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BIOTECHNOLOGY OF COCONUT

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ABSTRACT

Biotechnology of coconut (*Cocos nucifera* L.) is a relatively recent area compared to similar work in other crops. It started as tissue culture in the eighties, which led to the development of molecular markers in late nineties with the use of RAPDs. Since then, considerable research has been carried out and protocols for tissue culture regeneration almost perfected. Embryo culture is being very successfully applied to germplasm transfer. Molecular markers such as AFLP, SSR, etc., were used to develop QTL maps. The entire gamut of coconut biotechnology is under review in this paper.

Key words: Biotechnology, coconut, *Cocos nucifera*

INTRODUCTION

Research on coconut (*Cocos nucifera* L.) tissue culture started in the eighties following success in oil palm tissue culture. It was initially thought that application of tissue culture techniques in coconut would result in success but it proved otherwise. Culture medium developed for oil palm was indubitable for coconut and it was later proved that the coconut palm is highly recalcitrant to *in vitro* manipulations and every stage of the procedure brought its share of problems (Verdeil *et al.*, 1998). It was envisaged that this technique would also help in rapid propagation of elite hybrids. Success obtained in coconut embryo culture and its use in germplasm collection was one of the major achievements in this direction. Research on coconut tissue culture was thus aimed at solving problems of phenol production using anti-oxidants other than activated charcoal, and by production of embryogenic calli and regeneration of plants. This research is of paramount importance because, unlike in other crops, biotechnological research on coconut is being carried out intensively at present only at the Central Plantation Crops Research Institute (CPCRI), Kasaragod, Kerala, India and a few laboratories abroad, although sporadic attempts have been made in several other laboratories (Iyer, 1993, 1995). Any breakthrough resulting in coconut biotechnology would be of great importance to the country, in general, and coconut growing states in particular. Tissue culture of coconut was carried out in several countries besides India, including UK

(Wye College), France (IRHO/CIRAD), USA (Florida University), the Philippines, Australia, Indonesia and Sri Lanka. As a result of these programmes, only a few clonal plantlets could be produced over several years, and a repeatable and commercial protocol is yet to be developed (Iyer and Parthasarathy, 2000; Parthasarathy and Bose, 2001).

A viable micropropagation protocol for desired coconut hybrids/selections is thus fundamental to disseminating benefits of various breeding programmes to the farming community. The technique thus perfected could also be used for mass multiplication of disease resistant/tolerant types, especially, in the context of the epidemic and devastating nature of root (wilt) disease in Kerala. This disease is estimated as causing a loss of over 960 million nuts annually. Other international ramifications are deadly diseases like lethal yellowing, which is reported to be spreading at a rate of 100km/year in Mexico and would eventually wipe out all the country's estates (Veredeil *et al.*, 1998). Recently, a lot of interest is seen on molecular aspects of coconut and latest marker technologies including microsatellite and AFLP, are being used.

Molecular markers

The use of biochemical and molecular markers in coconut is a recent one. Biochemical markers like isozymes and molecular markers like Restricted Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic

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DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Inter Simple Sequence Repeats (ISSR) and Sequence Tagged Microsatellites (STM) are presently being studied. A comprehensive review on application of molecular markers was first presented by Rohde (1993) and later by Ashburner (1999). Fernando and Gajanayake (1997) have reported protocols for detection of isozyme polymorphism in coconut leaf tissue. They found esterases to be useful for studying the genotypic variations in coconut. Cardena *et al* (1998) used electrophoretic patterns of leaf peroxidases, endopeptidases and coomassie blue stained proteins in four cultivars and two hybrids. The polymorphism detected fitted the expression of two alleles of a dimeric peroxidase, two monomeric endopeptidase and a pair of active, and, null alleles of a coomassie blue stained protein. They concluded that protein markers would broaden the alternatives available to coconut breeders. Geethalakshmi *et al* (2000) observed limited polymorphism in esterases and polyphenol oxidase while polymorphism for peroxidase was absent. However, earlier attempts by Meunier *et al* (1992) and Carpio (1980) failed to achieve any success.

