17. PLANTATION CROPS

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In plantation crops, results of the conventional breeding methods are slow because of their long juvenile phase, heterogeneous outcrossing nature, narrow genetic base and large area needed for field testing. Biotechnology can only be considered as a new supplementary tool to evolve disease tolerant varieties and clonal propagation. Application of biotechnology alone is not expected to produce better crop plants, but combined with knowledge of related disciplines, it could be used as a powerful tool to improve these plantation crops.

Researchy on vegetative propagation, diploid production, protoplast culture, embryo rescue, cryopreservation, induction of somaclonal variation, *in vitro* screening and, molecular detection of pathogens are in progress at various centres in the country. Coconut, oil palm, arecanut, cashew nut, rubber, tea, coffee, and cacao are the major plantation crops cultivated in India.

Coconut

Micropropagation

Research on tissue culture in coconutis in progress at different research centres. However a repeatable and commercial protocol is yet to be developed. A viable protocol for embryo culture facilitating movement of coconut germplasm from distant places has been developed at the Central Plantation Crops Research Institute (CPCRI) Kasaragod (Plate.1) (Anitha Karun *e. al.*, 1993).

First plantlet was developed at to CPCRI Kasaragod from tender leaf explant taken from one-and-a-half-year-old West Coast Tall seedlings (Raju *et al.*, 1984). However it was not reproducible in subsequent trials. Profuse callus induction was achieved from immature zygotic embryos and further no regeneration into plantlets. Embryogenic capacity of leaf explants was found to be related to their physiological maturity in young palms (Karunaratne and Periyaperuma 1989) . Reports of success were published by using various explants (Blake and Eeuwens 1982; Shirke *et al.* 1993; and Verdeil *et. al.*, 1994; Hornung 1994, Chan *et. al.*, 1998)

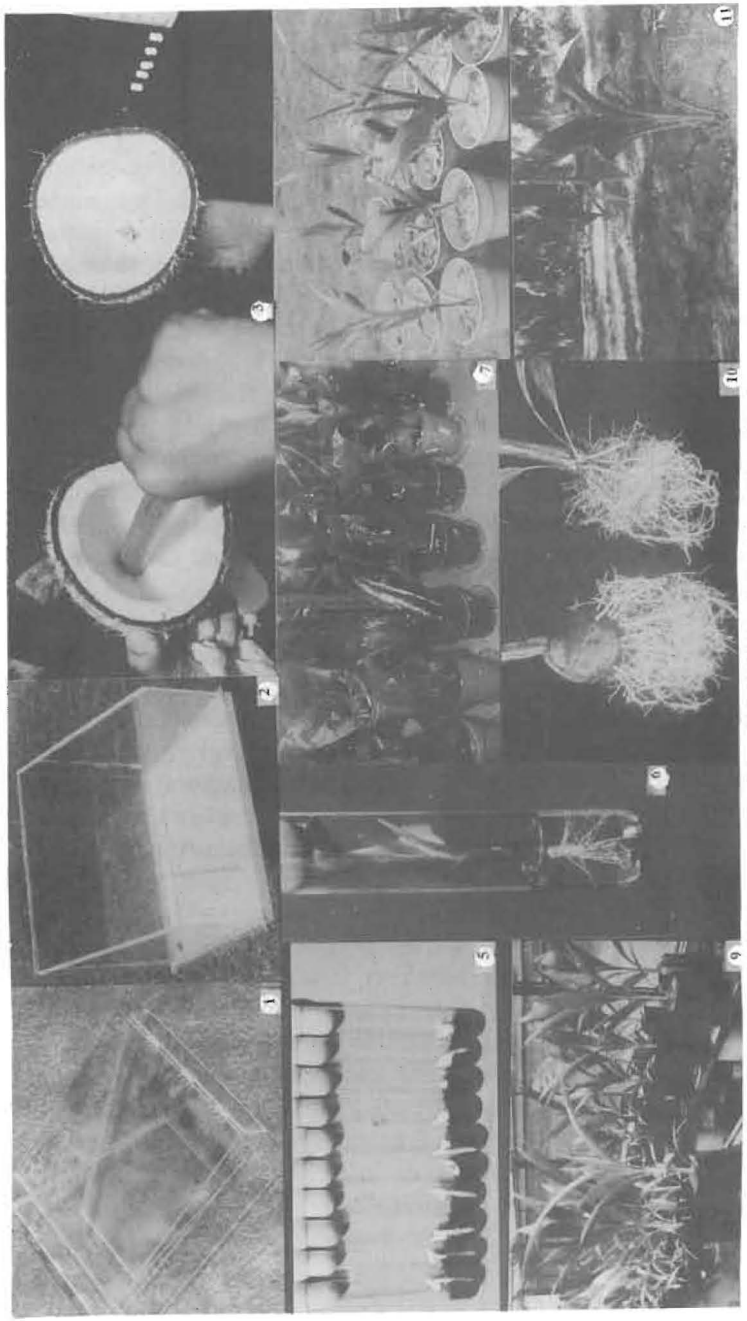


PLATE 1 Field collection, *in vitro* germination, *ex vitro* and field establishment coconut zygotic embryos.

