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Presidential Address[1]

BIOTECH-BREEDING FOR PLANTATION CROP IMPROVEMENT

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Esteemed General Chairman, Assistant Director General (Plantation Crops), Past Presidents, Members of the Executive Committee of Indian Society for Plantation Crops (ISPC), Members of the Standing and Working Committees of PLACROSYM- XI, Members of ISPC, Ladies and Gentlemen:

May I at the outset express my gratitude to all members of ISPC for having bestowed upon me the honour and privilege of delivering this Presidential Address to the 22nd Annual General Body Meeting of our august Society. Keeping in line with the theme of the current Symposium, namely "Plantation Technology for Productivity and Quality", I have chosen the topic of Biotechnology as a breeding tool for achieving sustainable genetic improvement of plantation crops.

Although conventional breeding procedures are rather slow and time-consuming for improvement of plantation crops, because of their long juvenile phase, heterozygous outcrossing nature, and the large area needed for experimentation, yet biotechnology can only be considered as a supplementary new tool to solve specific problems. Among other constraints, the breeder of plantation crops, in general, is beset with a narrow genetic base, lack of an inbred population since no systematic inbreeding has been done in the past, and the lack of adequate land area for field testing the selections and hybrids for yield and quality characters. Let us examine in what specific ways the tools and know-how of Biotechnology can help the plant breeder bring about greater precision in his approach towards realizing the goals of crop improvement research on Plantation Crops.

The word 'Biotechnology' in its broad definition connotes the use of technologies based on the living system to develop commercial processes and products, which include the techniques of recombinant DNA, gene transfer, embryo manipulation and transfer, plant tissue, cell and organ culture and regeneration, monoclonal antibodies, and bioprocess engineering.

Present day biotechnology is often referred to as a 'combination of power and ignorance': Power over genes, but relative ignorance of interactions between genes at cell-level, between cells at the organism-level, and between organisms in the ecosystems. Nevertheless, biotechnology and genetic engineering offer tremendous potential and opportunities for bringing about precision in genetic analysis of these complex group of woody perennials which are difficult to manipulate through conventional breeding procedures.

The specific area where tools of modern biotechnology and related fields of molecular genetics and plant tissue culture can profitably be employed for upgrading plantation crops may be grouped under the following heads:

1. Rapid clonal propagation of elite, disease-free selections, through cell, tissue and

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organ cultures, especially *via* somatic embryogenesis.

2. Conservation of native and exotic genetic resources, through *in vitro* gene banks, under both short-term limited growth conditions as well as long-term cryogenic storage.
3. Use of molecular markers like RFLP or RAPD to characterize cultivars, hybrids and elite materials, and estimate genetic distances within and between populations.
4. Production of haploids and isogenic (inbred) lines through anther/pollen culture for use in heterosis breeding and linkage analysis.
5. To identify molecular marker loci linked to desirable traits such as disease/drought resistance/tolerance for effecting marker assisted selections and subsequent gene cloning and transfer.
6. Use of molecular markers to construct saturated molecular linkage maps and identify genomic regions for yield related and other desirable traits.

The above aspects will be briefly considered in relation to the current status of achievements in each of our plantation crops, and future directions of research indicated.

PALMS

Although breaking the yield barrier still remains our priority objective in coconut, arecanut and oilpalm, the problem of disease and pest resistance/tolerance should now receive top priority attention in all the palms. The recent finding that the causal agent (MLO) which incites root(wilt) disease (RWD) in coconut, yellow leaf disease (YLD) in arecanut, and spear-rot disease in oilpalm can cross-infect each other, makes the problem more complicated and a cause for alarm (Anon., 1995). Our internal quarantine being what it is, with little or no restriction for movement of planting materials between states, utmost caution is called for in detecting one or the other disease in areas where oilpalm has been introduced amidst coconut or arecanut plantations. Vector control being a very tedious, costly and impracticable operation, constant vigil is needed to eradicate palms where spear-rot, YLD or RWD is detected in any one of them, in order to reduce the inoculum level and prevent further spread of the disease. Resistance breeding work also assumes prime importance, and the sooner we develop DNA-probes and RFLP markers for these three diseases of the three palms, the better it would be for planning future strategies.