Preliminary studies of Rohde (1993) helped in molecular characterization of the nuclear genome, which provided evidence for existence of truncated, copia-like repetitive sequences indicating that retro-elements may have played a role in generation of genetic diversity in coconut. Rohde *et al* (1995) described a novel approach for analysis of coconut germplasm using coconut specific primers complementary to the copia-like *EcoRI* elements. PCR amplification of spacer regions for a sub-set of tandemly arranged repeats detected polymorphisms which allowed analysis of biodiversity within coconut populations. Rohde (1996) subsequently described Inverse Sequence Tagged Repeat (ISTR) analysis. Duran *et al* (1997) analyzed 48 coconut genotypes using different DNA marker techniques, namely, RAPD, microsatellite primed PCR and ISTR. All three approaches detected a large amount of DNA polymorphism among genotypes and allowed identification of genotypes by individual specific fingerprint. Use of polymorphic microsatellites for assessing genetic diversity in coconut is gaining popularity of late (Karp, 1999). CIRAD, in collaboration with COGENT, developed a set of 14 microsatellite markers with sufficient discrimination power for practical identification of coconut cultivars. These projects culminated in developing standard protocols without the use of radioactive probes as well as in development of dedicated statistical software, Gene Class

2, adapted to use in coconut producing countries (Baudouin and Lebrun, 2002). Hautea *et al* (2000), Perera *et al* (1999) and Perera (2001) used microsatellites (simple sequence repeat - SSR) to assess genetic diversity of selected germplasm. SSR data indicated high degree of allelic diversity for microsatellite markers within the tall populations. Diagnostic SSR markers were identified for use in hybridity testing and two diagnostic markers were identified for use in hybrid test. Perera *et al* (2000a) used eight pairs of SSR primers to analyze genetic diversity in 130 individuals of coconut comprising 75 tall and 55 dwarf individuals, representing 94 different coconut ecotypes from around the world. Fifty-one alleles were detected, with an average of 6.4 alleles per locus. Fifty alleles were detected in tall coconuts (mean alleles/locus 6.3) compared with only 26 in dwarf (mean alleles /locus 3.3). The average diversity value in talls (0.589) was also significantly higher than that in dwarfs (0.348). Using eight SSRs, they were able to uniquely discriminate 116 of the 130 individuals. A phenetic tree based on D_{AD} (absolute distance) values clustered individuals into five groups, each mainly composed of either talls or dwarfs. Perera *et al* (2000b and 2000c) also used SSR to study polymorphism. They used 39 coconut-specific microsatellite primers developed from an enriched small insert genomic library. Eighteen of these were used to assay Sri Lankan coconuts. The outbreeding Tall variety (*Typica*) accounted for most of the diversity, in contrast to inbreeding varieties, nana (dwarfs) and intermediate (*aurantiaca*) types. Partitioning of genetic variability revealed that, for dwarf and intermediate forms, most variation was observed between rather than within forms. In contrast, tall forms exhibited as much variation within as between forms. A reduction in allelic variability was observed in Dwarfs compared with Talls and the pattern of allelic distributions suggested that Sri Lankan dwarfs were introductions. They used twelve pairs of microsatellite primers to screen a collection of global coconut germplasm. Eighty four alleles were detected in Talls as compared to 42 in Dwarfs with average diversity value at 0.703, which was significantly higher than that detected in the dwarf sample (0.374). They concluded that dwarfs were a sub set of the tall coconuts and directly evolved from talls and from 'Niu vai' types of tall types (Southeast Asia and Pacific origin). Genetic diversity in coconut populations across the entire geographic range was assessed using SSR and AFLP by Teulat *et al* (2000).

Merrow *et al* (2003) used 15 simple sequence repeat (SSR) microsatellite DNA loci to analyze genetic

variation within eight coconut cultivars from Florida. Parentage analysis of 'Fiji Dwarf' cultivar was also carried out using these loci. Red Malayan Dwarfs were found to be genetically distinct from Green and Yellow ones. Also, genetic identity of 'Red Spicata' was found to be more towards 'Fiji Dwarf'.