Fig. 1. Folding type of portable inoculation hood. Fig. 2. Front view of inoculation hood. Fig.3. Scooping of embryos from splited nut with the help of cork borer. Fig. 4. Extracted embryos. Fig. 5. Embryos in retreaval medium after 35-45 days of inoculation. Fig. 6. A complete plant Fig. 7. *Ex vitro* establishment. Fig. 8 & 9. Pot and poly bag establishment. Fig. 10. Comparison of root intensity of seed nut sown and embryo cultured seedling. Fig. 11 Well established seedlings in the field after 1 year.

Screening of *in vitro* 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 (PEG-6000), mannitol and Sodium chloride into culture medium. PEG and mannitol were found to have growth inhibitory action even at low concentration. 21% of embryos of Sri Lankan Tall died before reaching water stress level of 170mM NaCl. 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 2 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 170 mM NaCl concentration and none of either cultivar survived 230 mM salt concentration alone. However, embryos originated from two putative drought tolerant cultivars showed a higher survival rate when subjected to salt stress.

At 170 mM NaCl concentration all the embryos developed into seedlings. In fact, germination percentage of embryos was higher at 170 mM NaCl concentration than the control devoid of NaCl. Survival percentage gradually dropped with increase in salt concentration and about 18% survived the 330mM Nacl level.

Molecular genetic characterization

Researches in coconut on biochemical markers - isozyme and molecular markers like Restricted Fragment Length Polymorphism (RFLP), Random Amplified Polymorhic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Sequence Tagged Microsatelites (STM) - being taken up only recently. Protocol for detection of isozyme polymorphism in coconut leaf tissues was reported by Fernado and Gajanayake, (1997). They reported that esterase is useful for studying the genotypic variation in coconut. Rhode *et.al.* (1992) described a novel approach for the analysis of coconut germplasm with the use of coconut specific primers complementary to the *copia* like *eco* R1 elements. The PCR amplification of spacer regions for a sub-set of tandemly arranged repeats detected polymorphisms which 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 South Pacific region. They found moderate level of genetic diversity although very few RAPD markers were unique to specific populations.

As part of DNA fingerprinting of coconut germplasm using RFLP/RAPD markers, the DNA extraction protocol and RAPD protocol has been standardized at the CPCRI, Kasaragod (Anuradha *et. al.*, 1998). For DNA extraction SDS proved better than CTAB. Significantly higher yield was obtained at pH 8.0.

Molecular detection and diagnosis of coconut lethal yellow, root (wilt), cadangcadang and foliar decay diseases

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 Mosambique have killed millions of palms causing severe economic loss. Using the polymerase chain reaction, the 16S rRNA genes and the 16S-23S spacer regions of phytoplasmas associated with these lethal coconut diseases in Africa have been amplified. Following sequencing of the rDNA products, 2 primers have been designed which are 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 Caribbian or DNA from a variety of periwinkle-maintained phytoplasmas. Sequences analysis of the coconut 16S rDNA has shown that the phytoplasmas found in West Africa have greater than 99% similarity, while similarity between the phytoplasmas 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).

Cadang-cadang

Cadang-cadang is a major lethal disease of coconut in the Central Philippines. Diagnosis of disease on the basis of symptoms alone is, unreliable specifically in the early stage of infection as there are hardly any discernible symptoms. The causative agent is the coconut cadang-cadang viroids (CCCVd), a smallest known viroid 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 a combination of several molecular techniques. An effective molecular indexing for coconut pathogens requires to analyse the nucleic acid or protein component of the pathogen . Then the 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 (radiolabel, DIG, biotin and auto radiography, chemilnuiscence, fluorescence or calorimetric detection)

Coconut cadang-cadang viroid (CCCVd), viroid related to 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 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. The CFDV DNA comprises 1291 nucleotides and contains open reading frames for 6 potential proteins of molecular weight larger than 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 primes 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 the extract.(Randles *et al.*, 1997).

Stem bleeding

Electrophoretic protein patterns of coconut isolates of stem bleeding pathogen *Thielaviopsis paradoxa* (de Seynes) Hohnel. was studied at the CPCRI Kasaragod (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 (Phast system, Pharmacia, LKB Sweden) . Considerable variations in the protein band patterns were noticed in 12 isolates. Based on the similarity coefficient determined from the

protein patterns, these isolates were grouped into 2 main clusters. The data on virulence of these isolates were made use of to study the relationship between protein patterns and virulence.

Embryo Culture for Safe Movement of Coconut Germplasm

Germplasm collection should be maintained free from important pests and pathogens. The biotechnology offers techniques that can help to reduce the risk of spreading the pests and diseases. The technical guidelines finalized recently by the FAO/IPGRI for the safe movement of coconut germplasm emphasises collection and transportation through embryos instead of seed nuts (Diekmann, 1997). Collection and exchange of coconut germplasm through seednuts are difficult and costly due to their considerable bulkiness, their rapid germination and stringent phytosanitary regulations. Embryo culture has been commercially exploited in the Philippines to rescue and culture the Macapuno nuts (endosperm in semisolid and whose germination cannot occur under natural conditions) (De Guzman, 1970, Del Rosario and De Guzman, 1976, 1981). There are several protocols developed elsewhere in the world to collect coconut embryos directly from the field and their retrieval *in vitro*. (Assy-Bah *et al.*, 1987; Sossou *et al.*, 1987; Rillo and Paloma, 1992).

