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Review Article

Research potentialities of coconut biotechnology

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Introduction

The productivity of coconut needs to be increased substantially to have a chance in competition with other vegetable oils such as palm, soyabean, rapeseed and groundnut oils. The cloning of high yielding individuals or super palms could contribute to raising the productivity to the highest level possible. Commercial exploitation of coconut hybrids with high productivity has had limited success due to low seed multiplication coefficients, long juvenile period and lack of a repeatable protocol for cloning high yielding palms.

Standardization of a viable protocol for clonal propagation through *in vitro* approaches opens up tremendous possibilities of meeting the requirements for quality planting materials and breaking down productivity barriers.

In vitro culture of vegetative explants has been attempted at several coconut research centres from the early seventies. Research on embryo culture, cryopreservation, molecular characterization, protoplast culture and *in vitro* screening for biotic and abiotic stresses has been undertaken during the last 10 years.

Tissue culture

Attempts are being made to achieve a commercially viable protocol for coconut tissue culture at various research centres of coconut growing countries. The major problems in palm tissue culture are difficulties in acquiring explants, microbial contamination, browning of explants, delayed callus induction, low somatic embryogenesis and poor germination of regenerated plantlets (Reynolds, 1982).

Explants

The choice of explant is pivotal in tissue culture of mature tree species. Cloning of 'proven' adult trees for their genetic superiority is to be preferred over cloning of embryos or seedlings for obvious reasons. However, the sampling of meristematic tissues from adult trees is a difficult process and may even result in the death of the tree. Non-destructive sampling of juvenile tissues from adult coconut palm is carried out by excising the tissues immediately above the meristematic region. The fibrous leaf sheaths encompassing the stem of the coconut are extremely tough and can only be removed with great difficulty. Raju *et al.* (1988) described a method for extraction of tender spindle tissues of mature palms. Two whorls of leaves outside the spindle were first removed and the central column of the spindle was exposed by cutting away the spindle 15-20 cm above the shoot meristem. Leaf lamina and leaf bases up to 5 mm in size were then utilized as explants.

The inflorescence is also being used as a source of explants in coconut. Non-destructive sampling of the spadix from coconut palms was described by Blake and Eeuwens (1978) and Rillo (1989). The first 4-5 infloresences from the spear leaf are removed after trimming the leaves to reduce heaviness and breakage, and the abaxial surface of petioles is cut at about the level of the inflorescence. Petiole tissues are then removed carefully to expose the inflorescence. Best response is reported when the central portion of the infloresence is used by excluding the top and basal portion of the fourth and fifth inflorescence (Sugimura and Salvana, 1989).

The explants from juvenile palms include tissues from apices, tender leaves, leaf bases and the leaf sheath. Immature zygotic embryos (about 7 months after anthesis) are also used as explants.

Culture media and conditions

Two basic media have generally been used for *in vitro* culture of palms, viz. Eeuwens' (1976, 1978) Y3 medium and Murashige and Skoog's (1962) MS medium. For coconut, better response was reported in Y3 medium (Avril *et al* . 1986). The growth regulators 2,4-D, NAA, IBA, 6-γ-γ-dimethylallylaminopurine (2-iP), kinetin and benzyl aminopurine (BAP) [benzyladenine] are commonly used. Generally solid or semi-solid media are used for coconut tissue culture; agar is added for solidification.

Following surface sterilization, the explants are inoculated in the culture medium and kept in the dark until callus induction (about six months). Once the calli are induced, the cultures are incubated under illuminated conditions (up to 2500 lux). The temperature and relative humidity are to be kept constant in the culture rooms (27± 2°C; 60-70% RH). Periodic subculturing may be practiced at monthly intervals.

Severe browning of explants (up to 80%) immediately after inoculation or during the early period of culture is a major cause of concern in coconut tissue culture. The oxidation of polyphenols, which leads to the formation of highly toxic quinones, is responsible for browning (Nair *et al.*, 1995). Browning is found to be greater using explants of adult palms. The addition of activated charcoal in the nutrient medium is beneficial, but often that alone is not sufficient. The following procedures are suggested to reduce browning: (a) pretreating explants in sterile water/antioxidant medium, (b) pre-culture of explants in liquid medium to leach out the polyphenols, (c) preculture of explants in a medium containing phenol adsorbents like PVP, AC and polyphenol oxidase inhibitor, DIECA and (d) use of a reducing agent in the medium (cysteine hydrochloride). Rillo and Ebert (1993) reported the role of activated charcoal in preventing browning with inflorescence as the explant.

Callus induction and regeneration

Callus induction takes about 4-5 months of incubation in the dark in a medium supplemented with high concentration of auxins. Callus initiation is generally from the meristematic/cambial areas of explant tissue, viz. vascular tissues of the leaf, basal meristems of young pinnae, floral meristems of young inflorescences and embryonic tissues. Type of calli initiated is determined by physiological maturity of explant as well as level and type of auxins used. The calli derived from the various explants were reported to be similar; they were nodular, cream-coloured and quite friable. Gupta *et al.* (1984) obtained globular calli from explants of the leaf base, leaf lamina and infloresence after 10 weeks of inoculation.

With immature zygotic embryos the callus induction was reported to be within one month of culturing. The callus was derived from the periphery of the cotyledonary sheath. The types of calli observed were nodular, knobby (irregular in shape) and granular. Rillo and Ebert (1993) obtained callus after 3 weeks of inoculation in 2,4-D medium. D'Souza *et al.* (1980) reported protocorm and bud-like outgrowths from cotyledonary regions of the embryo; upon subculturing these buds turned green and developed shoot-like internal structures. Similar results were obtained by Karunaratne and Periyapperuma (1989) and Shivashankar and Sajini (1994).

Exposing the callus to light, and reducing the level of auxin (Thanh-Tuyen and Apruillo, 1994; Verdeil, 1993) and introducing cytokinins (Hornung, 1995; Chan *et al.,* 1998) in the medium, generally resulted in somatic embryogenesis. Percentage of calli forming embryos in a 12-h photoperiod was greater than that of calli forming embryos in the dark (Chan *et al.,* 1998). In coconut, once the initial phase of embryo induction has been achieved, the auxin level must be reduced in a gradual and controlled manner to achieve balanced development of plantlets with a well organized shoot meristem and normal adventitious root formation. Sudden removal of auxin results in massive development of haustorial tissue and precocious root proliferation at the expense of continued shoot meristem development (Branton and Blake, 1983). Specific nutritional requirements of coconut inflorescence calli during somatic embryogenesis induction were studied in detail by Magnaval *et al.* (1997).