Devakumar *et al* (2006) carried out assessment of genetic diversity of 21 Indian and 24 exotic coconut accessions using eight SSR primers. The eight coconut microsatellite loci studied distinguished a total of 48 alleles, with an average of 6 alleles per locus. Genetic diversity values were low for most dwarfs and high for the tall accessions, which was in accordance with their breeding behaviour. However, Kulasekharam Orange Dwarf showed genetic diversity higher than many tall. 'Within population' variation (58%) was found to be higher than 'among population' variation (42%).

Microsatellite analysis of lethal yellowing disease tolerant genotypes (Vanuatu Tall and Sri Lankan Green Dwarf) and the susceptible genotype (West African Tall) was carried out by Konan *et al* (2007). A total of 58 alleles were detected by the 12 microsatellite loci analyzed. Genotypes of the susceptible West African Tall cultivar were found to be less genetically clustered to the genotypes of the (two) tolerant cultivars. Fingerprinting based on microsatellites aided in identification of suitable parents for use in crossing programmes to develop a segregating mapping population for marker-assisted selection of lethal yellowing resistance genes.

Nagaraju *et al* (2002) were the first to standardize DNA amplification fingerprinting (DAF) in coconut. They used DAF and AFLP markers to study phylogenetic relationships among coconut accessions grown in India. AFLP approach was found to be more efficient as the number of primer combinations that detected polymorphic DNA markers were more in contrast to DAF. However, the number of polymorphic bands identified using selected primers in both the techniques was comparable. Genetic similarities among the accessions were determined. In DAF, out of 300 primers screened, 28 (9.33%) detected polymorphism producing an average of 5 polymorphic bands, while, in AFLP, 55 (86 %) primer combinations generated polymorphic bands (6.42). Dendrogram of the coconut accessions by UPGMA cluster analysis indicated grouping of all the dwarf accessions as one in DAF as well as AFLP analysis. DAF technique was later used by Jayadev *et al* (2005) to identify molecular markers which could

differentiate between coconut root (wilt) disease tolerant and susceptible palms. Of the 16 primers screened, three primers viz., UBC 66, UBC 84 and UBC 729 could differentiate resistant from susceptible coconut palms.

Use of RFLP and RAPD has also been reported. RFLP markers were used by Lebrun *et al* (1998a, 1998b, 1999) to study the spread and domestication of coconut through genetic diversity. They used 289 palms, representative of 26 tall and 16 Dwarf ecotypes, originating from major coconut areas. Twenty cDNA probes from oil palm, rice, maize and coconut, and one cytoplasmic probe from wheat, were hybridized on digested DNA using four restriction enzymes. Based on molecular polymorphism, they defined two main groups of tall coconut palms, originating from South East Asia and Pacific Ocean, and another as originating from the Indian subcontinent and from West Africa. Cultivars from East Africa and from the Andamans shared markers in both the groups, whereas Panama Tall appeared to be derived from the first one. All the Dwarfs (except Niu Leka) formed a highly homogenous group related to the first group of Talls. Lebrun *et al* (1999) reported RFLP analysis to be an efficient and powerful technique to obtain a precise picture of coconut diversity and of the ways in which the crop has spread and evolved. Everard *et al* (1996) and Ashburner *et al* (1997), and later, Wadt *et al* (1999) described the use of RAPD in coconut. Ashburner *et al* (1997) studied diversity in coconut in the South Pacific region. They reported a moderate level of genetic diversity, although very few RAPD markers were found to be unique to specific populations. Upadhyay *et al* (2002) screened 100 random primers and found only 53 primers to amplify coconut DNA and 34 primers detected polymorphism between West Coast Tall and Chowghat Orange Dwarf. Analysis of genetic distances revealed that all dwarf accessions grouped together whereas tall accessions showed much heterogeneity. Dewi Hayati *et al* (2000) used RAPD to analyze genetic diversity in four dwarf populations from East Java. They found that variability of coconut population grown outside East Java was higher than that at grown in East Java, since these coconut populations were collected from seeds of open pollinated plants.

