Chapter 5

Arecanut

☆ *Anitha Karun, Krishna Prakash, M.K. Rajesh and P. Chowdappa*

1. Introduction

Arecanut (*Areca catechu* L.) is commonly known as betel nut or 'Supari'. The importance of this nut is due to its use for masticatory purposes and is grown widely in most of the tropical Pacific, Asia, and parts of East Africa. The genus *Areca* belongs to the family Arecaceae (2n = 32) under the tribe Areceae are native to South and South-East Asia and the Pacific islands and is reported to contain about 76 species, of which *A. catechu* is the only cultivated species, the nuts of which are chewed as a mild stimulant (Bavappa *et al.*, 1982). Apart from its popularity as a masticatory nut, indigenous communities traditionally also use it in religious and social functions and it finds use as an ingredient in traditional medicines. The pharmaceutical importance of arecanut is due to the presence of an alkaloid, arecoline. Tannins extracted from tender arecanut are considered to be an excellent source of natural dye, tanning agent and adhesive (Bavappa *et al.*, 1982).

Arecanut palm is very widely cultivated plant in countries like India, Bangladesh, China, Indonesia, Vietnam, the Philippines, Thailand, Malaysia, Sri Lanka and Japan. India ranks first in both area (49 per cent) and production (50 per cent) of arecanut and at the same time, is the largest consumer also (FAOSTAT, 2013). In India, arecanut is cultivated in an area of 453 thousand hectares with an annual production of 6.22 lakh tonnes (NHB, 2013-14). Its cultivation is localized in southern and north-eastern states of India with the major states cultivating this crop being Karnataka, Kerala, Assam, Tamil Nadu, Meghalaya and West Bengal.

Arecanut palm is a slender, unbranched, single-trunked monoecious palm that can grow up to 100 ft, commonly growing in hot, humid tropical regions. Seed is the only propagule of arecanut as is the case of many other palm species. This, together with the long juvenile phase, heterozygous and outbreeding nature of the crop, makes the populations highly heterogeneous. Requirement of large area for experimentation further limit the scope of genetic improvement programmes in arecanut. Since the demand for quality planting material exceeds the supply and capacity, the additional requirements is being met from the unselected local cultivars. As compared to seed production, tissue culture of areca palm offers several advantages; mainly, it allows rapid multiplication of elite genotypes with desired characteristics.

Palm trees have been considered recalcitrant in tissue culture, although successful regeneration of palms through *in vitro* clonal propagation have been achieved using several explants such as roots, young leaves, shoot tips, immature inflorescence and zygotic embryos, mainly through direct and indirect somatic embryogenesis (Wang *et al.*, 2006). Somatic embryogenesis is the process by which embryo forms and develops from bipolar structures from somatic cells that parallel the developmental path of zygotic embryos. Indirect somatic embryogenesis has an intermediate callus phase whereas direct somatic embryogenesis does not have these. Successful protocol for clonal propagation via somatic embryogenesis has been reported for several palms such as African oil palm (*Elaeis guineensis;* Rabechault *et al.*, 1970), date palm (*Phoenix dactylifera;* Tisserat and Demason, 1980), peach palm (*Bactris gasipaes;* Valverde *et al.*, 1987), coconut (*Cocos nucifera;* Verdeil *et al.*, 1994), bottle palm (*Hyophorbela genicaulis;* Sarasan *et al.*, 2002), arecanut (Karun *et al.*, 2004) and interspecific crosses *Elaeis guineensis* × *E. oleifera* (Angelo *et al.*, 2013).

2. Arecanut Tissue Culture

Reports of arecanut tissue culture are rather limited. Arecanut tissue culture work was initiated for the first time by Ganapathi *et al.* (1997); *in vitro* germination studies of excised mature embryos of arecanut was carried out. Mathew and Philip (2000) first reported the protocol for *in vitro* propagation *via* direct adventitious shoot bud differentiation from cotyledon explants. A repeatable protocol for arecanut somatic embryogenesis and plantlet regeneration from leaf and immature inflorescence explants was developed by Karun *et al.* (2004) and Radha *et al.* (2006) at ICAR-Central Plantation Crops Research Institute, Kasaragod. Optimization of culture media, type of explant, plant growth regulators and their concentrations, subculturing periods, other additives, have paramount significance in developing a reproducible tissue culture protocol.