Raju *et al.* (1984) obtained few clonal plantlets via callus from leaf tissue of young coconut seedlings. Seedling tissues from apices, tender leaves, leaf bases and leaf sheaths were first cultured in Y3 medium supplemented with various combinations of auxins (NAA and 2,4-D) and cytokinins (kinetin, BAP and 2-iP). The cut surfaces of the tender leaf bases of one-year-old seedlings showed profuse callusing in medium supplemented with 2, 4-D (10 mg/litre) and kinetin (0.5 mg/litre), whereas tender leaf lamina segments showed only limited callusing. Subsequently these leaf cultures showed profuse rooting. Single leaf explants could produce up to 48 somatic embryoids. The individual embryoids had a tripolar structure with root and shoot poles and an incipient haustorial bulge. Initially, shoot growth was promoted by cutting away the root pole and a part of haustorium. Subsequently, the embryoids gave normal green shoots with scale leaves and sheathing leaf bases, primary roots with root caps, and an incipient haustorium. Embryoid development from the callus initiated from coconut leaf tissues was reported by Pannetier and Buffard-Morel (1982) and Bhaskaran (1985).

Immature rachillae explants excised from the leaf axils in Y3 medium supplemented with cytokinins (2 mg/litre each of BAP and 2-iP) resulted in the formation of shoot-like structures, some of which turned green but produced only roots (Kuruvenashetty and Iyer, 1980). Branton and Blake (1983, 1984) reported somatic embryogenesis and plantlet regeneration from tissues of coconut inflorescence. Neera-Bhalla-Sarin and Suman-Bagga (1988) used different explants like rachillae and anthers, etc., but a repeatable protocol could not be obtained. Among different media combinations and explants tried, Mascarenhas *et al.* (1988) obtained the best response in Y3 medium with 2,4-D (4.52×10^{-4} µM), calcium-pantothenate (2.1 µM) and biotin (4.10 µ M) and activated charcoal (0.25%) using inflorescence tissues as the explant.

Verdeil *et al.* (1994) achieved plantlet regeneration through somatic embryogenesis when immature inflorescences of coconut were cultured in a solid medium. The nutrient medium was Y3 modified with inorganic nutrients and vitamins (Morel and Wetmore, 1951) and supplemented with 116.8 mM sucrose, 2, 4-D ($1.5\text{-}3.5 \times 10^{-4}$ M) and 2 g/litre activated charcoal. Somatic embryogenesis was observed from the globular white callus from floral meristem. Induction and growth of callus derived from rachilla explants of young inflorescences of coconut palm was reported by Sugimura and Salvana (1989).

The feasibility of *in vitro* regeneration from immature inflorescence of tall cultivars was investigated at the Albay Research Center, Philippines (Cueto *et al.,* 1997). Nodular calloids were observed from the base of the floral buds. Somatic embryo maturation and germination was observed after 16-17 months in media containing 2,4-D (3×10^{-5} M) and BAP (5×10^{-5} M) and 2-iP.

Verdeil *et al.* (1998) reported the production of *in vitro* plantlets by somatic embryogenesis from immature inflorescence explants. Three concentrations of 2,4-D (44, 55 and 66 mg/litre) were used to ensure reproducible callogenesis. Calli isolated after 4-6 months were subcultured on a medium containing thidiazuron (TDZ) and a small quantity of 2,4-D. After two subcultures, TDZ was replaced by isopentenyladenine (iPA). The somatic embryos formed were matured in a medium containing only cytokinins (iPA or BAP). The limitations of this protocol were poor embryo germination rate, slow development of *in vitro* plantlets and difficulty in acclimatization.

Plantlet regeneration from zygotic embryos (8-10 months old) as explants was reported by Neera-Bhalla-Sarin *et al.* (1986), but the plantlets could not be established in soil due to poor root growth. Callus induction was obtained in B-5 medium with IAA-amino acid conjugates (IAA-aspartate and IAA-alanine, 2 mg/litre). Shoot and root differentiation occurred when the calli were transferred to B-5 medium with 2 mg/litre each of IAA-aspartate, kinetin or NAA, and three plantlets were derived in B-5 medium with NAA (0.5 mg/litre), BAP (2 mg/litre) and PVP (1.0 mg/litre).

Immature embryos, possessing high regenerative potentials, were found to be excellent material for *in vitro* propagation. A complete process of plant regeneration through callus induction and somatic embryogenesis from cultured zygotic embryos of two cultivars of coconut from the Philippines (Baybay Tall and Dwarf) was established by Thanh-Tuyen and Apruillo (1994). Callus initiation and regeneration media were macro-elements of Murashige and Skoog (1962), micro-elements of Y3, and vitamins of Nitsch (1969), 100 mg/litre *myo*-inositol, 300 mg/litre casein hydrolysate, 5% sucrose, 0.25% activated carbon, supplemented with 20-40 mg/litre 2,4-D, 1-5 mg/litre BAP and 1-5 mg/litre 2-iP.

Micropropagation of *Cocos nucifera* from plumular tissues excised from mature zygotic embryos was described by Hornung (1995). Calloids were obtained from plumular tissues excised from embryos extracted nine to eleven months after pollination. Embryogenic calloid production was assessed after pulse and continuous exposure of the tissue to varying amounts of 2,4-D. Plumular tissue gave calloid consistently in over 75% of the cultures which were exposed to media containing 4×10^{-4} M 2, 4-D. None of the remaining parts of the embryo produced calloid. After removal of the plumule, 7-month-old immature nuts from 25-year-old adult palms of West Coast Tall (WCT) in MS medium supplemented with 2,4-D (100 mg/litre), adenine sulphate (50 mg/litre) and NaH_2PO_4 (100 mg/

litre) gave rise to tiny nodular structures near the cotyledonary node which gradually proliferated further into a compact, white transparent callus mass in 4-5 weeks. The haustorial tissue which had expanded considerably was cut and removed to allow rapid growth of the callus. Transferring to the same basal medium supplemented with 10 mg/litre NAA and 2 µM thidiazuron resulted in the formation of roots with suppressed shoot formation (Ajit and Rajesh, 1996).

Calloid formation and plantlet regeneration from plumular tissue of mature zygotic embryo of coconut was studied by Hornung (1995). Pulse treatment of plumular explants with 2,4-D produced calloid at 5 or 8×10^{-4} M and 1, 2 or 5×10^{-3} M for 48 or 96 h. Chan *et al.* (1998) were able to regenerate plantlets from plumule explants through somatic embryogenesis. The culture medium for callus induction was comprised of Eeuwens Y3 medium supplemented with 0.1 mM 2,4-D. After 9-12 months of culture, calli bearing embryos were formed in a medium containing 1 µM 2,4-D and 50 µM 6-BAP.

Protoplast isolation and culture

The successful isolation of protoplasts from coconut embryos has been reported by Haibou and Kovoor (1982). An enzyme mixture consisting of 20 mg/ml pectinase, 10 mg/ml cellulase (*Aspergillus niger*) and 30 mg/ml cellulase (*Trichoderma viride*) in 0.5 M mannitol and the antibiotic carbenicillin (0.3 mg/ml) was used. Incubation at pH 5.5 and 20°C for 18 h released viable protoplasts which were carefully seperated from the numerous needle-like raphides by immediate sedimentation using a percoll gradient. Cell wall regeneration and cell division were observed together with microcallus formation, and no development was observed past the microcallus stage.