At the CPCRI, Kasaragod a simple and efficient protocol for field collection and *in vitro* culture, storage and subsequent field establishment has been standardized (Anithna Karun *et al.*, 1993, Anitha Karun and Sajini, 1994; Anitha Karun *et. al.*, 1997; (Anitha Karun *et al.*, 1998). Recently same protocol was utilized to collect the 15 exotic coconut germplasm from Mauritius, Medagascar and Sychelles (Kumaran *et al.*, 1998) by an expedition team from the CPCRI, Kasaragod.

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 option for the safe, medium and long term conservation of coconut germplasm. Engelmann *et al.* (1995) studied in detail these techniques. They could successfully cryopreserve and retrieve plantlets from nuts 7-8 months after pollination . Performance of immature embryos are good due to their small size (2-3 mm long 10-20 mg in weight) and low degree of

differentiation . Immature embryos could withstand rapid freezing in liquid nitrogen after 4 hours of pretreatment on a semi solid medium containing 600 g/litre sucrose and 10-15 % glycerol or sorbitol. In mature embryos, because of their large size according to cryopreservation standards (upto 10mm long, 80-160 mg in weight) and their advanced differentiation rendered their potential use problematic. The technique developed for immature embryos were successfully applied to mature embryos of four varieties (hybrid PB 121, Cameroon Red Dwarf, Indian Tall and Renell Tall. After 4 hr. of desiccation period in the air current of 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 compared with the control embryos.(Engelmann, 1997).

Use of RFLP (Lebrun *et al.*, 1997), RAPD (Wadt *et al.*, 1997), microsatellite (Rivera, 1997) and AFLP (Perera *et al.*, 1998) markers for the study of genetic diversity of coconut and the first linkage map by using DNA marker technology Rhode *et al.* 1997 have been reported.

Oil Palm

Micro propagation

Oil palm (*Elaeis guineensis* Jacq) is an open pollinated outbreeder with minimal possibility of selfing. Natural clonal propagation is not possible as the axillary buds give rise to inflorescence, either male or female. *In vitro* multiplication of superior selected types would be highly advantageous for obtaining better yield, improved oil quality, stress tolerance, faster growth and other desirable traits. Considering the slow growth of the palm, tissue culture could also greatly accelerate the regeneration rate. About 8-10 years period is required before assessing the fruit yield and oil content in the population. The advantages of good clonal population of selected high-yielding trees in oil palm have been described (Corley *et al.*, 1981). In addition to the advantages of growing uniformly high yielding clones of individual trees, clonal propagation will also facilitates to propagate *dura* and *pisifera* parents to produce desirable *tenera* combinations. This is particularly important to a country like India which is poised for

expansion of oil palm cultivation and availability of quality planting material is the major constraint.

Major research on *in vitro* culture of oil palm has been carried out in France (Institute Recherche de Huiles et Oleigeneux - IRHO), the UK (Wye College, University of London), Unifield TC (Unilever/Harrison and Cross field), Malaysia (Palm Oil Research Institute of Malaysia - PORIM), Indonesia (Marihat Research Station) and Nigeria (Nigerian Institute for Oil Palm Research - MIFOR).

The yields of palm oil through clonal propagation would be increased by as much as 30% over the seedling progeny with parents being the same. Progress in commercialization of clonal propagation suffered a setback when abnormal flower development was reported in palms transferred to field (Corley *et. al.*, 1986). The French group (IRHO-ORSTOM) experiments at La Me, Cote-d'Ivoire have now resulted in reducing floral abnormalities to only 3.1 % (Durand-Gasselin *et al.*, 1990). Using the same technique Lubis *et. al.*. (1993) observed normal vegetative growth and flowering of clones in Indonesia. The Fresh fruit bunch (FFB) yields were about 20 % higher in clones than those of seedling origin.

Successful vegetative propagation in oil palm through tissue culture has been reported by Jones (1984), Rabachault and Martin (1976), Paranjothy (1984, 1986) among others from the UK, Ivory Coast, France and Malaysia. The first attempt in India to develop a protocol of clonal multiplication of oil palm from tender leaf explants through somatic embryogenesis was done by Thomas and Rao (1985) at Bhabha Atomic Research Centre, Mumbai. They excised young leaves from 6 months old *tenera* seedlings. Meristematic portions of the leaf base were cut into explants measuring 10mm x 15 mm. After surface sterilization, the explants were cultured on a modified MS basal medium with modified vitamins, and supplemented with growth regulators. Best callus response was obtained on MS basal medium supplemented 50-70 mg/litre of 2,4-D. Sub culturable callus tissues were produced within 6-8 weeks. These callus tissues were periodically transferred to basal medium with reduced levels of 2,4-D and these cultures were maintained as a continuous source for induction of embryogenesis. They could obtain embryogenesis in both liquid and solid medium. Shoots of 10-12 cm size with 2-4 leaves were isolated individually and maintained in tubes with filter paper bridges containing MS liquid medium supplemented with 1 mg/litre

NAA and 1 mg/litre GA_3 to initiate root growth. Plantlets containing 3-4 leaves with roots were transferred to paper cups with pre-sterilized soil compost. Raju *et al.* (1989) reported successful culturing of explants from tender leaf of 3 year-old *tenera* seedlings.