2.1. Explant

The initial step in tissue culture is the selection of suitable explants from the mother plants. Several explants sources have been tested for the establishment of tissue culture in arecanut. Ganapathi *et al.* (1997) studied in *vitro* germination of excised mature zygotic embryos of arecanut. Mathew and Philip.(2000) first reported the protocol for *in vitro* propagation via direct adventitious shoot bud differentiation from cotyledonary explants. A protocol for *in vitro* germination of zygotic embryos, which facilitates rapid development of plantlets, was reported by Karun *et al.* (2002). A technique for vegetative propagation through *in vitro* shoot bud regeneration from shoot tip derived callus from four-week old sprouts of arecanut was described by Wang *et al.* (2002). Plant regeneration through somatic embryogenesis from zygotic embryo-derived callus was first reported by Wang *et al.* (2003). A repeatable protocol for arecanut somatic embryogenesis and plantlet regeneration from leaf and immature inflorescence (size 10–25 cm) explants was developed by Karun *et al.* (2004). Plantlet regeneration of arecanut through somatic embryogenesis using root, leaf and stem derived callus was reported by Wang *et al.* (2006). Radha *et al.* (2006) reported plantlet regeneration *via* direct and indirect somatic embryogenesis from immature inflorescence (length size 18.5–39cm) explants of arecanut palm. This occurrence of direct somatic embryogenesis is the first report in arecanut palm tissue culture.

2.2. Culture Method and Media

The basic technique in micropropagation of arecanut involves culturing the meristematic tissues in a callus induction media followed by subculture to induce embryogenic callus and subsequent production of embryoids. The preferred pathway of regeneration is indirect somatic embryogenesis through callus phase. For this, the first step is the induction of callus and the callus is induced to form somatic embryos. Role of basal medium and plant hormones in the culture medium is very important in induction of callus and further development into somatic embryos and plantlets.

In vitro propagation of arecanut *via* direct adventitious shoot bud differentiation from cotyledon explants on Murashige and Skoog (MS), White's, Branton and Blake's (BB) medium was reported by Mathew and Philip (2000). Results obtained showed combinations of auxins and cytokinins with activated charcoal, 2,4-D and high levels of phosphate in BB medium were critical for the differentiation of additional shoots from the cotyledon. The darkening effect of activated charcoal induced rooting in shoot cultures. Synergistic action of abscisic acid and auxins in the rooting medium enhanced the frequency of rooting.

Plantlet formation through shoot formation from shoot tip derived callus of arecanut was reported by Wang *et al.* (2002). Greenish soft callus was formed on shoot tip explants within four weeks, when cultured on MS basal medium supplemented with BA and TDZ (best result at 0.2 mg l^{-1} each). Most of calli proliferated during subculture on the same medium for callus induction, and 50–60 per cent of them formed shoots. About 90 per cent of shoots formed roots on medium containing 0.1 mg l^{-1} NAA after four weeks in culture. Plants have also been obtained through somatic embryogenesis from zygotic embryo derived callus of arecanut (Wang *et al.*, 2003). When segments of zygotic embryos were cultured on MS medium supplemented with dicamba (2, 4, 6 and 8 mg dm^{-3}) for 7 to 8 weeks in darkness, wounded regions of explants formed callus like structure. After 7- 8 weeks, callus showed pale yellow, compact, nodular structures which converted into somatic embryos within 2 to 4 months. These somatic embryos developed into plantlets after 10 weeks of culturing on hormone-free basal medium. After subculturing every month for three months, the plantlets were ready for transfer for acclimatization in the green house, 24 per cent survival rate was reported.