Anther culture

The heterogeneity of coconut palm results in greater variability in hybrid progenies. The success of experimental haploid production in many species and the natural occurrence of haploid coconut embryos has prompted efforts to produce haploid plants by anther culture. However, these attempts have had limited success, since embryos originating from microspores never developed beyond the torpedo stage.

Kovoor (1981) reported the development of callus from cultured coconut anthers; the response rate was only one out of thousands of anthers cultured. Haibou (1981) attempted to obtain direct embryogenesis from microspores. A significant percentage of cultured anthers showed dehiscence and extrusion of globular pollen, typical of the first proembryoidal stages in direct embryogenesis. The globular embryos were formed with extremely low frequency. Thanh-Tuyen and de Guzman (1983) reported formation of structures similar to heart-shaped torpedo stages of embryo development from pollen grains taken from coconut inflorescence before splitting of the spathe. Of several media combinations tested, the most suitable for embryoid formation appeared to be modified Blayde's and Keller's media supplemented with 6-9% sucrose,15% coconut water, 0.5% activated charcoal, and 2 p.p.m. NAA.

Endosperm culture

Kumar *et al.* (1985) obtained a fast growing friable callus from coconut endosperm which was in contact with zygotic embryo, and maintained by repeated sub cultures on Y3 medium with lowered auxin levels. Callus showed high aneuploidy and abundant oil globules when maintained for over eight weeks without subculturing. Hence, this system could be used for extraction of edible oil and other metabolites like cytokinins and amino acids that are known to be present in the coconut endosperm tissue.

Embryo culture for coconut germplasm exchange

Large size of the seed, water content and stringent phytosanitary requirements are the major problems in germplasm collection, transportation and storage of coconut seednuts. The technical guidelines finalized recently by the FAO/IPGRI for the safe movement of coconut germplasm emphasizes collection and transportation through embryos instead of seed nuts (Diekmann, 1997). A commercial application of embryo culture is the rescue and culture of Macapuno variety in the Philippines (de Guzman 1970; del Rosario and de Guzman 1976, 1981). The three components of an embryo culture protocol for germplasm movement are field collection of embryos, *in vitro* active conservation and retrieval and *ex vitro* establishment of the seedlings.

Field collection techniques

Zygotic embryos of coconut are collected by split opening of the dehusked nut. Simple and portable equipments can only be used for collection and direct inoculation of embryos in the field. Sossu *et al.* (1987) used a portable collapsible glove box instead of a laminar flow hood. The endosperm with seed were surface sterilized with 5% calcium chloride for 15-20 minutes and the excised embryos were treated with 2% calcium hypochloride for 2-5 minutes. Embryos were transferred to the culture flasks, whose inner surface was swabbed with alcohol.

Raju and Bavappa (1987) used simple portable equipment to collect embryos aseptically in the field in a culture tube and subsequently transferred them to fresh media. The risk of contamination of embryos can be reduced if they are inoculated individually. The field collection procedure was therefore modified by Anitha Karun *et al.* (1993). The embryos were scooped out with a portion of endosperm in the field by using a cork-borer from the dehusked and split open nut. The extracted embryo from the endosperm was placed in distilled water followed by surface sterilization with 50% chlorine water for 20 minutes. Embryos were washed with sterile water 3-4 times and directly inoculated individually to vials containing 5-10 ml of Y3 medium/sterile water. The entire operation was carried out in the field using a folding type portable inoculation hood made of plexiglass, surface sterilized with alcohol.

In vitro active conservation

In vitro active conservation (short-term storage) of coconut zygotic embryos becomes necessary when the collection sites are located in far-off places. Assy-Bah *et al.* (1987) could store the endosperm cylinders in KCl solution (16.2 g/litre) for a period of 14 days. Various studies indicated that germination of coconut embryos and their further development depended greatly on the levels of activated charcoal and sucrose. The absence of either charcoal or sucrose is therefore expected to inhibit germination of embryos and their further growth (de Guzman *et al.*, 1971; Karunaratne *et al.*,1985; Assy-Bah *et al.*, 1987 and Rillo and Paloma, 1990).

Karunaratne (1988) stored the embryos in slow growth phase for 5 months in an agar based medium containing nutrient barely sufficient for their survival. After 2, 3 and 5 months of storage, embryos were transferred to retrieval medium for resumption of growth, and germination was found to be 53, 40 and 32%, respectively, against 61% germination in embryos without storage.

Assy-Bah and Engelmann (1993) stored dwarf coconut embryos for 6 months in a medium containing 2 g/litre activated charcoal without sucrose and obtained 77% germination. They also found that increased levels of sucrose in the absence of charcoal affected the viability of embryos and that germination was negligible in the absence of charcoal and sucrose.

Anitha Karun and Sajini (1994a) observed that zygotic embryos can be stored in sterile water for 2 months. Compared to nutrient media, the chances of contamination of cultures are minimal in sterile water. About 80% of the stored embryos germinated when cultured in retrieval medium. Germination dropped to 13% when stored for 6 months. Embryos can also be stored for 2 months in sterile coconut water (Anitha Karun *et al*., 1997).

Prolonged storage of cultures at 10°C and 4°C for 3 months caused irreversible damage to the coconut embryos and no plants were recovered when transferred to the germination medium (Karunaratne, 1988). Mature and immature embryos of WCT stored at 10°C for one month did not germinate upon transfer to retrieval medium (Anitha Karun *et al*., 1997).

Cryopreservation of coconut germplasm

Coconut genetic resources are traditionally conserved *ex situ* in gene banks. The use of *in vitro* culture techniques including slow growth and cryopreservation represents an important additional option for the safe medium and long term conservation of coconut germplasm. These techniques were studied in detail by Engelmann *et al.* (1997). They could successfully cryopreserve and retrieve plantlets from nuts of 7-8 months after pollination. Performance of immature embryos is good due to their small size (2-3 mm length, 10-20 mg in weight) and low degree of differentiation. Immature embryos can withstand rapid freezing in liquid nitrogen after 4 h of pretreatment on a semi-solid medium containing 600 g/litre sucrose and 10-15% glycerol or sorbitol. Large size and advanced differentiation of mature embryos render their potential use in cryopreservation problematic. The technique developed for immature embryos was successfully applied to mature embryos of four varieties (Hybrid PB 121, Cameroon Red Dwarf, Indian Tall, Renell Tall) (Engelmann,1997). After a 4-h desiccation period in the air current of a laminar flow cabinet, embryos were pre-treated for 11-20 h on a medium containing 600 g/litre sucrose and 15% glycerol, then rapidly immersed in liquid nitrogen. Whole plants could be produced from 73-93% of cryopreserved embryos, depending on the variety, with only a slight delay in their regrowth rate in comparison with the control embryos.

