance. Unfortunately none of the cultivars of *A. catechu* has shown resistance to *P. meadii*. Challenge inoculation on harvested immature nuts of *A. concinna* and *A. triandra* showed their resistance to *P. meadii* (Prathibha *et al*., 2015).

Embryo rescue is a form of *in vitro* culture techniques by which non-viable embryos could be turned to viable (Sage *et al*., 2010). Interspecific hybrids of wild *Areca* spp. with cultivated *A. catechu* could lead to a genotype with possible resistance to *P. meadii.* One of the main problems faced in interspecific crosses is the higher abortion rate of embryos. In such situations, the embryos usually have to be rescued following a proper *in vitro* procedure for its rescue. The

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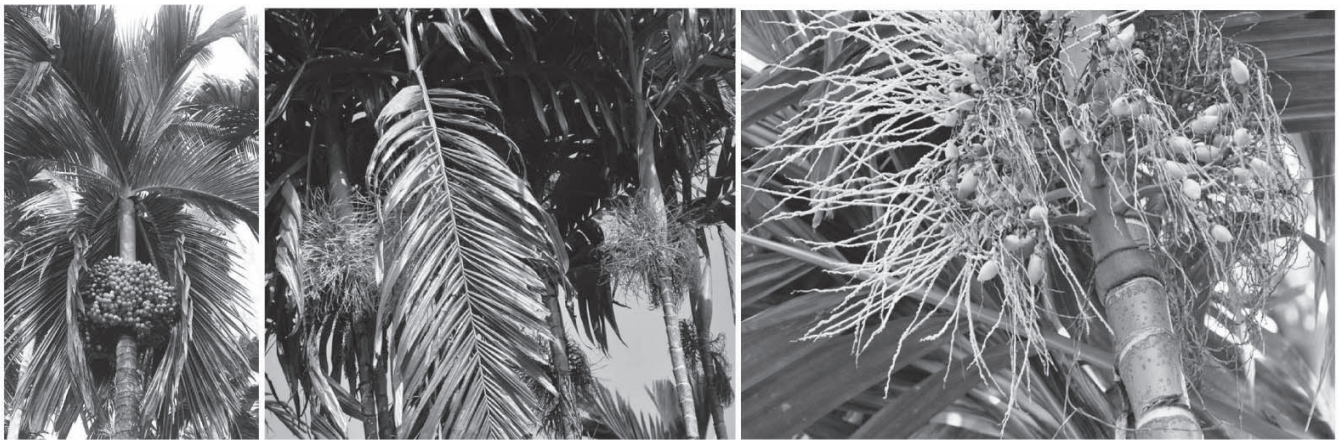

Fig. 1: View of mature bearing palms of (a) *Areca catechu, (b) Areca concinna* and (c) *Areca triandra*

most widely used embryo rescue procedure involves excising plant embryos and placing them onto artificial culture media (Miyajuma, 2006). Embryo rescue has been successfully applied in plant breeding for raising hybrids in many plant species (Iyer and Subramanyam, 1971; Sharma *et al*., 1996) including makapuno coconut (Assy Bah *et al*., 1987; Rhillo and Paloma, 1992) and interspecific hybrids of palms (Tzec-Simá *et al*., 2006; Alves *et al*., 2011; Angelo *et al*., 2011). With this background, the aim of the present study was to establish a protocol for isolation, sterilization of zygotic embryo from immature nuts of *A. concinna* and *A. triandra* and *in vitro* plant recovery.

METHODOLOGY

Sample collection, sterilization and inoculation

Immature nuts (four months) of *A. concinna* and *A. triandra* were harvested from the research field of ICAR-CPCRI RS, Vittal, Karnataka. Nuts were washed with Tween 20 under running tap water for 1 hour. Calyx was removed from the nuts and sterilized in $HgCl_2$ (0.01%) for 3 minutes and followed with a washing in distilled water for three times. Husk was removed in laminar air flow and the kernels were sterilized in 20% sodium hypochlorite for 10 minutes followed by rinsing in sterile water three times. Embryos were excised from the kernel aseptically and inoculated on to four different basal media *viz*., *Y3 (*Eeuwens, 1976), MS (Murashige and Skoog, 1962), B5 (Gamborg *et al*., 1968) and White (White, 1963). All basal media were supplemented with 3 % sucrose and 0.1 % charcoal. The sequence of sub-culturing, duration and concentration of growth regulators and other additives is represented in Fig. 2.