The heartening feature in our efforts to locate resistance/field tolerance for the dreaded root (wilt) disease (RWD), is the indication of field resistance/tolerance of the Chowghat Green Dwarf (CGD) parent and its F_1 hybrid with disease-free West Coast Tall cultivar located in the 'hot spots' of Kerala where the disease is endemic (Anon., 1994). If the F_1 is showing field tolerance, its genetic implications need to be understood in terms of the number and nature of the gene(s) involved. Being a long-duration perennial, it is not conceivable to attribute single gene control for continued resistance/tolerance to MLO infection, since the microbial pathogen will most certainly have a faster rate of mutation for its survival in the host which lives in the farm for several decades (50-80 years for Talls and 40-50 years for Dwarfs). Only the polygenic control of this character can afford the internal buffering in the host against the onslaught of a rapidly mutating MLO. Molecular markers constitute an excellent tool for characterizing such polygenic traits, since only a single population is sufficient for identifying different genomic regions imparting the disease resistance/tolerance. The strainal similarity of the MLOs causing the above three disease-symptoms as revealed on cross-inoculation between the three host-palms, makes the situation alarming. Here is a strong case for constructing RFLP/RAPD markers for these three diseases and the resistant/tolerant hosts.

Meanwhile, progress on the tissue culture front is also encouraging, protocols

for oilpalm and coconut being worked out, and the IRHO having commercialized oilpalm clones derived from leaf/inflorescence culture. Although success in coconut is limited to a few clones obtained at CPCRI from seedling leaf tissues (Raju *et al.*, 1984), both IRHO (Verdeil *et al.* 1989, 1992, 1993, 1994) and Philippines (Rillo & Ebert, 1993) are closing in towards achieving success, and by the turn of this century it is hoped that coconut clones will also become a routine possibility, with advancement in our knowledge on basic aspects of this recalcitrant palm.

Success has been achieved on the routine use of embryo culture for field collection, storage (Anitha Karun & Sajini, 1994) and retrieval of coconut (Assy-bah *et al.*, 1989) and oilpalm germplasm. The future priority will be to standardize cryo-storage methods using liquid nitrogen, so that *in-vitro* gene banks will be a distinct possibility. All the above effort, particularly RFLP/ RAPD mapping is going to cost a lot of money and questions of economy will be raised. However, considering the enormous losses of over 960 million nuts incurred from RWD alone, the above lines of work would be worthwhile and cost effective. Sources of finance will not be wanting, since organizations like Govt. of India's Dept. of Biotechnology are eager to support such inter-disciplinary and even inter-institutional research, across all bureaucratic barriers.

Our future research on *in-vitro* culture of palms should be directed towards finding answers for (1) reduced response of adult palm vs. juvenile seedling tissues, (2) trigger for callus-mediated *vs.* direct somatic embryogenesis, from leaf/inflorescence tissues, (3) role of antioxidants and activated charcoal (AC), in prevention of browning *vs.* adsorption of growth factors and inhibitory chemicals like ethylene, or 5-hydroxymethyl furfural produced by sucrose dehydration, and of growth promoting auxins like 2, 4-D. There is evidence from work done in Philippines that AC adsorbs 99.5% of the ^{14}C labeled 2, 4-D by 4th day, leaving only 0.5% available in medium (Ebert & Taylor, 1990), especially in liquid as against semi-solid media. Higher temperatures (20-30°C) and low pH of medium accelerated adsorption of 2, 4-D by AC. Similar studies are needed to track the fate of other growth factors like cytokinins and vitamins to ascertain if they become growth limiting due to adsorption by AC.