A viable protocol for somatic embryogenesis and plantlet regeneration from immature inflorescence explants of arecanut was reported for the first time at CPCRI, Kasaragod (Karun *et al.*, 2004). The protocol was standardized with leaf explants excised from one-year-old seedlings and later modified for immature inflorescence (spadix length 10–15 cm) sampled from adult palms of different arecanut varieties *viz.* Mangala, Sumangala and Mohitnagar (Figure 5.1). The basal medium used was MS medium and picloram as most suitable callogenic agent. Serial transfer of explants from higher to lower auxin concentration (from 200µM – 100µM – 50µM –10µM – 5µM to hormone free medium) at 30–35 days interval was essential for sustained growth of callus and somatic embryo induction. Hormone free MS medium was used for somatic embryo formation. Germination of somatic embryo was achieved in half strength MS medium supplemented with cytokinins; 20 µM 6-benzylaminopurine (BA) was found to be the best. For rapid germination of somatic embryos, MS liquid medium supplemented with 5 µM BAP was used. Subsequent plantlet development was achieved in MS medium supplemented with 10 mg l^{-1} BA, 5 mg l^{-1} indole-3-butyric acid (IBA) and 0.5 mg l^{-1} naphthaleneacetic acid (NAA). Plantlets with 2-4 leaves and fairly good root system were transferred to potting mixture, consisting of sterilised sand and soil in the ratio of 5:1 and kept inside poly house for hardening for 12-18 months.

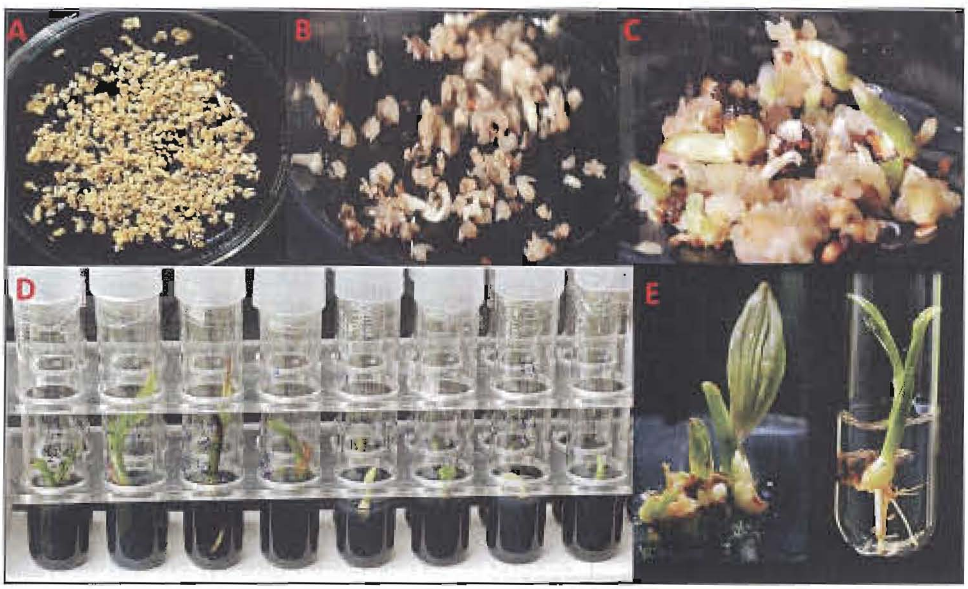

Figure 5.1: Arecanut Clonal Propagation through Somatic Embryogenesis: (A) Inflorescence explants, (B) Callusing and formation of somatic embryos, (C) Germination of somatic embryos, (D) and (E) Plantlet development.

This protocol for mass multiplication of elite arecanut palms has been successfully applied for propagation of field resistant Yellow Leaf Disease (YLD) palms (Karun *et al.*, 2005). Direct and indirect somatic embryogenesis from inflorescence explants of arecanut was reported by Radha *et al.* (2006). However the number of direct somatic embryos formed in Eeuwens Y3 medium was very few and the plantlet development was completed within 9-12 months. Five cytokinins *viz.*, thidiazuron (TDZ), BA, kinetin, N6-(2-isopentenyl) adenine (2iP) and zeatin were tested for their effect on regeneration. Amongst these, TDZ at a concentration of 2 mg l^{-1} gave maximum shoot length, number of leaves and root growth and was found to be the most efficient cytokinin for maturation and conversion of direct somatic embryos of arecanut into complete plantlets.