Application of AFLP in coconut was reported by Perera *et al* (1998). They generated 322 amplification products from 42 genotypes with eight pairs of primers (*Eco* RI and *Mse* I). Overall, maximum variation was detected in the tall (*Typica*) rather than the intermediate (*Aurantiaca*) and dwarf (*Nana*) forms. A hierarchical analysis of

molecular variance (AMOVA) was used to quantify and partition levels of variability as between and within form components. They found that for inbreeding dwarf and intermediate forms, maximum variation was observed between, rather than within, forms. In contrast, outbreeding tall forms exhibited as much variation within, as between, forms. These observations have important implications for maintenance and collection of coconut germplasm. Morphologically, *Aurantiaca* group is considered to be intermediate between tall and dwarf accessions. Estimation of genetic relatedness based on AFLP analysis identified *Aurantiaca* group as being more similar to dwarf rather than tall group. In addition, putative duplicate accessions were identified in *Aurantiaca* group.

Thirty three coconut accessions representing different geographical regions of the world, maintained at the International Gene Bank in India, were analyzed using 19 ISSR primers (Manimekalai and Nagarajan, 2006). A total of 199 ISSR markers were generated, out of which 154 were polymorphic. Least similarity was found between Nicobar Tall and Chowghat Orange Dwarf, both accessions from India. Coconut accessions from Southeast Asia, South Asia and South Pacific formed separate groups, which was generally in accordance with origin and their dispersal.

The first linkage map on coconut was reported by Rohde *et al* (1999) first using a population of 52 F₁ plants from the MYD 20 x LAG 07 (Laguna Tall) using ISTR. Initial analysis of this mapping population identified 51 polymorphic ISTR markers, 43 of which could be arranged into 12 linkage groups comprising a total of 542 recombination units. Subsequently, Herran *et al* (2000) and Lebrun *et al* (2001) constructed the linkage map. Herran *et al* (2000) work was identical to that of Rohde *et al* (1999) using identical mapping populations while Lebrun *et al* (2001) used the Rennell Island Tall (RIT) population. They reported total genome length to be 1971 cM for the RIT map, with 5-23 markers per linkage group. QTL analysis for yield characters in two consecutive sampling periods identified nine loci, while, three and two QTLs were detected for the number of bunches and one and three QTLs for the number of nuts. Their study indicated that co-segregation of markers with these QTLs provided an opportunity for marker-assisted selection. Baudouin *et al* (2006) investigated genetic factors that controlled fruit characters in coconut. QTL analyses was performed for fruit component weights and ratios in the segregating progeny of a Rennell Island Tall genotype, complemented by the linkage map previously constructed by Lebrun *et al* (2001).

Of the 52 putative QTLs identified for the 11 traits studied, 34 grouped in six small clusters. Interestingly, QTLs for fruit component weight, endosperm humidity and fruit production were found at different locations in the genome, suggesting the need for selecting QTLs for individual traits, for efficient marker-assisted selection for yield.

Cardena *et al* (1999) described prospects for marker assisted breeding of lethal yellowing resistant coconuts.

Shalini *et al* (2007) reported identification of molecular markers based on mite resistance in coconut. Mite resistant and susceptible accessions were collected and analyzed using RAPD and SSR primers. Nine SSR and four RAPD primers were identified with mite resistance using single marker analysis. When stepwise multiple regression analysis of RAPD and SSR data was done, a combination of five markers could account for 100% of association with mite resistance.

Tissue culture

Coconut is a difficult crop to manipulate *in vitro*. However, after Eeuwens (1976) initial standardization of media and successful report of callus induction from various explant sources like stem, leaf, and inflorescence, a few laboratories around the world initiated intensive research. Till 1995, work on coconut tissue culture was carried out sporadically in quite a few laboratories. Unlike many crops, coconut was posing several problems. Besides, the number of laboratories working on this crop was also less. Appreciating this, an international collaborative project was initiated in 1995 consisting of researchers from France, Cote d'Ivoire, U.K., Germany, Philippines and Mexico. Results of this collaboration led to solving a large number of problems encountered in coconut tissue culture (Hoche *et al*, 1998). The most commonly used basal medium at present is the Y3 formulation (Eeuwens, 1976). However, del Rosario (1984) found no difference between Murashige and Skoog (1962) and Y3 media. Her work indicated that glucose was better than sucrose for callus growth. A major problem in coconut tissue culture has been browning of tissue and its consequent death. To address this problem, the antioxidant used is activated charcoal (AC), which adsorbs even auxins and kinetins such as 2,4-D and Benzyl Amino Purine to the tune of 99.4% and 97.8%, respectively, at 5 days after culture media preparation (Ebert *et al*, 1993). This kind of inactivation of media supplements results in excessive use of auxins and cytokinins in the media in the media. Oropeza and Taylor (1994) used radio labelled 2,4 - D to study uptake by the coconut inflorescence