Another aspect of *in-vitro* culture that deserves our intensive attention is the production of haploids through anther/pollen culture, where preliminary success was reported both in India, (Iyer, 1982) and in the Philippines (Thanh Tuyen & Guzman, 1983; Thanh-Tuyen, 1985), and in France (Monfort, 1985) upto the pollen embryoid stage. The production of haploids and isogenic diploids in coconut and other palms will greatly accelerate the pace of our heterosis breeding and genetic engineering programmes to evolve rare recombinants.

Oil quality: So far this aspect has not received the attention it deserves from coconut breeders, but in *tenera* oilpalm hybrids, variation has been recorded in the ratio of saturated vs. unsaturated fatty acids. In the annual oilseeds like rape (*Brassica rapa*) and *Arabidopsis*, possibilities are indicated, of evolving "designer oilseed crops" through the induction of useful oil-quality mutations in *Arabidopsis thaliana* and their subsequent transfer into rape species by a process known as 'shuttle mutagenesis' (Röbbelen, 1991; Murphy, 1991; Ashri, 1992; 1993). Mutants for fatty acid composition have already been induced in *Arabidopsis* (Browse, 1989). Another possible way of integrating mutation breeding and genetic engineering is being considered in *Sesamum*, whose oil contains a natural phenolic anti-oxidant, 'sesamol' derived from sesamolin, which is being considered for reducing oil oxidation and prolonging shelf life of all vegetable oils. If genes controlling sesamol biosynthesis in *Sesamum* could be identified through RFLP/RAPD analysis,

transferred to other oilplants and expressed, their oil stability can be greatly improved. Willmitzer and Toepfer (1992) have reported that several DNA sequences coding for enzymes of plant lipid metabolism have already been cloned, and most of the enzymes of plant lipid metabolism are expected to be mapped in the next couple of years. Several commercial Biotech. Companies have major programmes in this area (Cubitt, 1991). Genetic engineering in rape (*Brassica napus* & *B. rapa*) leading to a modified fatty acid composition has also been achieved, besides transferring anti-sense genes from rat into rape for medium chain hydrolase.

Callus cultures of oilpalm have been used to study the formation of a storage lipid whose biosynthesis was monitored by incubating the cells with ^{14}C - acetate and determining radioactivity in the triacyl glycerol fraction of labeled lipids formed (Turnham & Northcote, 1984). Sharp increase in radioactive triacyl glycerol 5 weeks after culture, indicated the production of embryoids. Studies on developing somatic and pollen derived embryos of *Brassica napus* and other oil crops show that embryo formation in culture parallels embryogenesis in developing seeds, e.g., in storage lipid assembly and fatty acid elongation. Specific activities of the Kennedy pathway (whose enzymes assemble the Triacyl glycerols in the endoplasmic reticulum) are found to be much higher in cultured somatic and microspore (haploid) embryos than in zygotic embryos. Such embryos could hence be used as models to help studies on assembly of storage lipids and regulation of corresponding genes in oilseeds. Thus, genetically modified embryos in culture may be useful for the prognosis of alteration in fatty acid composition of seed-oils at a very early stage (Weber & Taylor, 1990).

Thus, it will become possible to develop within a few years, genotypes that will produce 'tailor made' oils of specific fatty acid ratios to meet the needs of the edible (for human consumption), and the industrial consumers for soap manufacture and other chemical industries. Let us hope that in coconut and oilpalm too we will witness a shift from "petrochemistry" to "botanochemistry" as predicted by Pryde & Rothfus (1989), so that both developed and developing countries will stand to benefit from such a shift.

Breeding for stress tolerance: Biotic and abiotic

Among the biotic stress factors, MLO-incited diseases of palms, such as lethal yellowing (LY) in Jamaica, root(wilt) disease (RWD) in Kerala, spear rot in oilpalm, yellow leaf disease in arecanut (YLD), and Tatipaka disease in Andhra Pradesh, are the major diseases to engage our concerted effort to evolve strategies to contain the spread of these diseases, screen cultivars, hybrids and natural populations for possible sources of field resistance/tolerance, and finally, to develop DNA probes as diagnostic tools as also for genetic engineering studies.