Establishment of plantlets

Plantlets with well developed root system were hardened

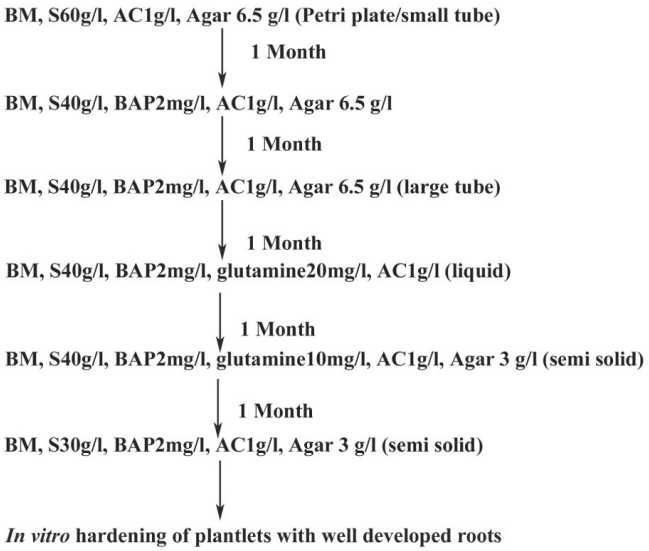

Fig. 2: The protocol followed to culture immature embryos of *Areca triandra* and *Areca concinna in vitro*. BM- basal media, S- sucrose, BAP- Benzyl Amino Purine and AC- activated charcoal.

in potting mixture consisting of sterilized sand, soil and coir dust in 3:1:1 ratio.

Statistical analysis

Data on embryo germination and growth of the plantlets in terms of shoot and root lengths were compared among different treatments and tested for their significance through DMRT.

RESULTS AND DISCUSSION

Raising plantlet from immature embryo *in vitro*

Surface sterilized immature embryo of *A. concinna* and *A. triandra* were inoculated on to four basal medium. Germination of the embryo was initiated after three weeks of incubation in dark. Mortality and contamination of the embryos were

negligible or nil indicating the efficiency of the sterilization and isolation of the embryos from immature nuts. Embryo was considered as germinated once the plumular portion emerged (Fig. 4a). Cultures were kept in dark till the shoot emerges from the immature embryo. Improved germination was reported when embryo was incubated in darkness for a month which mimics the dark natural status of the embryo in the seed nut (Batugal and Engelmann, 1998; Karun *et al*., 1999; Muhammed *et al*., 2013).

Percentage of germination varied significantly among the different media tested. Percentage of germination was more in case of *A. triandra* as compared to *A. concinna* irrespective of the media tested. Highest germination percentage in both the species were obtained when embryos were cultured in Y3 media as compared to rest of the basal media. However difference in germination was not significant between Y3 and MS (Fig. 3; Fig 4b). The percentage of embryo germination was significantly lower in Whites medium (Fig. 3). Y3 medium has been reported to be suitable in many of the palm species for culturing zygotic embryos (Karun *et al*., 1999; Padua *et al*., 2014) as well as for the somatic embryos (Muniran *et al*., 2008).