explants. The tissue took up most of the radioactivity within 24 h. At this time, the volume of the explant was only about one tenth of that of the external medium and the uptake of 2,4 -D occurred against a concentration gradient. Thus, uptake of radio-labelled 2,4 -D by coconut inflorescence cannot be explained by simple diffusion. Alternately, 2,4 -D may be taken up by facilitated diffusion. They emphasized the importance of pH for 2,4 -D uptake by coconut explants. Another auxins used was 2,4,5 -T which led to formation of nodular calli on the inflorescence explants (Buffard Morel *et al*, 1988). NAA and IAA resulted in direct embryogenesis in leaf explants (Raju *et al*, 1984).

Plantlet development was first achieved at CPCRI, Kasaragod, from tender leaf tissue explants taken from 1-2 year old WCT seedlings (Raju *et al*, 1984). However, this was not reproducible in subsequent trials. Profuse callus induction was achieved from immature zygotic embryos. Regeneration of somatic embryos from the embryogenic callus has been achieved but plantlet differentiation is not regular. Several experiments in this direction are in progress. Somatic embryogenesis is usually indirect in coconut and has to pass through callogenesis. Raju *et al* (1984) observed direct embryogenesis and embryoids were reported to arise from vascular tissue but Blake (1989) reported this to be unusual as this area normally gives rise to root primordia. Karunaratne and Periyaperuma (1989) reported that the embryogenic capacity of leaf explants was related to their physiological maturity in young palms of coconut. Leaf tissues from 12 to 24 month old palms were embryogenic but their potential was quickly lost with onset of juvenility. Even in young palms, explants of tender leaves responded differently as per their maturity. Only a particular leaf in a particular physiological state produces embryogenic cells and only a portion of this leaf yielded embryogenic explants (Karunaratne *et al*, 1991). This may be one of the reasons why experiments of Raju *et al* (1984) were difficult to reproduce. Sporadic reports of success were reported with leaf ex-plant by other workers too (Blake and Eeuwens, 1982; Shirke *et al*, 1993; de Siqueira and Inoue, 1992; Verdeil *et al*, 1993, 1994). Buffard Morel *et al* (1992) reported successful production of somatic embryos from leaf explants. Their study was supported by detailed histological observation. According to them, the primary formations resulted from mitotic divisions of perivascular cells and differentiation of a cambium-like layer insured the growth of nodular calli.

Tissue culture with other explants such as zygotic embryos, leaf base, apical meristem and endosperm was

also tried (Verdeil and Buffard Morel, 1995). Calli initiated from embryos, leaves, leaf bases, and the apical meristem could not be regenerated (Neera Bhalla Sarin *et al*, 1986). Callus induction from anthers and rachilla did not give repeatable response (Sarin and Suman Bagga, 1988). But, root explants (Jones, 1983), and sub-apical and leaf explants due to their limited embryogenic potential (Karunaratne *et al*, 1991) are of limited use. Immature inflorescences and immature embryos have been found to be promising. Blake and Eeuwens (1978) reported initial success using inflorescence tissue for callus production. They used immature rachillae on Y3 medium (Eeuwens, 1976) supplemented with 0.5µM NAA. Branton and Blake (1986) produced plantlets in 9 months from immature rachilla explants through somatic embryogenesis of nodular callus by reducing 2,4-D concentration in Y3 medium to 100µM 2,4-D, with 5µM each of 2ip and BAP, and 0.25% AC. Areza *et al* (1993) soaked the inflorescence tissue in antioxidants, viz., Citric acid (50mg/l) and Ascorbic acid (100mg/l), prior to slicing and culturing in Y3 medium supplemented with activated charcoal (AC), which resulted in reduced browning. Verdeil *et al* (1994) reported successful embryo maturation via somatic embryogenesis from inflorescence explants, which further regenerated into plantlets. They cultured immature inflorescences of coconut belonging to three different genotypes (PB-121, PB-111 & MYD) on agar medium supplemented with AC (0.2%) and a range of 2, 4-D (0.15 to 0.35 mM). Globular, white callus emerged from immature floral meristems, depending on inflorescence age and 2, 4-D levels. Immature inflorescences were most successful among the various explants tried, and plantlet regeneration was successful even though transfer of plantlets to nursery is yet to be achieved. Use of plumular tissues taken from germinating embryos was another source from where success was forthcoming, because of the juvenile nature of the tissue (Hornung, 1995). Buffard Morel *et al* (1995) used young, non-chlorophyllous leaves and immature inflorescences in Eeuwens inorganic nutrients supplemented with Morel and Wetmore vitamins, 30g/l sucrose, 2 g/l activated charcoal and 40 to 60 g/l 2,4 - D. They observed calli 6 - 8 months after culture initiation. They observed a multicellular pathway, which led to formation of meristematic and epidermised structures with low 2,4 - D (40 to 60 g/l). The first stage of development of these structures was characterised by fragmentation of the cambium-like zone and formation of complex meristematic structures, followed by their epidermisation. They observed a unicellular pathway, which led to the appearance and individualization of embryogenic cells