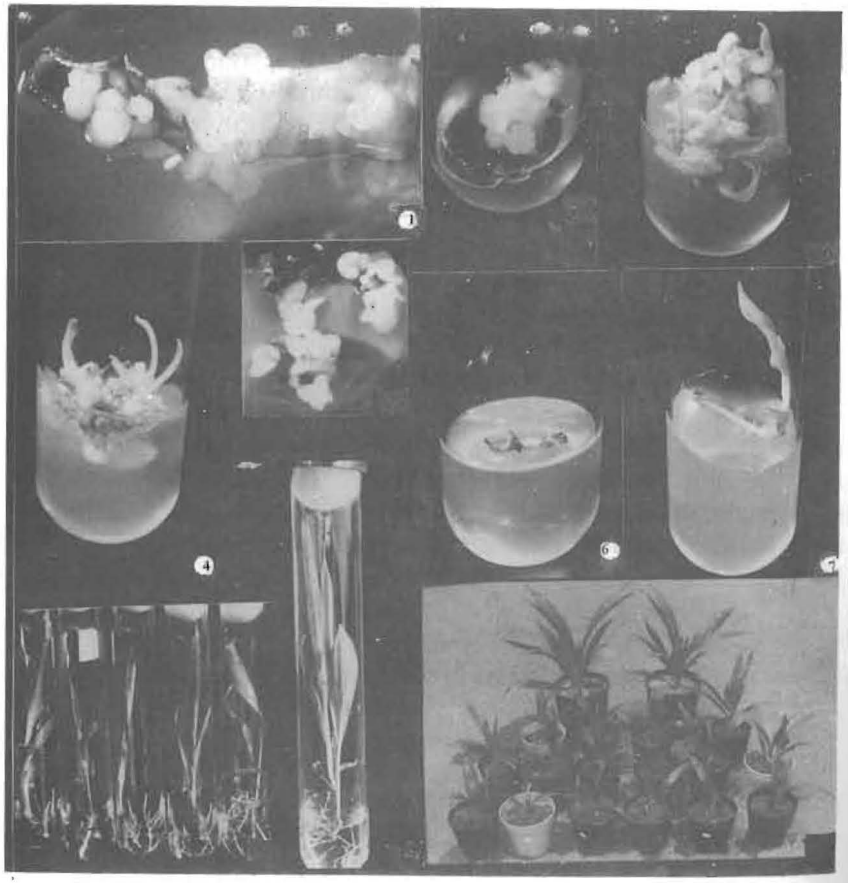
Plantlet development was achieved by Anitha Karun and Sajini (1996) from leaf explants of 18 month old *dura* and 6 month old *tenera* oil palm seedlings. Callus induction was noticed after 100-120 days in *dura* (Plate 2, Fig.1) and 150-180 days in tenera on culturing in half strength MS basal medium supplemented with 25 mg/litre 2,4-D. About 7% of the culture explants in *dura* and 10 % in tenera produced embryogenic calli (Plate 2,Fig.2).. These were transferred to regeneration medium containing 1 mg/l zeatin riboside. Plantlet development from calli was achieved through both embryogenesis (Plate 2, Fig.5) and organogenesis (Plate 2, Fig.2-4). It was seen that a larger share of plantlets were derived through organogenesis than somatic embryogenesis. After 18 months of experiments, there were 175 plantlets established in pots. Comparable results were observed on repeating the experiments in 3 *dura* seedlings and 2 *tenera* seedlings.

Histological studies on *dura* palm cultures were conducted to investigate the developmental stages of regeneration (Anitha Karun and Sajini, 1998). The primary calli were nodular and originated from the perivascular region of the vein and it was observed that the origin of somatic embryos/meristemoids was from a group of cells (multicellular). Multiple shoot induction was obtained (2-3 shoots) by using several *tenera* crosses in MS basal media supplemented with high cytokinin (CPCRI, Kasaragod, 1994). Since oil palm cannot be readily transformed by *Agrobacterium*, protoplast offers an alternative transformation system.

Oil palm being a monocotyledon and a woody plant has been recalcitrant to protoplast isolation and culture. Bass and Hughes (1984) reported the isolation and generation of viable protoplast of oil palm. Sambanthamurthy *et al.*. (1996) reported the isolation of highly viable protoplast from various oil palm tissues and formation of microcallusis from protoplast of polyembryogenic cultures. Studies on protoplast cultures and regeneration, rDNA technique for manipulation of desirable traits including oil quality, resistant to biotic and abiotic stresses are the major areas need to be focussed.