Bajaj (1984) studied the survival of frozen WCT zygotic embryos of 1-1.5 cm in size. Following storage for one month, the embryos were partially dehydrated and then cut into two transverse halves. After pretreatment with cryoprotectants (7% DMSO + 7% sugar in MS liquid), the embryos were blotted dry, wrapped in sterile aluminium foils, and frozen by gradually lowering into liquid nitrogen for 5 minutes. They were thawed in warm water (35 to 40°C), washed and cultured on MS + 2,4-D (0.2 mg/litre) + NAA (0.5 mg/litre) + kinetin (0.1 mg/litre). The retrieved coconut embryos and their segments showed a lag period of 4 months without any sign of growth. This indicated the possibility of long-term conservation of coconut germplasm.

In vitro retrieval

A number of reports have appeared on the use of liquid nutrient medium for *in vitro* retrieval of coconut embryos (Sossou *et al.*, 1987; Karunaratne, 1988; Ashburner and Thompson, 1991; Rillo and Paloma, 1992). However, Anitha Karun *et al.* (1993) observed better germination and survival in solid medium. Lack of adequate rhizogenesis was one of the main reasons for the poor performance of embryo culture-derived seedlings. Increase in rooting induction has been achieved by transfer of cultures from liquid to solidified medium (Balaga and de Guzman, 1971), increasing the sucrose levels (de Guzman *et al.*, 1971; del Rosario and de Guzman, 1976), supplementing the medium with NAA (Ashburner *et al.*, 1993) and transferring the cultures to liquid medium supplemented with IBA (5 mg/litre) and NAA (1 mg/litre) (Anitha Karun *et al.*, 1993). Inorganic constituents of culture medium have been used by Balaga and de Guzman (1971); del Rosario and de Guzman (1976) and Miniano and de Guzman (1978).

Abraham and Thomas (1962), D'Souza (1980), Jagadeeshan and Padmanabhan (1982), Gupta *et al.* (1984), Padmanabhan (1982, 1988) Mascarenhas *et al.* (1988) and Kalamani and Sree Rangaswamy (1990) have also attempted to develop protocol for coconut embryo culture.

Most of the aforesaid experiments were carried out using mature embryos (about 11 months old). Such a restriction, however, may limit the number of available nuts during germplasm expeditions. Anitha Karun *et al.* (1993) therefore investigated the feasibility of collecting embryos of 8 to 10 months old. Germination percentage of 8-month-old embryos was lower than that of matured ones. Better responses could be obtained when sucrose was replaced with glucose in the medium.

Ex vitro studies

The rooting of the germinated plantlet was observed to be a major problem in *in vitro* retrieval. In a number of experiments the *in vitro* retrieved seedlings were reported to have high mortality upon transplantation to soil due to poor root development. A positive correlation was observed between root intensity and survival in pots (Anitha Karun *et al*., 1993; Anitha Karun and Sajini 1994b). This protocol uses a potting mixture consisting of sterile soil, sand and coir dust in equal proportions. As a precautionary measure before transplanting to the pots, the seedlings were treated with Bavistin [carbendazim] (1 g/litre) and thereafter with IBA solution (1000 p.p.m.) for one hour each. The leaves were trimmed before transplanting to reduce transpiration. Higher humidity condition was maintained initially by covering plantlets with polythene bags. Humidity was reduced gradually by providing perforations to the polythene bag and later lifting the bag during night time and thereafter completely. Hoagland's solution was given to the plantlets once a month. The establishment of the plantlets in the pots, polybags and field was found to be very satisfactory and comparable with establishment of true seedlings (Anitha Karun and Sajini, 1994b).

Applications

The protocol for embryo culture developed by Anitha Karun and Sajini (1994b) was successfully used in two germplasm expeditions. The first was the collection and transportation of 87 embryos of six Pacific Ocean accessions maintained at the World Coconut Germplasm Center (CPCRI), Andaman Islands, India. The embryos were retrieved *in vitro* at Kasaragod and successfully transplanted to the field (CPCRI, 1994-95). The same protocol was followed for the subsequent collection of 15 exotic coconut germplasm from Mauritius, Madagascar and Seychelles (Kumaran *et al*., 1998).

In vitro *screening for drought tolerance*

Karunaratne *et al.* (1991) studied the feasibility of developing an *in vitro* technique for screening drought tolerant coconut germplasm. Water stress in the culture system was progressively increased with each passage, by incorporating polyethylene glycol (PEG-6000), mannitol and sodium chloride into culture medium. PEG and mannitol were found to have growth inhibitory action even at low concentrations. Some 21% of embryos of the Sri Lankan Tall variety died before reaching water stress levels of 170 mM NaCl or above; about 78% survived at 170 mM NaCl concentration and only 12.6% were able to resist a concentration of 320 mM NaCl. When zygotic embryos derived from two known drought susceptible cultivars of coconut, CRIC-65 and Dwarf (from *Pumila)*, were tested using the technique, 29 and 73% of embryos, respectively, died due to stress damage at the 170 mM NaCl concentration, and no plants of either cultivar survived at salt concentrations of 230 mM. However, embryos that originated from two putative drought tolerant cultivars showed a higher survival rate when subjected to salt stress. At 170 mM NaCl all the embryos developed into seedlings. In fact, germination percentage of embryos was higher at 170 mM NaCl concentration than in the absence of NaCl. Survival percentage gradually dropped with increasing salt concentration, with about 18% of plants surviving at the 330 mM NaCl concentration.

DNA marker technology for characterization of genome

Molecular marker technology has potential uses for germplasm identification and genetic mapping of coconut palms. The construction of the first linkage map in coconut was reported by Rhode *et al*. (1997).

Investigations in coconut pertaining to biochemical markers such as isoenzymes and molecular markers (restricted fragment length polymorphism (RFLP), randomly amplified polymorhic DNA (RAPD), amplified fragment length polymorphism (AFLP) and sequence tagged microsatelites (STM)) have only recently been undertaken. A protocol for detection of isoenzyme polymorphism in coconut leaf tissues was reported by Fernado and Gajanayake (1997). They proposed that esterase was useful for studying the genotypic variation in coconut. Rhode *et al.* (1992) described a novel approach for the analysis of coconut germplasm using coconut specific primers complementary to the copia-like *Eco*R1 elements. PCR amplification of spacer regions for a subset of tandemly arranged repeats detected polymorphisms that allowed an analysis of biodiversity within coconut populations. Ashburner *et al.* (1997) reported the first successful use of RAPD analysis in coconut to identify diversity of coconut in the South Pacific region. They found moderate levels of genetic diversity, although very few RAPD markers were unique to specific populations.

As a part of DNA fingerprinting of coconut germplasm using RFLP/ RAPD markers, the DNA extraction protocol and RAPD protocol were standardized at CPCRI, Kasaragod (Anuradha *et al.,* 1998).