The disturbing factor is the wide host range of the lethal yellowing (LY) MLO which not only affects coconut but several other palm genera - thus threatening the valuable collection of palms at the Fairchild Botanic Gardens in Florida. Thus, in a study conducted in Mexico, MLOs were detected in coconut palms of Yucatan Province, using radiolabelled cloned DNA probes. They found that two probes LYI-43 and LYD-9 containing DNA segments from a Florida LY MLO, hybridized at moderate frequency with DNA extracted from 6 yucatan LY-diseased but not healthy palms, thus suggesting similarities between LY MLO from Yucatan, Mexico, and LY MLO from Florida, USA. The MAYPAN hybrids replanted in LY-ravaged gardens of Jamaica are now said to be in full bearing and show no significant losses (Harries, 1991). Commercially, losses in both Malayan Yellow Dwarf (MYD) and its hybrids are reported to be less than one percent.

In India as stated earlier, the MLO's of Coconut RWD, Arecanut YLD and Oilpalm Spear-rot appear to be similar as they can cross-infect all the three hosts. (Anon., 1995). This makes it all the more difficult to keep constant vigil of mixed plantations, or contiguous plantations of coconut, arecanut and oil palm, to detect any possible disease appearing. Only through RFLP/RAPD analysis one can unravel the real identity of the three MLO's and the nature of homology/ dissimilarities among them.

In fact, Harrison *et al.*, (1992) have cloned and identified 5 Eco RI restriction fragments comprising chromosomal DNA of MLO associated with lethal yellow (LY) disease of Manila Palm (*Veitchia merrillii*) occurring in Florida. When used individually as (^{32}P)d ATP - labeled probes in dot and Southern hybridizations at high stringence, four or five probes consistently hybridized to DNA extracts derived from LY-affected palms only. However, at moderate stringency, all probes hybridized with DNA of other MLO's that occur in Flórida, and 3 probes also hybridized to DNA of several *Acholeplasmas* and/or *Spiroplasma* species. In addition to Manila palms, the probes also detected the presence of LY MLO DNA in DNA samples extracted from heart tissues of LY-affected true date (*Phoenix dactylifera*), cliff date (*P. nupicola*), chinese fan palm (*Livistona chinensis*), and five coconut palm cultivars. Probes also hybridized to DNA from symptomatic *Caryota rumphiana* and *L. rotundifolia*, two palm species previously not known to be affected by LY mycoplasma. This extends the known host range of LY MLO and increases our understanding of epidemiology of the disease. These probes may be useful in identification of primary and alternate plant hosts and insect vector(s) as both the probes and the disease are further characterized.

Similarly, molecular diagnosis has been developed for the viroid agent of Cadang-Cadang disease (CCCVD) of coconut in southern Luzon of Philippines (Hanold & Randles, 1991a, b; Maromorosch, 1993). This disease develops very slowly and cannot be unequivocally identified on the basis of visual symptoms in a single observation. Another disease of Guam coconuts called "tinangaja" is also caused by a related viroid (CTiVD). Oilpalm also develops bright orange leafpots under natural infection or artificial inoculation, resulting in loss of nuts and the palm. Other palms inoculated successfully with CCCVD are arecanut, golden palm (*Chrysalidocarpus herbaceous lutescens*). date palm, royal palm (*Roystonea regia*) and Manila palm. Several other herbaceous monocots growing near coconut also contain viroid-like molecules with similar DNA sequence as CCCVD. These authors have cautioned against unrestricted movement of germplasm following their recent identification of CCCVD-like sequence in the Pacific region, far away from Cadang Cadang area. Even embryo/ tissue cultures of coconut and oilpalm should be derived only from material tested for freedom from viroid. In fact, Rillo *et al.* (1988) have used *in-vitro* cultured embryos to screen coconut populations for resistance to cadang-cadang.