PLATE 2. Somatic embryogenesis /organogenesis from oil palm leaf explant

Fig.1. Callus induction after 100 -120 days in 25 mg/l 2, 4-d, 3 mg/l 2-iP, and 40 mg/l adenine sulphate on ½ strength MS medium. Fig.2. Friable calli separated from the leaf explant in regeneration medium with zeatin riboside 1 mg/l. Fig.3. & 4 Fast growing calli after 4 weeks in regeneration media gave large nos. of meristemoids and multiple shoots.. Fig.5. Formation of somatic embryos as well as fast growing calli in regeneration medium. Fig.6. Germination of separated somatic embryo. Fig.7. Somatic embryo developed into plantlet. Fig.8.& 9. Formation of roots in rhizogenesis medium. Fig .10. Established plantlets in pots.



Rajanaidu *et al.* (1997) emphasised immediate prospect of tissue culture techniques to develop biclonal seeds. This involves multiplication of parents of elite *dura* and *pisifera* crossess through tissue culture and then crossing these parental clones to produce D x T seeds.

Embryo rescue

Pisifera palms are generally sterile and low percentage of seeds produced show poor germination and producing weak seedlings owing to very small kernel and absences of shell. The *Pisifera* embryo-rescue culture medium has been standardized at the CPCRI, Kasaragod. The embryos extracted 4 months after fertilization were sterilized with 0.1% $HgCl_2$ and inoculated directly to MS basal medium supplemented with adenine sulphate (40 mg/litre), CH (500mg/ litre), Thiamine Hcl 2mg/litre. About 28% germination was noticed and further experiments are in progress.

Molecular markers (RFLP)

Use of molecular markers for oil palm breeding was attempted by Jack and Mayes (1993) at IRHO, France. The RFLP/RAPD, DNA molecular marker techniques are being widely used in plant breeding for genetic fingerprinting, determination of genetic distance, genome analysis and identification of markers linked to desirable breeding traits. Work at the IRHO has confirmed the identity of tissue culture derived clones with the original mother palm. The DNA markers with high levels of polymorphism e.g. probe (pOpg54) generated at least 50 distinct banding patterns amongst 124 genotypes. Hypervariable clone (pSMP60) was identified and sequence analysis revealed a complex mosaic of interspersed repetitive domains. To construct an oil palm RFLP linkage map for the identification of markers linked to shell thickness, *Fusarium* wilt resistance, oil quality and yield were also studied by these workers. Mapping was accomplished in population including selfed material, crosses between distinct *Elaeis guineensis* accessions and *Elaeis olifrea* accessions and *Elaeis guineensis X E.olifera* interspecific crosses. Widely differing values of DNA polymorphism were obtained ranging from 15% in self, 50 % in intraspecific crosses and 95% in interspecific crosses.

The application of RFLPs for genetic finger printing of 111 elite oil palm has been studied at Cambridge, the UK, using highly variable RFLP probes for palm identification. (Mayes *et al.*, 1996). Suitability

of RAPD analysis for detection of somaclonal variants in oil palm has been reported by Rival *et al.* (1998).

Amplified Restriction Fragment Polymorphism (AFLP)

AFLP is an efficient method for the identification of a large number of molecular markers essential for genetic mapping. This is based on the selective amplification of a subset of genomic restriction fragments using PCR. Singh *et al.* (1998) studied the AFLP techniques to develop markers for mapping the oil palm genome, segregation pattern and inheritance of AFLP bands in the progenies of a selfed and an interspecific cross.

Elecrophoretic analysis of genetic variability in oil palm derived from leaf tissue culture was studied by Budiana and Tahardi (1991). Leaf tissues from a *tenera* variety of oil palm propagated either by tissue culture or by seed were analysed for electrophoretic variability in respect of proteins and esterase and peroxidase *isoenzymes*. Patterns derived from the tissue cultured` plants proved to be less variable than those from the seed derived material, indicating greater genetic uniformity.

Protein markers were identified to differentiate 2 types of callus of the same clones of oil palm (Marmey *et al.,* 1991). In their studies electrophoresis of proteins extracted from primary (nodular) calluses and fast growing calluses revealed the presence of 22.2 k Da band only in the fast growing calluses. A 21.5 k Da band was also less pronounced in these calluses. The perturbation of the cytokinin metabolism was found only in the fast growing calluses. Radioimmuno assay of endogenous cytokinins in callus and embryoids was studied in detail by Jone (1990).

The use of flow cytometric analysis on 2 different crosses of *dura* X *pisifera* oil palm to get an accurate estimation of nuclear DNA content was made (Rival *et. al.* 1997). The genome size of *Elaeis guineensis* was found to be 2C=3.76+(-) 0.009 pg and therefore ca.3.4 X 10^{9}. Embryogenic calli and plants showed the same ploidy level but the measured`2C DNA values differred significantly . Since fast growing callus (FGC) already identified as a source of 'mantled' phenotype variants did not show any difference in their ploidy level, this proved the hypothesis that this is an epigenetic origin of a somaclonal variant.

Genetic transformation study was reported by Chowdhury *et al.* (1997). This work indicated efficiency of GUS gene expression in embryogenic callus and young leaflets of mature and seedling palms after microprojectile bombardment with GUS gene with *Emu, Ubil,* Act1, 35S, Adh1. The *ubil* or *Emu* promoter should facilitate the expression of desired genes in oil palm and help in development of an efficient, stable transformation. .