Wang *et al.* (2006) reported somatic embryogenesis and plantlet regeneration using arecanut root, leaf and stem parts as explants. They were able to induce and maintain embryogenic calli on MS basal medium contained full-strength macro- and micro-elements of MS salts supplemented with 2, 4-dichlorophenoxyacetic acid (2, 4-D) or 3,6-dichloro-2-methoxybenzoic acid (dicamba) at concentrations of 2, 4, 6 and 8 mg dm^{-3} in darkness. Somatic embryos were formed in the presence of 2-4 mg dm^{-3} dicamba on primary callus and during subculture on 2-8 mg dm^{-3} 2, 4-D or 2-4 mg dm^{-3} dicamba-containing media. Earlier, Wang *et al.* (2003) also reported formation of somatic embryos from zygotic embryo-derived callus after 2-3 passages of subculture (at an interval of 8 weeks) on 2-8 mg dm^{-3} dicamba-containing medium. Plantlet conversion from embryos was successfully achieved on growth regulator-free medium kept under a 16 h photoperiod condition.

Assessing the genetic fidelity of tissue culture derived plantlets is very much important for establishing the genetic uniformity in perennial crops, as these crops will remain in the field for a long time. Among the different molecular markers available, RAPD markers are preferred due to their cost effectiveness, technical simplicity and non-requirement of sequence information of template DNA. To evaluate clonal fidelity of somatic embryogenesis derived plantlets from Yellow Leaf Disease (YLD) resistant arecanut palms, Random amplified polymorphic DNA (RAPD) markers were used by Karun *et al.* (2008). Pair wise genetic similarities generated by Jaccard's coefficient between each mother palm and its progenies (eight plantlets/palm) were showing high similarity (99 per cent in one case and 98 per cent in another).The low level of genetic variability using RAPD markers in plantlet derived from direct somatic embryogenesis of inflorescence culture shown by plantlets of direct somatic embryogenesis can be exploited for large-scale multiplication of elite arecanut palms with desirable qualities.

3. Future Aspects

Several approaches have come afore in order to enhance the productivity of *in vitro* culture depending on the final product desired and the species investigated. One such innovative approach is the use of cell suspensions and bioreactors for plant culture. Cell suspension culture is the method of culturing and maintaining the embryogenic calli obtained from the explants in a liquid medium with appropriate nutrients under stable microclimatic condition.The technique enables the production of individualized embryos in synchronous growth with both root and shoots; compared to routine process with polyembryonic cultures and compulsory rooting treatment (De Touchet *et al.*, 1991). The embryogenic cells produced in cell suspension culture could be used in bioreactors to enhance somatic embryogenesis.

Zouine *et al.* (2005) demonstrated a procedure for the rapid development of a high number of somatic embryos from embryogenic suspension culture in date palm. This method might be efficient for mass propagation of arecanut. The adaption of bioreactors in the plant tissue culture is considered as a major milestone since they offer several advantages *viz.*, short time and labour-saving, relatively easy to scale-up, allow enhanced growth and multiplication and improved nutrient availability due to the use of liquid medium over traditional tissue culture techniques. A system named as temporary immersion systems (TIS), wherein explants are flooded with nutrient medium containing growth regulators at regular time intervals, has been successfully used in scaling up of somatic embryogenesis. This system offers the possibility of automating some culture stages. This would be an interesting strategy for up-scaling plantlet regeneration potential in arecanut tissues under *in vitro* conditions.

References

Angelo, P.C., Steinmacher, D.A., Lopes, R., da Cunha, R.N.V. and Guerra, M.P. (2013). Histological analysis and transcription profiles on somatic embryogenesis in interspecific hybrids of *Elaeis guineensis* × *E. oleifera*. *Agricultural Sciences*, **4**:1–11.

Bavappa, K.V.A., Nair, M.K. and Premkumar, T. (1982). The Areca palm (*Areca catechu* Linn.). *Central Plantation Crops Research Institute*, Kasaragod, Kerala, India.

De Touchet, B., Duval, Y. and Pannetier, C. (1991). Plant regeneration from embryogenic suspension cultures of oil palm (*Elaeis guineensis* Jacq.). *Plant Cell Reports*, **10**:529–532.