Lebrun *et al.* (1997) studied the genetic diversity of coconut by using RFLP markers. A total of 289 palms of 26 tall and 16 dwarf cultivars originating from the major coconut cultivation areas were analysed using 20 nuclear probes from coconut, oil palm, rice and maize and cytoplasmic probes from wheat, in combination with 4 restriction enzymes. The results showed two main groups of tall coconuts originating from South-East Asia and Pacific Ocean and from the Indian Continent. Cultivars from East Africa and from Andamans shared markers of both groups. All Dwarfs (excepts Niu Leka) formed a very homogeneous group.

Genetic characterization of coconut has been based on vegetative and yield traits. Wadt *et al.* (1997) evaluated the genetic divergence and the variability of the tall coconut found in different locations using RAPD markers. Three ecotypes were used: Praia do forte (GBrPF) from Brazil, a tall coconut from West Africa (GOA) and a tall coconut from Rennell (GRL), all maintained in the coconut active germplasm bank (BAG-Coco) at EMBRPA/CPATC-Sergipe, Brazil. Leaf samples from 21 individuals from each ecotype were obtained for DNA extraction and amplification. RAPD analyses were performed using 18 different decamer primers of which 6 were selected for analyses. The six primers defined 49 loci, of which 43 were polymorphic among accessions. These results suggested that the analysed populations have an elevated degree of inbreeding. The ecotype GRL is distant from the other two; GOA and GBrPF are relatively close to one another, yet still distinct. The mode of dissemination of coconut in West Africa and South America may be responsible for the similarities between GOA and GBrPF.

Perera *et al.* (1998) studied the genetic relationships between indigenous coconut accessions from Sri Lanka by means of AFLP profiling. They conducted the PCR-based DNA profiling of coconut palms indigenous to Sri Lanka by AFLP. A total of 322 amplification products were generated from 42 genotypes with 8 pairs of primers (*Eco*R1 and *Mse*1). 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 into between- and within-form components. This revealed that for the inbreeding dwarf and intermediate forms most variation was observed between rather than within forms. According to AFLP analysis, the *Aurantiaca* group is closer to the dwarf group than the tall group.

Development of coconut microsatellite primers and high degree of polymorhphism was reported by Rivera *et al.* (1997) in coconut populations maintained by the Philippines Coconut Authority.

Molecular detection and diagnosis of pathogens

Coconut improvement in the past has been achieved mainly through the introduction of new germplasm followed by evaluation and selection. While transporting the germplasm there is a risk of the introduction of new plant pathogens such as viruses, viroids, phytoplasma, bacteria,fungi nematodes, protozoa and algae. To detect the pathogen at a molecular level, a single specific molecular test or a combination of molecular techniques are needed for valid detection. Molecular analysis involves detection and diagnosis of pathogens based on pathogen specific nucleic acids or pathogen-specific antigens such as proteins produced upon binding of an antibody probe(s) to specific epitope(s) on pathogen protein.

Molecular techniques for the detection and differentiation of pathogens in the case of lethal yellow (Continental Africa, America and the Caribbean region), cadang-cadang (Philippines), Tinangaja (Guam Island), foliar decay diseases (Vanuatu) and stem bleeding diseases are currently being developed.

Coconut lethal yellow

Coconut lethal diseases such as Cape St. Paul wilt in Ghana, Awka disease in Nigeria and coconut lethal disease in Tanzania, Kenya and Mozambique have killed millions of palms causing severe economic losses. Using the Polymerase Chain Reaction (PCR), the 16S rRNA genes and the 16S-23S spacer regions of phytoplasma associated with these lethal coconut diseases in Africa were amplified. Following sequencing of the rDNA products, two primers were designed which were specific for the diseases found in either East or West Africa. Neither of these primers, when paired with a universal primer, produced PCR amplification products from healthy coconut DNA, infected coconut DNA from the Caribbean or DNA from a variety of periwinkle-maintained phytoplasma. Sequence analysis of the coconut 16S rDNA has shown that the phytoplasma found in West Africa have greater than 99% similarity, while similarity between phytoplasma from a primary cluster within the phytoplasma clade and the pathogen causing the diseases in West Africa formed a new sub-clade within this cluster (Jones and Tymon, 1997). Cardena *et al.* (1997) identified RAPD markers linked to lethal yellowing resistance by three-stage selection of primers based on their ability to amplify RAPDs in (i) the DNA pool of either the Malayan Yellow Dwarf (resistant) or the West African Tall (susceptible) genotypes, (ii) all individuals that originated from the respective DNA pools, and (iii) all individuals from other resistant or susceptible populations.

Cadang-cadang

Cadang-cadang is a lethal disease of coconut in central Philippines. Diagnosis of the disease on the basis of symptoms alone is unreliable, particularly in the early stage of infection as there are hardly any discernible symptoms. The causative agent is the coconut cadang-cadang viroid (CCCVd), one of the smallest known viroids which is only 246 nucleotides in size. Unlike other viroids, sequence variation has been observed in CCCVd and is related to the stage of disease development. Detecting the pathogen types requires either a single specific molecular test or combination of several molecular techniques. An effective molecular indexing for coconut pathogens requires an analysis of the nucleic acid or protein component of the pathogen, development of an optimal extraction for the pathogen and specific analysis procedures (nucleic acid hybridization, PCR, sequencing, immunology) in conjunction with development of a sensitive probe/primer labelling and detection procedure (radiolabelling, DIG, biotin and autoradiography, chemiluminiscence, fluorescence or colorimetric detection).

Coconut cadang-cadang viroid (CCCVd), the viroid associated with CCCVd, and viroid-like sequences (VLS) related to CCCVd and Tinangaja disease were detected and differentiated in the hybridization assay using diagnostic oligonucleotide-probes (DOP) and by reverse-transcriptase polymerase chain reaction (RT-PCR). (Hodgson and Randles, 1997).

Foliar decay disease: (CFD)

Coconut foliar decay is a lethal disease of introduced coconut palm cultivars in Vanuatu. Small amount of a disease-specific single-stranded DNA of low electrophoretic mobility in polyacrylamide gels can be detected in palms with clear symptoms. CFDV DNA comprises 1291 nucleotides and contains open reading frames for six potential proteins of MW > 5 kDa. Diagnosis of CFDV is most readily done by hybridization using randomly primed radioactive cDNA probes, or non-radioactive cRNA probes transcribed from a plasmid vector. With PCR, a pair of oligonucleotide primers representing the linear amplification of CFDV DNA and the PCR product are analysed on 1% agarose gels and detection of a single band of size 1.3 kb is considered to be diagnostic for the presence of CFDV DNA in the extract (Randles *et al.,* 1997).

Stem bleeding

Electrophoretic protein patterns of coconut isolates of the stem bleeding pathogen *Thielaviopsis paradoxa* were investigated at CPCRI, Kasaragod by Ramanujam *et al.* (1996). Mycelial proteins of twelve isolates of *T. paradoxa* isolated from stem bleeding-affected coconut palms in Kerala, Karnataka and West Bengal were studied using SDS-PAGE. Considerable variations in the protein band patterns were noticed in the twelve isolates. Based on the similarity coefficient determined from the protein patterns, the twelve isolates were grouped into two main clusters. The data on virulence of these isolates have been made use of to investigate the relationship between protein patterns and virulence.