isolated by thick wall, with dense cytoplasm, a high nucleocytoplasmic ratio, and single, large nucleolus, and, starch and protein reserves. This pathway was the result of presence of high 2,4 -D concentration (80 - 120 g/l). Chan *et al* (1998) developed a protocol using plumules of zygotic embryos. They used Y3 medium supplemented with 0.1mM of 2,4-D, 2.5 g/l AC, solidified with 3g/l gelrite. Cultures were incubated for 3 months in darkness at 27°C. Calli bearing embryogenic structures were cultured on the same medium with 1µM 2,4-D and 50µM BAP under a photoperiod of 12-hour light at 27°C, and subcultured every three months. Plantlets were produced at 6 to 9 months. A procedure for regeneration of complete plantlets via organogenesis from plumular tissues of coconut was outlined by Rajesh *et al* (2005). Callus was induced from plumular tissues in Y3 media supplemented with either 2,4-D (74.6µM) alone or 2,4-D (74.6 µM) in combination with TDZ (4.54 µM). The frequency of callus induction increased and browning of explants decreased when cytokinin (TDZ) was added along with auxin (2,4-D) in callus induction medium. Calli were subcultured at monthly intervals on media containing low levels of 2,4-D and a constant level of either cytokinins (BA and TDZ) or polyamines (spermine and putrescine). Higher percentages of embryogenic calli, somatic embryoids and meristemoids were obtained on Y3 media supplemented with either spermine or BA. Plantlets with balanced shoot and root formation were transferred to pots and established in the greenhouse. Histological studies of differentiated tissues confirmed development of shoot buds (organogenesis) and typical bipolar embryoids (somatic embryogenesis).

Induction of somatic embryogenesis and plantlet regeneration from callus cultures of unfertilized ovaries isolated from immature female flowers of coconut was reported by Perera *et al* (2007). Ovary explants, when cultured on a medium containing 100 µM 2, 4-D and 0.1% activated charcoal gave rise to callus. Embryogenic calli were sub-cultured onto somatic embryogenesis induction medium containing 5µM abscisic acid, followed by subculture on plant regeneration medium (supplemented with 5 µM 6-benzylaminopurine). Somatic embryos formed were bipolar and germinated into normal plantlets.

Griffis and Litz (1997) used anthers and filaments, unfertilized ovaries and immature leaf pieces. Both callus initiation and direct initiation of somatic proembryos could be stimulated with addition of 2,4 -D to the culture medium. In a few cases, somatic embryos arose directly on filaments attached to immature anthers after several months in culture.

Unfertilized ovaries cultured in media supplemented with 2,4 -D and diethylstilbestrol (DES) monitored for 24 months indicated substantial fresh weight gain and numerous unusual morphogenic changes in ovaries on Y3 medium supplemented with 5 or 15 mg/l DES , 25 or 50mg/l 2,4 -D and 3 mg/l 2iP. Several unfertilized ovaries formed callus and adventitious roots but not somatic embryos. On similar media, some immature leaf tissues from seedling formed callus at the cut ends while others formed roots, or numerous somatic proembryos directly. Some pro embryos also developed haustoria like tissues or roots with obvious bipolarity, but further shoot apical development did not take place except in one case.