In Ivory Coast, Franqueville and colleagues (1991) have located resistance/ tolerance to the bud-rot pathogen, *Phytophthora heveae*. Whereas the West African Tall (WAT) was sensitive to bud rot, it was tolerant to immature nut fall. Within PB-121 hybrid (MYD × WAT) MAWA, there was considerable variability, some trees showing vertical resistance. MYD was tolerant to this disease. Other hybrids like Cameroon Red Dwarf (CRD) × Rennel Tall (RLT), MYD × RLT and D × T of Vanuatu showed tolerance to both budrot and immature nut fall. These findings need to be confirmed in other countries where bud rot is prevalent due to *Phytophthora palmivora*.

Shauna Somerville (1994) at the Carnegie Institution of Washington has proposed a gene-for-gene hypothesis for molecular

mechanism of disease resistance in barley against powdery mildew. The resistance genes encode receptor proteins that intercept a pathogen signal/and activate defensive responses.

Breeding for Drought-Tolerance in coconut:

The abiotic stress that adversely affects palms is drought that occurs frequently in many areas in the country. Although there is no authentic data on the extent of production losses caused by drought in coconut and arecanut, it was the unprecedented drought of 1982-83, particularly in North Kerala that led to a systematic approach to screen germplasm collections for drought-tolerance in coconut. Having identified the coconut cultivars and hybrids possessing desirable traits to with-stand drought, like stomatal regulation (Rajagopal *et al.*, 1990), biochemical characterization of enzymes (Shivashankar *et al.*, 1991), and lipid peroxidation (Chempakam *et al.*, 1993), the next logical step would be to understand the genetic basis of tolerance to drought. Application of modern techniques like RFLP/RAPD for identification of genes controlling either stomatal regulation, wax deposition, or specific enzyme markers would help in opening up new vistas in drought research in coconut. For instance, the location of genes controlling the production of wax components like hydrocarbons or β-diketones as also the synthesis or activation of enzymes like Superoxide Dismutase, in response to field stress might prove useful. Transfer of such genes to less tolerant or susceptible palms could result in evolving transgenic plants in coconut for drought prone areas. Karunaratne *et al.* (1991) have also developed an *in-vitro* assay for drought tolerant coconut germplasm.

CASHEW:

One of the major objectives in cashew improvement is to achieve stability of performance in diverse environments. Being a highly heterogeneous, outcrossing population, the selections and hybrids need to be tested for particular locations in order to evolve region-specific cultivars and hybrids, since their performance over locations is highly inconsistent. Nevertheless, we have exceptionally adapted varieties like M44/3 which has performed consistently well in all locations. However, it is not possible to pinpoint the specific gene combination or complex which has contributed to the consistently stable performance of this variety over diverse locations. The use of modern tools of biotechnology such as RFLP/RAPD can certainly help in identifying specific Quantitative Trait Loci (QTLs) which determine the high performance of varieties. Also, the RFLP mapping of varieties will clarify many doubts regarding correct identity of germplasm collections in molecular terms. Selections of parents for breeding programme will acquire a new dimension of precision, so that predictable hybrids with heterotic expression of characters of qualitative and quantitative inheritance can be realized.

Kernel quality of cashew in particular is becoming increasingly noticed in international markets and hence if we have to have this edge over other exporting countries, we must precisely identify desired genotypes possessing the premium quality of kernels, not only in terms of size and weight, but also in protein value and essential amino acids. This screening can be elegantly achieved through RFLP markers.

Use of tissue, cell and anther culture in cashew, should not be looked upon merely for generating clones of elite trees, both as rootstock as well as scion material. Efforts should also be directed towards developing a cell-suspension system, for induction and multiplication of somatic-embryos. The problem of browning particularly of adult tree tissues can be overcome to a great extent, by the use of liquid cultures under dark incubation, and use of chemicals like phenyl-3 methyl-5 pyrazolone, ammonium chloride, urea and sugar to remove inhibitors secreted by explants, which on reaching a

certain threshold value stop the growth of tissues, bud-break and elongation.