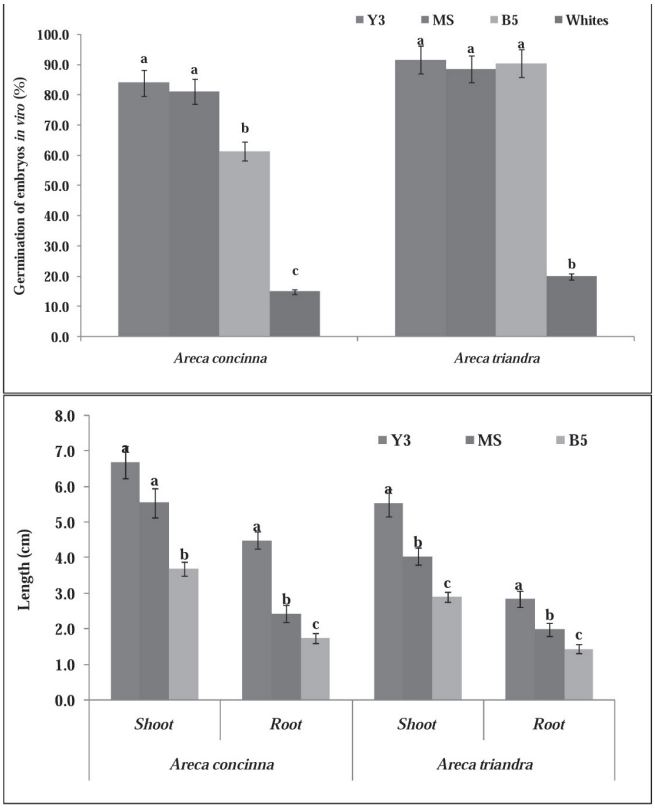

Fig. 3: Effect of different basal media on culturing of immature embryos of *A. concinna* and *A. triandra*, wild relatives of arecanut. Percentage of germination observed in these species in different media combinations (top); shoot and root length of the *in vitro* raised plantlets raised from culturing of immature embryos for three months (bottom). Values represented with similar alphabets for a parameter did not differ significantly according to DMRT.

After one month, germinated embryos were transferred to medium containing 2 mg l^{-1} BAP as growth hormone and subsequently transferred to liquid medium. Shoot and root length measured after three months of culturing indicate significant differences among the media tested. Supplementation of the media with glutamine improved the growth in plantlets. In general *A. concinna* plantlets were lengthier as compared to *A. triandra*. Similarly root length was more in *A. concinna*. Among the media, plantlets in Y3 performed better as indicated by longer shoot and roots (Fig. 3 and 4). Y3 medium has been earlier reported to be a potential basal medium for the growth and development of *in vitro* plantlets (Karun *et al*., 1999; Padua *et al*., 2014). Media containing BAP reported to initiate healthy shoot from *in vitro* cultures (Chaturvedi *et al*., 2004; Karun *et al*., 2004) and the potential of BAP was attributed to its ability to induce endogenous production of natural hormones like zeatin (Zaerr *et al*., 1982). Culture medium consists of

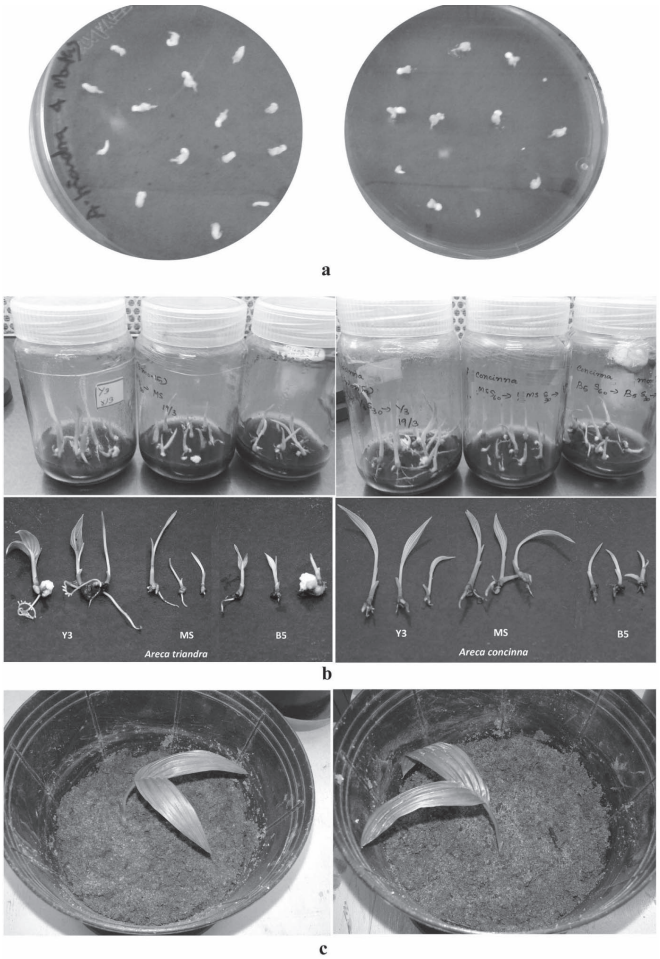

Fig. 4: Plantlets derived from immature embryos of *A. concinna* and *A. triandra*. Embryos in early stage of germination (a) and formation of shoot and roots (b) during a period of three months of culturing in *Y3*, MS and B5 medium. Plantlets with well developed roots were pot established (c) in a mixture comprising of sterilized sand, soil and coir dust in 3:1:1 ratio.