Cryopreservation

Engelmann *et al.* (1998) were able to store the embryoids for 15 months in liquid nitrogen and then regenerate plantlets from the cryopreserved materials.

Rubber

In vitro plantlets were regenerated for several clones of rubber by Carron *et al.* (1989). Regeneration by somatic embryogenesis paves the way for genetic engineering (Carron *et al.*, 1998).

The ability to introduce foreign genes into plant cells and tissues by plant transformation techniques and to regenerate viable, fertile plants have been widely employed for both basic research and introduction of new traits to modify and improve crop plants. Arokiaraj *et al.* (1996) established with agrobacterium mediated transformation of *Hevea brasiliensis* anther calli and their regeneration into plants using GV2260 (p35S GUSINT) and LBA 4404 (pAL 4404/p MON 9793), β-Glucaronidase (GUS) gene and the neomycin phospho transferase (npt II) gene. The presence of GUS gene in transformed plantlets was confirmed by using PCR from transformed calli and embryiods. Gene insertion into *Hevea brasiliensis* has been reported by Arokiaraj *et al.* (1994).

Use of RAPD markers for clone identification and analysis of genetic relationship among *Hevea* clones have been reported by Varghese *et al.* (1996) and Seguin *et al.* (1996). Attanayaka *et al.* (1997) described the expression of rubber elongation factor protein in *E. coli.* Genomic Southern blot analysis of different *Hevea* to genotype with the REF (rubber elongation factor) cDNA probe revealed the presence of restriction fragment length polymorphism (RFLP)

Studies conducted by Arokiaraj *et al.* (1995) showed that increased HMGR activity (3-hydroxy-3-methyl glutaryl co-enzyme A reductase) could enhance rubber biosynthetic rate by pushing the carbon flux

through the isoprenoid pathway. It showed that increased HMGR activity can indeed be induced by genetic transformation. A gene for latex specific (RT-PCR) technique cloned into binary vector pART27 and then transformed into *Hevea* anther calli via particle bombardment. The HMGR activities ranged from 70 to 410 % increase in kanamycin resistant calli generated in the light and from 110 to 580 % increase in kanamycin resistant calli grown in the dark compared to the control values. The HMGR activities in transformed embryoids grown in the light ranged from the basal level to 250 % and from 120 to 300 % increase in transformants grown in the dark compared to the control values.

Cocoa

Digestion patterns of DNA of 3 species of *Phytophthora* responsible for black pod disease of cocoa (*Theobroma cacao* L) was investigated by Chowdappa and Mohan (1996). The extent of intraspecific DNA diversity was studied in isolates of *Phytophthora palmivora, P. citrophthora and P.capsi* causing black pod disease of cocoa in India. Dendrograms were constructed from similarity coefficients following the analysis of restriction digestion fragment patterns of total DNA that were generated by endonucleases such as Sal 1 and Hind 111. Isolates within the species of *Phytophthora* had distinct patterns. Similarly coefficients were identical among isolates within a species while they were distinct among different species. Dendrograms obtained after unweighted pair grouping with arithmetic averaging cluster analysis revealed that the isolates belonging to some species formed a single cluster whereas different species formed separate clusters. Thus DNA polymorphisms can be used in differentiating species of *Phytophthora* associated with cocoa.

Vegetative propagation such as budding, grafting could provide only a limited number of propagules, while through tissue culture rapid multiplication can be achieved. Figueira *et al.* (1991) from Purdue University have reported that high CO_2 (470 ppm-28000 ppm) environment promoted shoot and root development in cotyledon-derived somatic embryos.

In India, Philomina *et al.* (1992) and Indulekha and Iyer (1993), Mallika *et al.* (1992) have produced few plantlets through micropropagation, However, a repeatable protocol for commercial propagation is yet to be invented.

Coffee

Viable protocol for plantlet regeneration in coffee from different explants has been developed both directly and indirectly at Central Coffee Research Institute, Balehonur (India).

Mass somatic embryogenesis is a possible tool for large-scale propagation of coffee. Petiard *et al.* (1992) have reported that about 5,000 embryos/liter can be produced daily. They have also studied the economics of mass somatic embryogenesis (artificial seed production) of coffee. Somatic embryogenesis and use of protoplast in plant breeding and their transformation, investigation on haploids and germplasm preservation are reviewed by Baumann and Neuenschwander (1990).

Naveen and Sreenath (1998) have standardized protocols for isolation of nuclear DNA from coffee leaves. These DNA were used for PCR, RAPD, AFLP and other molecular experiments. Anthony *et al.* (1997) conducted a detailed study on molecular biology in support of *Arabica* coffee genetic improvement. The application of molecular techniques such as RAPD analysis and RFLP markers to the genetic improvement of *Coffea arabica* has also been attempted. Isozyme and RAPD analysis have contributed to evaluating genetic diversity and defining related groups to aid the selection of crossing material. The RFLP markers have aided the study of coffee phylogeny and identification of genetic markers linked to the desirable traits.