Abscisic acid is also reported to induce somatic embryogenesis in coconut. Recently, Fernando and Gamage (2000) induced nodular callus from 7-9 month old immature zygotic embryos in BM72 medium supplemented with 24 µM 2,4-D. This callus was subcultured onto a medium supplemented with 2.5 - 7.5 µM abscisic acid for 3 - 7 weeks, with subsequent subculture at 5 week intervals on media containing gradually reduced concentrations of 2,4 -D. They found incorporation of ABA to enhance production of somatic embryos. These embryos formed normal plants. Studies by Samosir *et al* (1999) indicated that development and maturation of coconut somatic embryos could be improved by using ABA alone, or, with any of the osmotically active agents preferably Poly Ethylene Glycol (PEG).

Immature zygotic embryos were more likely to undergo somatic embryogenesis than mature explants. Samosir *et al* (1998) used longitudinally sliced mature zygotic explants cultured on medium supplemented with 125-µM 2,4-D and 2.5 g/l activated charcoal. Plantlets successfully produced by application of NAA (10µM) which allowed for normal seedling growth to occur. Control of ethylene and polyamines was found to improve somatic embryogenesis in coconut. Adkins *et al* (1998) used cotyledonary slices from embryos cultured on medium with additives like aminoethoxyvinylglycine (AVG) and silver thiosulphate (STS) which could reduce ethylene production, or used polyamines such as spermine, putrescine and spermidine. Somatic embryogenesis was promoted by supplementation with AVG (2µM) or STS (3µM) or by the addition of putrescine (7.5µM) and spermine (1µM). STS also aided somatic embryo proliferation, maturation, and germination.

Use of zygotic embryo culture for germplasm collection, storage and retrieval was standardized and put

in to practice in India (Karun and Sajini, 1994; Karun *et al*, 1996). Koshy and Kumaran (1997) collected 15 accessions from the Indian Ocean Islands of Mauritius, Madagascar and Seychelles (Anon, 1998), and later, Parthasarathy (2001) used this technique to collect four accessions from Sri Lanka.

Sucrose might be important in early stages of coconut embryo cultures to maintain high chlorophyll concentration and a high number of chloroplasts. Continuous growth of the resulting plantlets in sucrose containing medium, however, can hamper development of photoautotrophy and, in turn, affect plantlet performance upon its transfer to soil.

***In vitro* conservation**

Coconut is a recalcitrant species where nuts do not undergo maturation drying and are shed at relatively high moisture content level (Parthasarathy, 1999). One of the earliest reports on cryopreservation of coconut embryos was reported by Chin *et al* (1989). They found that embryos cryoprotected with 10% DMSO showed highest per cent survival when cryopreservation was followed by 10% glycerol treatment. Earlier, Bajaj (1985) reported only elongation of whole embryos or proliferation of cut ends of transverse halves of the embryo after cryopreservation. He did not observe normal development. He used 7% DMSO and 4% sucrose as cryoprotectants and found the percentage of survival to be low (17 - 25%). Karunaratne *et al* (1985) reported one of the earliest attempts to preserve coconut embryos in culture in a dormant state. They devised a special 'survival medium', which suppressed the growth of embryos for a period of 5 months. Assy-Bah and Engelmann (1992a) found that immature embryos of coconut (7 to 8 months after pollination) could withstand rapid freezing in liquid nitrogen after 4 h of pregrowth on semisolid medium containing 600g/l glucose and 10% to 15% glycerol or sorbitol. In these conditions, survival ranged from 10% to 43% and one embryo developed into a rooted plantlet 2.5 months after freezing. While, in a later study, (Assy-Bah and Engelmann, 1992b) observed mature embryos (10 - 12 months after pollination) of four varieties of coconut to withstand cryopreservation in liquid nitrogen, which developed into plants. Pretreatment consisted of 4 h desiccation in the air current of a laminar flow cabinet followed by 11 to 20 h culture on medium containing 600g/l glucose and 15% glycerol. They carried out freezing and thawing with recovery rates between 33% and 93% of frozen embryos, depending on the variety. Assy-Bah and