References

Abraham, A.; Thomas, K.J. (1962) A note on the *in vitro* culture of excised coconut embryos. *Indian Coconut Journal* **15,** 84–88.

Ajit, K.S.; Rajesh, M.K. (1996). Callus induction and regeneration from immature zygotic embryos of coconut (*Cocos nucifera* L.). Paper presented *at National Symposium on Horticulture Biotechnology* held at Bangalore during 28-30 October 1996, Abst no. II-78, pp. 30.

Anitha Karun; Sajini, K.K. (1994a) Short term storage of coconut embryos in sterile water. *Current Science* **67** (2) 118–120.

Anitha Karun; Sajini, K.K. (1994b) Coconut embryo culture: *In vitro* germination and *ex vitro* establishment. Paper presented at *National Development in Plant Molecular Biology and* Genetic *Engineering*, 29-31 December 1993, TNAU, Coimbatore, pp. 23.

Anitha Karun; Sajini, K.K.; Iyer, R.D. (1997) *In vitro* active conservation of coconut zygotic embryos. *Journal of Plantation Crops* **24** (Suppl), 586–593.

Anitha Karun; Shivashankar, S.; Sajini, K.K.; Saji, K.V.(1993) Field collection and *in vitro* germination of coconut embryos. *Journal of Plantation Crops* **21** (Suppl), 291–294.

AnuradhaUpadhyay; Parthasarathy, V.A.; Seema, G.; Anitha Karun (1998) A protocol for DNA extraction from coconut leaves. Paper presented at *Commerecial Aspects of Plant Tissue Culture, Molecular Biology and Medicinal Plant Biotechnology*, held at Jamia Hamdard (Hamdard University), New Delhi, 25-27 Feb, 1998. Abstract No GE-16, pp.102.

Ashburner, G.R.; Thompson, W.K. (1991) Coconut embryo culture for the international transfer of germplasm. In: *Advances in Coconut Research and Development* [edited by Nair, M.K.; Khan, H.H.; Gopalasundarum, P.; Rao, E.V.V.B.]. New Delhi, India: Oxford and IBH Publishers, New Delhi. pp. 43–49.

Ashburner, G.R.; Thompson, W.K.; Halloran, G.M. (1997) RAPD analysis of South Pacific coconut palm populations. *Crop Science* **37**, 992–997.

Ashburner, G.R.; Thompson,W.K.; Burch,J.M. (1993) Effect of α-naphthalene acetic acid and sucrose levels on the development of cultured embryos coconut. *Plant Cell Tissue, and Organ Culture* **35**, 157–163.

Assy-Bah, B.; Durand-Gasselin, J.; Pannetier, C. (1987) Use of zygotic embryo culture to collect germplasm of coconut (*Cocos nucifera* L.). *FAO/IBPGRI Plant Genetic Resources Newsletter* **71**, 4–10.

Assy-Bah, B.; Engelmann, F. (1993) Medium term conservation at mature embryos of coconut. *Plant Cell, Tissue and Organ Culture* **33**, 19–24.

Avril, L.B.; Richard, L.B.; Blake, J. (1986) Regeneration in Palms. *Cell Culture and Somatic Cell Genetics of Plants* **3**, 207–222.

Bajaj, Y.P.S. (1984) Induction of growth in frozen embryos of coconut and ovules of citrus. *Current Science* **53**, 1215–1216.

Balaga, H.Y.; de Guzman, E.V. (1971) The growth and development of coconut Makapuno embryos *in vitro*. II. Increased root incidence and growth in response to media composition and to sequential culture from liquid to solid medium. *Philippines Agriculturist* **55**, 551–565.

Bhaskaran, S. (1985) Tissue culture technology for higher vegetable oil production. In: *Oil seed Production Constraints and Opportunities* [edited by H.C. Srivastava, S. Bhaskaran, B. Vatsya and K.K.G. Menon]. New Delhi Oxford and IBH publications, pp. 537–544.

Blake, J.; Eeuwens, C.J. (1978) Inflorescence tissue as source material for vegetative propagation of coconut palms. In *Proceedings of International Conference on Cocoa, Coconuts*, Kuala Lumpur. pp. 549–556.

Branton, R.L.; Blake, J. (1983) Development of organized structures in callus derived from explants of *Cocos nucifera* L. *Annals of Botany* **52**, 673–678.

Branton, R.L.; Blake, J. (1984) Clonal propagation of coconut palm. In *Proceedings of International conference on Cocoa, Coconut,* pp. 1–9.

Branton, R.L.; Blake, J. (1986) Clonal propagation of coconut. In: *Cocoa and coconuts: Progress and outlook* [edited by E.P. Pushparajah, C.P Soon], pp. 771–780.

Cardena, R.; Oropeza, C.; Sipson, J.; Ashburner, R. (1997) Search for random amplified polymorphic DNA markers linked to lethal yellowing resistance. Abstract in Genetic Improvement. Paper presented at *International Symposium on Coconut Biotechnology,* Merida, Mexico, 1-5 December 1997, pp. 17.

Chan, J.L.; Saenz, L.; Talavera, C.; Hornung, R.; Robert, M.; Oropeza, C. (1998) Regeneration of coconut (*Cocos nucifera* L.) from plumule explants through somatic embryogenesis. *Plant Cell Reports* **17**, 515–521.

CPCRI (1994-95). Annual Report. CPCRI, Kasaragod, pp. 43.

Cueto, C.A.; Ebert, A.W.; Rillo, E.P.; Orens, O.D. (1997) *In vitro* regeneration of coconut (*Cocos nucifera* L.) from immature inflorescence of tall cultivars. *Acta Horticulture* **447**, 121–122.

D'Souza, L. (1980) Hypocotyl budding in cultured coconut embryos. In: *Plant Tissue Culture, Genetic Manipulation and Somatic hybridization of Plant Cells* [edited by Rao, P.S., Heble, M.R. and Chadha M.S.] Bombay, BARC. pp. 290–294.

de Guzman, E.V.; del Rosario, A.G.; Eusebio, E.C. (1971) The growth and development of coconut Makapuno embryo *in vitro*.III. Resumption of root growth in high sugar media. *Philippine Agricuturist* **53**, 566–579.

de Guzman,; E.V. (1970) The growth and development of coconut 'Makapuno' embryo *in vitro*. The induction of rooting. *Philippine Agriculturist* **53** (2), 65–78.

del Rosario, A.G.; de Guzman, E.V. (1976) The growth of coconut 'Makapuno' embryos *in vitro* as affected by mineral composition and sugar level of medium during the liquid and solid cultures. *Philippine Journal Science* **105**, 215–222.

del Rosario, A.G.; de Guzman, E.V. (1981) The status of tissue culture in Philippines. In: *Proceedings of COSTED Symposiump Tissue Culture of Economically Important Plants.* [edited by A. N. Rao]. COSTED/ANBS, Singapore. pp. 292–294.