Successful cryopreservation of zygotic embryos of *Coffea bengalensis* was achieved by Krishna and Sreenath (1998). Coffee Board. Embryos were excised from young green fruits and were air dried in the laminar flow for about an hour by keeping them on sterile dry germination paper. Air dried embryos were transferred to cryovials and cryopreserved for plunging them into liquid nitrogen and kept for a day. Later the embryos were fast thawed and cultured on MS medium resulting in good germination.

Muniswamy and Sreenath (1998) studied the short term storage of encapsulated zygotic embryos of *Coffea canephora* in room temperature. The preserved encapsulated embryos showed good germination upto 12 months and developed into plantlets on half strength MS medium with 0.2 mg/litre BAP by giving monthly subcultures, whereas the seeds of the same parent showed good

germination only upto 4 months beyond which the germination decreased.

Tea

Micropropagation of tea was well studied. Four types of responses have been observed. They are: a) direct shoot formation b) somatic embryogenesis c) callus induction and proliferation d) direct root formation from explants (Sood *et al,*. 1993). They also prepared synthetic seeds of tea by encapsulating somatic embryos and successfully germinated. The plantlets were transferred to soil. Progress on commercial propagation of tea by tissue culture technique was reported by Sarathchandra *et al.* (1990). Clonal variation in multiplication in tea through tissue culture was reported by Murali *et al.* (1996) and Pandidurai *et al.* (1996) reported the factors affecting *in vitro* shoot multiplication and root regeneration.

An efficient method for the *in vitro* propagation of tea [*Camellia sinensis* (L.) O. Kuntze] from nodal explants of clone TRI-2025 from somatic embryos was described by Akula and Dodd (1998). Direct induction of somatic embryos without callus formation occurs at 60 % frequency within 4 weeks of nodal explants. The production of embryos continued upon transfer of the cultures to fresh medium and 4-8 fold multiplication rate were obtained during each 6 weeks culture cycle.

At the United Planters Association of Southern India, a protocol is being standardized to produce somatic embryoids from immature cotyledons for evolution of drought tolerant elite clones. From a single cotyledon, 70 numbers of embryoids were obtained. Plantlet regeneration through leaf callus is also in progress at UPASI (UPASI, 1997).

Cashew

Multiple shoot induction has been achieved with MS medium containing either Thidiazuron alone or in a combination with other growth regulators BA and NAA and IBA. Elongation could be achieved on hormone free half MS medium containing 0.2% activated charcoal. Maximum *in vitro* rooting efficiency (80%) was noticed on liquid WPM medium supplemented with 1% sucrose and NAA and IBA. Rooted micro shoots after hardening both by *in vitro* and *ex vitro* method were planted in earthern pots (NRCC, Puttur, 1997). Twenty-

nine tissue culture plants produced from seedling explants were field planted. All these are surviving (NRCC Puttur 1998). At the NRC for Cashew, Puttur multiple shoot buds (1-13 buds/explant) could be induced from each nodal culture of seedlings (Thimapaiah and Sherly, 1996).

Premium quality kernel are essential for export purpose, with good protein and aminoacids. The RFLP markers can be used for screening these characters. The RFLP mapping of varieties would correct the identity of germplasm in molecular terms.

Biofertilizers for Plantation Crops

Due to continuous increase in fertilizer costs,, health hazards posed by them and stringent standards for export of agricultural products, there has been a lot of interest recently in biological systems supplying nutrients for plant growth . While there have been a number of studies on beneficial microbes associated with annual plants and formulations of bio- fertilizers based on these (Subba Rao, 1982), not much information are available on plantation crops (Subba Rao, 1983) But there is natural association among various plantation crops and beneficial micro-organisms. There is scope for selecting efficient strains for commercial production of biofretilizers, which may help to scale down the use of chemical fertilizers. Symbiotic nitrogen fixing bacteria can contribute a part to plant nitrogen needs. Asymbiotic nitrogen-fixing bacteria, *Beijerinekia* spp is common in coconut and rubber growing soils (Kothandaraman, 1979; Thomas *et al.*, 1991). Benzbaruah (1994) isolated various nitrogen fixers - *Derxia*, *Bacillus*, *Pseudomonas* and *Arthrobacter* - tea garden soils. *Azoctobacter* is known to occur in the rhizospere soils of a number of plantation crops (Subba Rao, 1983). The root associated nitrogen fixer *Azospirillum* is reported from coconut palm, arecanut palm, cashew, rubber, coffee and tea (Ghai and Thomas, 1989; Govindan and Purushothaman, 1985; George, 1990). Insoluble soil phosphates are rendered available either by plant roots or by soil microbes through secretion of organic acids (Banik and Dey, 1983). Rubber growing soils (Mathew *et al.*, 1987) and coconut soil (Bopaiah and Thomas, 1993) harbour phosphate-solubililzing microbes. The *Beijerinekia* sp., a nitrogen fixer associated from rubber growing soils, is capable of solubilizing phosphates (Kothandaraman, 1979).

Improved growth and nutrient uptake due to VA mycorrhizal association have been demonstrated in many horticultural crops (Menge *et al.*, 1978). The VAM colonizes the roots of coconut, coffee, tea and oil palm. (Hayman, 1982; Thomas and Ghai, 1987).