amino acids proved to stimulate the growth of the embryo (Bhojwani and Razdan, 1983). Glutamine was found most effective amino acid for cultured embryo growth (Monnier, 1978). Throughout the culturing period, media was supplemented with 1 g l^{-1} of activated charcoal. Activated charcoal has been a integral part of the *in vitro* culture techniques since it effectively negate the phenolic compounds which may lead to browning of the explants (Karunaratne *et al*., 1985; Abdelwahd *et al*., 2008). Plantlets with well developed root system comprising of secondary roots were hardened in pots with a potting mixture of sterilized sand, soil and coir dust. Notwithstanding the slow growth, plantlets were established in pots without contamination or death (Fig. 4c).

In vitro raised plantlets have been utilized successfully for the selection for drought tolerance and other stresses (Karunaratne *et al*., 1991). Since challenge inoculation on harvested immature nuts of *Areca concinna* and *Areca triandra* have shown resistance to *P. meadii* (Prathibha *et al*., 2015); the plantlets developed from the embryo culture can be tested from their resistance to *P. meadii* and its molecular mechanism can be delineated through gene expression studies.

CONCLUSIONS

The study resulted in standardization of sterilization and embryo excision from immature nuts of *A. concinna* and *A. triandra*. Eeuwens Y3 media with BAP as growth regulator is effective in culturing immature embryos. The present protocol needs to be refined to culture embryos of different stages of maturation. The protocol should also be validated in areca inter-specific hybrids.

REFERENCES

Abdelwahd, R., N. Hakam, M. Labhilili and S.M. Udupa. 2008. Use of an absorbent and antioxidants to reduce the effect of leached phenolics in *in vitro* plantlet regeneration of faba bean. *African Journal of Biotechnology*, **7(8)**: 997-1002.

Alves, S.A.O., O.F. de Lemos, B.G. dos Santos Filho and A.L.L. da SilvaIn. 2011. *In vitro* embryo rescue of interspecific hybrids of oil palm (*Elaeis oleifera* x *Elaeis guineensis*). *Journal of Biotechnology and Biodiversity*, **2(2)**: 1-6.

Angelo, P.C., L.A. Moraes, R. Lopes, N.R. Sousa, R.N. da Cunha and R.C. Quisen. 2011. In vitro rescue of interspecific embryos from Elaeis guineensis x E. oleifera (Arecaceae). Revisita De Biologia, **59**(3):1081-1088.

Assy Bah, B., T. Durand-Gasselin and C. Panetier. 1987. Use of zygotic embryo culture to collect germplasm of coconut (*Cocos nucifera* L.). Plant Genetic Resource Newsletter, **77**: 410.

Batugal, P.A. and F. Engelmann. 1998. Coconut Embryo *In Vitro* Culture. Proceedings of the First Workshop on Embryo Culture 27 –31 October 1997 Banao, Guinobatan, Albay, Philippines.

Bavappa, K.V.A. 1963. Morphological and cytological studies in *Areca catechu* L. and *Areca triandra* Roxb. M.Sc. (Ag) Thesis, University of Madras. pp 63.

Bavappa, K.V.A. 1968. A decade of research in arecanut. *Indian Farming*, **18**(4): 5-7.

Bhojwani, S.S. and M.K. Razdan. 1983. Plant tissue culture: Theory and practice. Elsevier, Amsterdam.

Chaturvedi, R., M.K. Razdan and S.S. Bhojwani. 2004. *In vitro* morphogenesis in zygotic embryo cultures of neem (*Azadirachta indica* A. Juss.). *Plant Cell Reports*, **22**: 801-809.

Eeuwens, C.J. 1976. Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera*) and cultured *in vitro*. *Physiologia Plantarum*, **36**:23-28.

FAO, 2014. http://www.fao.org/statistics/en/.

Gamborg, O.L., R.A. Miller and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, **50**:151-158.

Iyer, C.P.A. and M.D. Subramanyam. 1971. Possible role of embryo culture on mango breeding. *Indian Journal of Horticulture*, **29**:135–136.

Karun, A, K.K. Sajini and S. Shivashankar. 1999. Embryo culture of coconut: The CPCRI protocol. *Indian journal of horticulture*, **56**(4): 348-353.