Diekmann, M. (1997) The use of biotechnology for the safe movement of coconut germplasms. In: *International Symposium on Coconut Biotechnology,* Merida, Mexico, 1-5 December 1997, pp. 30.

Eeuwens, C.J. (1976) Mineral requirements for growth and callus initiation of tissue explants excised from mature tissues *in vitro*. *Physiologia Plantarum* **36**, 23–28.

Eeuwens, C.J. (1978) Effects of organic nutrients and hormones on growth and developmnt of tissue explants from coconut (*Cocos nucifera* L.) and date (*Phoenix dactylifera*) palms *in vitro*. *Physiolgia Plantarum* **24**, 173–178.

Engelmann, F. (1997) Cryopreservation of coconut germplasm. In: *International Symposium on Coconut Biotechnology,* Merida, Mexico, 1-5 December 1997, pp. 34.

Fernando, W.M.U.; Gajanayake, G. (1997) Patterns of isozyme variations in coconut (*Cocos nucifera* L.) populations used for breeding improved varities. *Plantation, recterche, development* **52**, 256–261.

Gupta, P.K.; Kendulkar S.V.; Kulkarni, V.M.; Shirgurkar, M.V.; Mascarenhans, A.F. (1984.) Somatic embryogenesis and plants from zygotic embryos of coconut *Cocos nucifera* L. *in vitro*. *Plant Cell Reports* **3**, 222–225.

Haibou, T.K. (1981) *In vitro* culture of coconut (*Cocos nucifera* L.). These de 3^{e} cycle, Universite Paris VIII.

Haibou, T.K.; Kovoor, A. (1982) Regeneration of callus from coconut protoplasts. In: *Tissue Culture of Economically Important Plants. Proceedings of International Symposium* (edited by Rao, A.N). COSTED/ANBS, Singapore, pp. 149–151.

Hodgson, R.A.J.; Randles, J.W. (1997) Detection of coconut cadang-cadang viroid-like sequences. In: *International Symposium on Coconut Biotechnology,* Merida, Mexico, 1-5 December 1997, pp. 25–27.

Hornung, R. (1995) Micropropagation of *Cocos nucifera* L. from plumular tissues excised from mature zygotic embryos. *Plantations, Recherche, Developpment* **2** (2), 38–41.

Jagadeesan, M.; Padmanabhan, D. (1982) Studies on coconut embryo culture. PLACROSYM-V (Abst-31). CPCRI Kasaragod, 15-18 December 1982, pp. 22

Jones, P.; Tymon, A. M. (1997) Detection and diagnosis of African lethal yellowing-like diseases. In: *International Symposium on Coconut Biotechnology* held at Merida, Mexico, 1-5 December 1997, pp. 22.

Kalamani, A.; Sree Rangaswamy, S.R. (1990) *In vitro* culturing of coconut embryos. *Indian Coconut Journal* **20** (11), 9–10.

Karunaratne, S.; Kurukularachchi, C.; Gamage, C. (1985) The culture of embryos of dwarf coconut. *Cocos nucifera* L. var. Nana *in vitro*. *Cocos* **3**, 1–8.

Karunaratne, S.; Periyapperuma, K. (1989) Culture of immature embryos of coconut (*Cocos nucifera* L): callus proliferation and somatic embryogenesis. *Plant Science* **62**, 247–253.

Karunaratne, S.; Sunil, S.; Kovoor, A.(1991) An in vitro assay for drought-tolerant coconut germplasm. *Euphytica* **53**, 25–30.

Karunaratne, S.M. (1988) Short term *in vitro* preservation of coconut seed material: A method to facilitate field collection and transport of coconut germplasm. *CORD* **4**, 40–47.

Kovoor, A.; (1981) Palm tissue culture: state of the art and its application to the coconut. In: *5th session of FAO Technical Working Party on Coconut Production and Protection*. Rome, FAO, pp. 37–52.

Kumar, P. P.; Raju, C.R.; Mini, C.; Iyer, R.D. (1985) Induction and maintenance of friable callus from the cellular endosperm of *Cocos nucifera* L. *Plant Science* **40**, 203–207.

Kumaran, P.M.; Koshy, P.M.; Anitha Karun. (1998) Germplasm exchange through embryo cultures. *Proceedings of 7th Swadeshi Science Congress*, 6-8 November 1997 held at CPCRI, Kasaragod, Kerala, pp. 35–39.

Kuruvinashetty, M.S.; Iyer, R.D. (1980) Tissue culture studies on coconut palm for its clonal propagation. In: *Proceedings of National. Symposium. on Tissue Culture, Genetic Manipulation and Somatic Hybridization of Plant Cells* [edited by P.S.Rao, M.R.Heble and M.S. Chadha]. Bombay, India: BARC, pp. 184–191.

Leburn, P.L.; Baudouin, L. (1997) Use of RFLP markers for the study of genetic diversity in coconut. In: *International Symposium on Coconut Biotechnology* held at Mexida, Mexico, 1-5 December 1997, pp. 13.

Magnaval, C.; Noirot,M.; Verdeil, J.L.; Blatters, A.; Huet, C.; Grosdemange, F.; Beule,T.; Buffard Morel, J. (1997) Specific nutritional requirements of coconut calli (*Cocos nucifera* L) during somatic embryogenesis induction. *Journal Plant Physiol* **150**, 719–728.

Mascarenhas, A.F.; Gupta, P.K.; Nair, S.; Khuspe, S.S. (1988) Studies on Plant Tissue Culture at the National Chemical Laboratory, Pune. In: *Proceedings of National Seminar on Plant Tissue Culture.* 2-4 March, 1983. CPCRI, Kasaragod, pp. 198–199.

Miniano, A.P.; DeGuzman, E V. (1978) Responses of non Makapuno embryos *in vitro* to chloride supplementation. *Philippine Journal of Coconut Studies* **3**, 27–30.

Morel, G.; Wetmore, R.M. (1951) Fern callus tissue culture. *American Journal of Botany* **38,** 141–143.

Murashige, T.; Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco cultures. *Physiologia Plantarum* **15**, 473–497.

Nair, M.K.; Shivashankar, S.; Anitha Karun (1995) Control of browning of explants. *NARP-II Comprehensive Report. Basic Research Sub project on Horticultural Crop Biotechnology -Coconut*, pp. 10–11.

Neera-Bhalla-Sarin; Suman-Bagga. (1988) *In vitro* culture of embryos and other parts of *Cocos nucifera*. In: *Proceedings of National. Seminar on Plant Tissue Culture*. pp. 132-139. Central Plantation Crops Research Institute, Kasaragod, 2-4 March 1983.