Though a number of efficient beneficial microbes are known, their large-scale use in plantation crops is restricted. In limited trials conducted, positive responses have been recorded. The *Azotobacter* has been shown to benefit coffee plants (Azizuddin and Rao, 1987). Similarly *Azospirillum* is reported to benefit cocoa. Hydroxaminate siderophose producing *Pseudomonas* has been reported to enhance the growth of tea plants (Benzbaruah, 1994). Sivaprasad *et al.* (1987) recorded better growth of cocoa plant inoculated with *Glomus fascienlatum* along with medium dose of phosphorous. Morita and Konishi (1989) found a relationship between VAM infection and soil P concentration in tea fields.

The combined inoculation of various microbes gives better results than single organism inoculation. Premkumari (1991) simultaneously inoculated coffee plants with VAM fungi, *Azospirillum* and phosphate solubilizers and observed improved growth of plants. Similar observations were made by Rajagopal and Ramarethinam (1997) in tea plants. Suresh *et al.* (1990) reported that a combined application of *Pseudomonas putida, P. fluorescens* and *Azotobacter* is beneficial for the rooting of layers in cashew.

The potential of beneficial microbes to be used on a large scale in plantation crops is very promising in the light of emerging interest and thrust on organically grown agricultural products for internal use and export. Even in cases, where inorganic fertilizers are used, biofertilizers may find applications, as it has been proved that their use results in enhanced nutrient use efficiency (Nayak *et al.*, 1986). The use of biofertilizers in plantation crops might also help to reduce the use of pesticides, as it has been shown that many nitrogen fixers (Pandey and Kumar, 1990; Mohan *et al*,. 1987) and plant growth promoting rhizobacteria (PGPR) (Benzbaruah, 1994) are capable of inhibiting plant pathogenic fungi and reducing pest incidence. However, more studies on field response of plantation crops to biofertilizers are needed to make recommendations.

Some preliminary studies (Iyer and Moosa, 1993; Thomas *et al.*, 1991) indicate that VA mycorrhizal associations in plantation crops are capable of enhancing drought tolerance. In drought-prone areas in India, this should be tested on a large scale and if conclusively proved, should be recommended as a technology.

While efforts have been made to improve nitrogen fixation through *Rhizobium* green manure plants systems (Thomas and Shantharam, 1984; Kothandaraman *et al.*, 1987) efforts should also be made to add more nutrients through microorganisms very closely associated with roots and whole plant system. It is interesting to note that obligate/facultative endophytic nitrogen fixing bacteria occur in plantation crops such as oil palm and coffee (Ferreira *et al.*, 1995; Baladani *et al*,. 1997). Future studies should be addressed to study their association with other plantation crops and their use as biofertilizers. As this group of bacteria are very closely associated with plants, we can expect more efficient nutrient transfer. There is a possibility of transferring genes for pest/disease resistance into these bacteria. It might be easy to transfer such engineered nitrogen fixers into plant system, as VAM fungi are common in plantation crops (Haymen, 1982). These are known to help endophytic nitrogen fixers to infect the plant system (Paula *et al.*, 1991).

Practically, it may not be possible to completely replace chemical fertilizers with organics or biofertilizers. In plantations, where the use of chemical fertilizers are less, biofertilizers might help to make the best use of applied chemicals. Nitrogen fixers, such as *Azospirillum* are known to excrete plant growth promoting hormones (Tien *et al.*, 1979) and promote root growth (Govindan and Chandy, 1985). Such biofertilized plants are shown to absorb nutrients more efficiently (Nayak *et al.*, 1986). It would be of interest to conduct such studies in plantation crops.

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18. HORTICULTURAL CROPS BIOTECHNOLOGY IN INDIA

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Biotechnology is a key technology for the future development in agriculture. It will provide better and healthier foods including fruits and vegetables, reducing reliance on toxic pesticides. The predominant countries concentrating on its commercialization are U.S.A., Japan and the European community. Canada, Israel, South Korea, Taiwan, Singapore, Brazil, India and China have also applied biotechnology for the development in plant sciences. Since research in biotechnology is highly cost and labour intensive, careful consideration is needed in its planning.

India is second in fruit and vegetable production in the world. However, India's share in global export of horticultural commodities is negligible due to low productivity, lack of infrastructural facilities and inadequate post harvest management. The varietal wealth available in the country can be exploited for production throughout the year. Biotechnology as a tool offers great scope to remove many such impediments. Realising the importance of biotechnology in national development, the Government of India established the National Biotechnology Board in 1982 which was upgraded to a full-fledged Department of Biotechnology (DBT) in 1986, under the Ministry of Science and Technology.

Keeping in view the future expansion of horticulture, biotechnology received priority attention for quality improvement and increasing productivity and production to compete in the world market for export. By establishing tissue culture and molecular biology laboratories at the ICAR institutes –The Indian Agricultural Research Institute(IARI), New Delhi; Indian Institute of Horticultural Research (IIHR), Bangalore; Central Plantation Crops Research Institute (CPCRI), Kasaragod; Central Tuber Crops Research Institute(CTCRI), Thiruvananthapuram; Indian Institute of Spices Research (IISR), Calicut and Central Potato Research Institute (CPRI), Shimla, biotechnology research in different horticultural