Karun, A., E.A. Siril, E. Radha and V.A. Parthasarathy. 2004. Somatic embryogenesis and plantlet regeneration from leaf and inflorescence explants of arecanut (*Areca catechu* L.). *Current Science*, **86**(12): 1623-1628.

Karunaratne, S. C. Kurukulaarachchi and C. Gamage . 1985. A report on the culture of embryos of dwarf coconut, *Cocos Nucifera* L. var. nana, *in vitro*. *COCOS*, **3**:1-8.

Karunaratne, S., S. Santha, and A. Kovoor. 1991. An in vitro assay for drought-tolerant coconut germplasm. *Euphytica*, **53**:25-30.

Miyajuma, D. 2006. Ovules that failed to form seeds in zinnia (Zinnia violacec Cav)". Scientia Horticulturae, **107**(2): 176-182.

Monnier, M. 1978. Culture of zygotic embryos, p. 277–286. In: T.A. Thorpe (Ed.). Frontiers of plant tissue culture. Univ. of Calgary Press, Canada.

Muhammed, N., R. Nyamota, S. Hashim and J.N. Malinga. 2013. Zygotic embryo *in vitro* culture of *Cocos nucifera* L. (sv. East African Tall variety) in the coastal lowlands of Kenya. African Journal of Biotechnology. 12(22): 3435-3440.

Muniran, F., S.J. Bhore and F.H. Shah. 2008. Micropropagation of *Elaeis guineensis* Jacq. 'Dura': Comparison of three basal media for efficient regeneration. *Indian Journal of Experimental Biology*, **46**: 79-82.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. *Plant Physiology*, **15**:473- 497.

Murthy, K.N. and K.V.A. Bavappa. 1960. Breeding in arecanut. *Arecanut Journal*, **11**: 60-61.

Murthy, K.N. and R.N.S. Pillai. 1982. Botany. In: Bavappa K.V.A., Nair, M.K., Prem Kumar, T. (Eds), *The Arecanut palm*, Central Plantation Crops research Institute, Kasaragod, pp-11-49.

Padua, M.S.S., L.V. Paiva, L.G. Texeira da Silva, L. Coutinho Silva and V.C. Stein. 2014. In vitro development and acclimatization of dendezeiro (Elaeis guineensis). Revista Árvore, **38**(6): 1095-1102.

Prabhakaran Nair, K.P. 2010. Arecanut. In: The agronomy and economy of important tree crops of the developing world. Elsevier publication, USA. pp. 1-19.

Prathibha, V.H., Vinayaka Hegde, K.M. Sharadraj, K. Nidhina, N.R. Nagaraja and M. Chaithra. 2015. Identification of sources of resistance against *Phytophthora* in arecanut. Abstract in 3rd International Symposium on *Phytophthora* held at Bengaluru, 9-12th September 2015. Pp40.

Raghavan, V. 1957. On certain aspects of the biology of arecanut (*Areca catechu* L.) and utilization of its by-products. D.Phi1. Thesis. Gauhati University. pp 186.

Rhillo, E.P. and M.B.F. Paloma. 1992. *In vitro* culture of Makapuna coconut embryo. *Coconut Today*, **9**:90-101.

Sage, T.L., F. Strumas, W.W. Cole and S. Barret. 2010. Embryo rescue and plant regeneration following interspecific crosses in the genus *Hylocereus* (Cactaceae). *Euphytica,* **174**:73-82.

Sharma, D.R., R. Kaur, and K. Kumar. 1996. Embryo rescue in plants-A review.

Euphytica **89**:325–337.

Tzec-Simá, M.A., R. Orellana and M.L. Robert. 2006. In vitro rescue of isolated embryos of Bactris major jacq. and Desmoncus orthacanthos mart., potentially useful native palms from the Yucatan Peninsula (Mexico). In Vitro Cellular & Developmental Biology-Plant, **42**(1):54-58.

White, P.R. 1963. The Cultivation of Animal and Plant Cells. 2nd Edn., Ronald Press Co., New York. ISBN: 0826093809, pp: 228.

Zaerr, J. B. and M.O. 1982. Mapes, In Tissue Culture in Forestry (Eds: Bonga, J. M. and Durzan, D. J.), Martinus Nijhoff/Dr. W. Junk Publishers, The Hague, Boston, pp. 231-255.