Neera-Bhalla-Sarin; Suman-Bagga; Sopory, S.K.; Guha Mukerjee, S. (1986) Induction and differentiation of callus from embryos of *Cocos nucifera* L. by IAA-congugate. *Plant Cell Reports* **5**, 322–324.

Nitsh, J.P. (1969) Experimental androgenesis in *Nicotiana. Phytomorphology* **19**, 389–404.

Padmanabhan, D. (1982) Experimental cloning in palms. *S.B.Ci News letter* **1** (6), 95–98.

Padmanabhan, D. (1988) Potentials of embryo culture in *Cocos nucifera* L. In: *National seminar on plant tissue culture*, CPCRI, Kasaragod, 2-4 March 1983. Madurai Kamaraj University, Madurai, Tamil Nadu, India, pp. 146–148.

Pannetier, C.; Bufford-Morel, J. (1982) Premiers resultats concernant la production d'embryons somatiques a partir de tissus foliaires de cocotier, *Cocos nucifera* L. (First result of somatic embryo production from leaf tissue of coocnut, *Cocos nucifera* L). *Oleagineux* **37**, 349–354.

Perera, L.; Russell, J.R.; Provan, J.; McNicol, J.W.; Powell, W. (1998) Evaluating genetic relationship between indigenous coconut (*Cocos nucifera* L.) accessions from Sri Lanka by means of AFLP profiling. *Theoretical and Applied Genetics* **96** (3/4), 545–550.

Raju, C.R.; Bavappa, K.V.A. (1987) Use of tissue culture for plant quarantine and exchange of germplasm and planting materials in coconut. Paper presented at expert consultation on use of tissue culture in plant quarantine for exchanging plant materials, NBPGR, New Delhi, 26 February-2 March 1987.

Raju, C.R.; Kumar, P. P.; Chandramohan, M.; Iyer, R.D. (1984) Coconut plantlets from leaf tissue cultures. *Journal of Plantation Crops* **12**, 75–78.

Raju, C.R.; Shyla, R.; Sajini, K.K.; Bavappa, K.V.A. (1988) Attempts at production of clonal coconut palms. In: *National Symposium on Coconut Breeding and Management*, KAU, Vellanikkara, Trichur, 23-26 November 1988, p. 23.

Ramanujam, B.; Chempakam, B.; Nambiar, K.K.N. (1996) Electrophoretic protein patterns of coconut isolates of *Thielaviopsis paradoxa* (deseynes) Hohnel. *Journal of Plant Pathology* (Communicated).

Randles, J.W.; Wefels, E.; Miller, D.C.; Hanold, D.; Morin, J.P.; Rhode,W. (1997) Detection and diagnosis of coconut foliar decay disease. In: *International Symposium on Coconut Biotechnology* held at Merida, Mexico, 1-5 December 1997, pp. 28.

Reynolds, J.F. (1982) Vegetative propagation of palm trees. In: *Tissue Culture in Forestry* [edited by J.M. Bonga and D.J. Durzan]. Martinus Nijhoff, The Hague, pp. 182-207.

Rhode, W.; Kullaya, A.; Rodriguez, J.; Ritter, E. (1997) Analysis of coconut germplasm biodiversity by DNA marker technologies and construction of a first linkage map. In: *International Symposium on Coconut Biotechnology* held at Merida, Mexico, 1-5 December 1997, pp. 15.

Rhode, W.; Salamini, F.; Ashburner, G.R.; Randles, J.W. (1992) An *Eco*R1 repetitive sequence family of the coconut palm *Cocos nucifera* L. shows sequence homology to *copia* -like elements. *Journal Genetics & Breeding* **46**, 391–394.

Rillo, E.P. (1989) A non-destructive technique for collecting immature coconut inflorescence for tissue culture. *Philippine Journal of Coconut Studies* **4**, 16–17.

Rillo, E.P.; Ebert, A.W. (1993) *In vitro* regeneration of coconut: Progress Report of the Philippines-German coconut tissue culture project. *Proceedings of International GTZ Workshop on Plant Biotechnology in Technical Cooperation programmes* held at Trinidad, Legaspi City, Philippines, 6-11October 1993, pp. 84-88.

Rillo, E.P.; Paloma, M.B.F. (1990) Comparison of three media formulations for *in vitro* culture of coconut embryos. *Oleagineux* **45** (7), 319–323.

Rillo, E.P.; Paloma, M.B.F. (1992) Storage and transport of zygotic embryos of *Cocos nucifera* L. for *in vitro* culture. *Plant Genetics Resources Newsletter* **86**, 1–4.

Rivera, R.; Edwards, K.J.; Karp, A.; Arnold, G.M.; Ayad, G.; Hodgkin, T. (1997) Isolation of polymorphic microsatellites in coconut. In: *International Symposium on Coconut Biotechnology* held at Merida, Mexico, 1-5 December 1997, pp. 16.

Shivashankar, S.; Sajini, K.K. (1994) Development of multiple shoots and plantlets from embryo derived callus of *Cocos nucifera* L. In: *National Symposium on Development in Plant Molecular Biology and Genetic Engineering* held at TNAU, Tamil Nadu, India, 29-31 December, pp. 49.

Sossou, J.; Karunaratne, S.; Kovoor, A. (1987) Collecting palm: *In vitro* explanting in the field. *Plant Genetic Resources Newsletter* No. 16, 7-18.

Sugimura, Y.; Salvana, M.J. (1989) Induction and growth of callus derived from rachilla explants of young inflorescences of coconut palm. *Canadian Journal of Botany* **67**, 272–274.

Thanh-Tuyen, N.; Apruillo, D.I. (1994) Plant regeneration through somatic embryogenesis from cultured zygotic embryos of coconut. *Philippines Journal of Coconut Studies* **17** (1), 1–7.

Thanh-Tuyen, N.; De Guzman, E.V.D. (1983) Formation of pollen embryos in cultured anthers of coconut (*Cocos nucifera* L.). *Plant Science Letters* **29**, 81–88.

Verdeil, J.L. (1993) Etude de la regeneration due cocotier (*Cocos nucifera* L) par embryogenese somatique a Partir d' explants inflorescence. Thes'e de doctorate de I' Universite Paris VI. 150 pp.

Verdeil, J.L.; Baudouin, L.; Hocher, V.; Bourdeix, R.; N'Cho, Y.P.; Sangare, A.; Rillo, E.; Oropeza, C.; Hamon, S. (1998). What are the possible applications for coconut (*Cocos nucifera* L.) micropropagation? *Plantations recherche, developpement* **5**, 339–342.

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

Wadt, L.H.O.; Sakiyama, N.S.; Pereira, M.S.; Tupinampa, E.A.; Riberiro, F.E.; Aragao, W.M. (1997) RAPD markers in the genetic diversity study of coconut. In: *International Symposium on Coconut Biotechnology* held at Merida, Mexico, 1-5 December 1997, pp. 14.