Ganapathi, T.R., Suprasanna, P., Bapat, V. and Rao, P.S. (1997). In vitro culture of embryos of arecanut (Areca catechu L.). *Fruits*, **52**:313–316.

Karun, A., Manimekalai, R., Priya, S. and Radha, E. (2005). Genetic fidelity of tissue derived plantlets and histology of somatic embryogenesis in arecanut (*Areca catechu* L.). *Poster paper presented at International Conference on Plant Genomics and Biotechnology*: Challenges and Opportunities, *Indira Gandhi Agricultural University*, Raipur, India.

Karun, A., Radha, E., Vijayan, P.S., George, J., Rajesh, M.K. and Ananda, K.S. (2008). Assessment of genetic fidelity of arecanut plantlets derived through direct somatic embryogenesis by RAPD markers. *Journal of Plantation Crops*, **36**(3):228–231.

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

Karun, A., Siril, E.A., Radha, E. and Parthasarathy, V.A. (2002). *In vitro* embryo retrieval technique for arecanut (*Areca catechu* L.). *Proceedings of PLACROSYM XV. pp.* 209 – 214.

Mathew, M. and Philip, V.J. (2000). *In vitro* adventitious shoot formation from embryos of *Areca catechu* L. *Phytomorphology*, **50**:221–227.

Rabechault, H., Ahée, J. and Guenin, G. (1970). Colonies cellulaires et forms embryoides obtenues in vitro a partir de cultures d'embryons de Palmier a huile (*Elaeis guineensis* Jacq. var. dura Becc.). *Comptes Rendus de l'Académie des Sciences* **270**:3067–3070.

Radha, E., Karun, A., Ananda, K.S. and Kumaran, P.M. (2006). Plantlet regeneration via direct and indirect somatic embryogenesis from inflorescence explants of arecanut palms. *Journal of Plantation Crops*, **34**(3):50–54.

Sarasan, V., Ramsay, M. and Roberts, A. (2002). *In vitro* germination and induction of direct somatic embryogenesis in "Bottle Palm" [*Hyophorbe lagenicaulis* (L. Bailey) H.E. Moore], a critically endangered Mauritian palm. *Plant Cell Reports*, **20**:1107–1111.

Tisserat, B. and Demason, D.A. (1980). A histological study of development of adventive embryos in organ cultures of *Phoenix dactylifera* L. *Annals of Botany* **46**:465–472.

Valverde, R., Arias, O. and Thorpe, T.A. (1987). Picloram-induced somatic embryo genesis in pejibaye palm (*Bactris gasipaes* HBK). *Plant Cell Tissue Organ Culture*, **10**:149–156.

Verdeil, J.L., Huet, C., Grosdemange, R. and Buffard-Morel, J. (1994).) Plant regeneration from cultured immature inflorescences of coconut (*Cocos nucifera* L.): evidence for somatic embryogenesis. *Plant Cell Reports*, **13**:218–221.

Wang, H.C., Chen, J.T. and Chang, W.C. (2006). Somatic embryogenesis and plant regeneration from leaf, root and stem-derived callus of *Areca catechu* L. *Biologia Plantarum*, **50**:279–282.

Wang, H.C., Chen, J.T., Wu, S.P., Lin, M.C. and Chang, W.C. (2002). Plant regeneration through somatic embryogenesis from zygotic embryo-derived callus of *Areca cathecu* L. (Arecaceae). *In Vitro Cellular and Developmental Biology - Plant* **39**:34–36.

Wang, H.C., Chen, J.T., Wu, S.P., Lin, M.C. and Chang, W.C. (2003). Plant regeneration through shoot formation from callus of *Areca cathecu* L. *Plant Cell Tissue Organ Culture*, **75**:95–98.

Zouine, J., El Bellaj, M., Meddich, A., Verdeil, J.L. and El Hadrami, I. (2005). Proliferation and germination of somatic embryo from embryogenic suspension cultures in *Phoenix dactylifera*. *Plant Cell Tissue Organ Culture*, **82**:83–89.