Engelmann (1993) developed optimal conditions for medium-term conservation of zygotic embryos. After 6 months of storage on medium devoid of sucrose and containing 2g AC/l, 100% embryos developed into whole plantlets within 5 months upon transfer to recovery medium. After a 12-month storage period on medium containing 15g/l sucrose and devoid of activated charcoal, 51% of the embryos germinated within 2 months upon transfer to recovery medium. Presence of sucrose in the storage media was reported to initiate embryonic response to cellular expansion and elongation as well as cell division in the epidermal layer to keep pace with expanding tissues (Mkumbo and Hornung, 1997). Engelmann *et al* (1995) studied factors affecting cryopreservation of coconut embryos. They concluded that embryos should be used only when they are in an optimal physiological state, notably, their maturity and metabolic status. Modifications in recovery conditions can greatly increase survival rate of the zygotic embryos.

Critical moisture content of 20% was found to be essential for successful cryopreservation of coconut zygotic embryos using the desiccation method (Karun *et al*, 2005). A simple cryopreservation technique involving pre-growth desiccation with sucrose was developed for coconut zygotic embryos by Sajini *et al* (2006). Results showed that 1M sucrose was insufficient for dehydration of embryos. Using 3M sucrose for 24 h, the moisture content of embryos was reduced to 27% which resulted in 29% plantlet recovery in pots.

Karun *et al* (2006) developed a protocol for cryopreservation of coconut pollen. Germinability and pollen-tube growth in fresh, oven-dried and cryopreserved pollen was studied. Germination medium consisted of 8% sucrose, 1% gelatin, 1% agar and 0.01% boric acid. Germination percentage of cryopreserved pollen was found to be 38.54, which was significantly less than that in fresh pollen (46.65%). However, pollen tube growth was more vigorous in cryopreserved pollen. There was also significant palm-to-palm variation with regard to germination and pollen-tube growth.

Recently, several basic studies on physiological and biochemical aspects of somatic embryogenesis and regeneration have been published. Based on the reaction of coconut callus to somatic embryogenesis induction medium (SEI), Magnaval *et al* (1997) observed 3 types of responses, namely, (i) the traits that were modified by SEI condition and varying over time; (ii) traits modified by the SEI condition but constant over (iii) traits unchanged

by the SI condition over time. They studied specific nutritional requirement of coconut callus during these phases. In another study, the same team (Magnaval *et al*, 1995) classified the calli into five groups based on amino acid composition by the clustering method. Dussert *et al* (1995) presented a detailed study on nutrient uptake and *in vitro* growth of coconut callus. Another aspect of research worth a mention is the photosynthetic ability of *in vitro* grown coconut plantlets. Triques *et al* (1997) studied various photosynthetic parameters using complementary approaches. Transmission electron microscopic (TEM) studies revealed complete ultrastructural organization of chloroplasts in plantlets at the end of the *in vitro* culture process (6 weeks under light). Studies by Rival *et al* (1999) proved that coconut belonged to a class of plants in which *in vitro* grown leaves may contribute to autotrophy, and then, play an active part in planted acclimatization as indicated by a dramatic decrease in PEPC/Rubisco activity ratio and an increase in photochemical activity of PSII.

Conclusion

The above review would show the ample effort that has been put into research on coconut biotechnology. Unfortunately, in India coconut biotechnology has so far been carried out only at CPCRI. If one goes by history, there were nearly a dozen laboratories involved in coconut biotechnology in the eighties. When it was discovered that biotechnology is difficult in this crop, many laboratories stopped their work on coconut biotechnology. Harries (1999) rightly stated “A devil’s advocate, asked to say if clonal coconuts really do have any use, would have to admit that the rate of progress has been disappointingly slow. The early aims, to clone high-yielding individual palms as farm plating material, are now seen to be naïve. Academic studies may have earned higher degrees for research scientists but they have not spawned financially successful industries enjoyed by some other crops”. But, recent developments, we hope, prove Harries wrong.

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