Multiple shoot formation

Bud culture in cashew has given only a limited number of shoots, and increase in dose of cytokinin often resulted in fasciated micro-shoots. However, lowering BA levels and increase of sucrose, has induced elongation of microshoots (D'Souza *et al.*, 1994).

Immature cotyledon segments have given direct somatic embryogenesis without any intervening callus, or a very incipient callusing. This system if augmented and made regular can be used for basic studies on somatic embryogenesis, as also in encapsulation studies for developing protocols for germplasm storage both under limited growth conditions as well as cryostorage.

Anther culture: This is a longstanding need in cashew for developing haploids and isogenic lines to facilitate heterosis breeding as well as for gene transfer experiments. The major limitation in achieving *in vitro* androgenesis is the availability of only a single fertile anther whose size is quite small at the uninucleate microspore stage of the buds. Tea mosquito infestation compounds the problem further resulting in severe browning and contamination. Screening of cultivars and hybrids against tea mosquito-incited toxin, and further multiplication of the resistant selections is a priority area where tissue and cell culture combined with molecular techniques will pay dividends. The greatest advantage is the natural propensity for obtaining cashew grafts in large numbers, for which bio-technology approaches would help to supplement the additional requirement of scion shoots as well as root stock materials generated *in-vitro*.

BEVERAGE CROPS

Tea, coffee and cocoa as a group form ideal model systems for combining conventional breeding with modern tools of biotechnology and genetic engineering for crop improvement, since the scientists working in these crops have nearly perfected the protocols for *in vitro* multiplication of elite materials. Both tea and coffee research institutions have developed necessary infrastructure for this work, and hence are well poised to enter the new era of Bio-tech breeding.

TEA: Protocols have been developed for micropropagation, somatic embryogenesis and their encapsulation to produce synthetic artificial 'seeds' of tea (Sood *et al.*, 1993; Manivel., 1993; Rajasekharan & Mohankumar, 1992; Rajkumar and Ayyappan, 1992). Tea plantlets have been successfully induced from cotyledon callus (Wu, 1976; Wu *et al.*, 1981) and from stem callus (Kato, 1985). Production of shoots and buds has been achieved using nodal explants (Phukan & Mitra, 1984). Tea being self-incompatible, it would be worthwhile producing haploids and homozygous diploids using anther/pollen culture technique, and preliminary success was reported upto the multicelled pollen stage and haploid callus formation (Raina & Iyer, 1974, 1983, Chen & Liao, 1987). The main areas of tea improvement where bio-technology can contribute significantly are:

1. *Low caffeine tea*: Evolving low caffeine tea with improved solubility, which involves the application of both conventional breeding as well as molecular tools like RFLP/RAPD, to unravel the molecular biology of cell wall polymers and architecture. Possibility of transferring low caffeine genes from other *Camellia* sp. should be explored both through conventional hybridization and transgenic approaches to the modification of the biosynthetic pathway for caffeine synthesis, using antisense RNA technology to selectively switch off individual genes and block biochemical pathways. The other approach is through accelerated particle gene delivery (using 'gene gun') which has brought many recalcitrant crops within range for genetic manipulation.

2. *Drought Tolerance in Tea:*

In vitro approaches for evolving drought tolerant teas are being followed at the UPASI Tea Research Institute, where, protocols for rapid clonal multiplication have been developed in four cultivars (Manivel, 1993). Induction of variation for selecting drought tolerant somaclones through irradiation or chemical mutagens, has been in force at the UPASI Tea Research Institute using UPASI-3 clone whose *in vitro* response was good and productive.

Somatic cell hybridization using protoplast fusion has been tried to transfer the Darjeeling tea flavour of 'China' clones to the Assam cultivars possessing strong and brisk liquors, (Banerjee, 1986).

Tea being a dicot species, it would be worthwhile pursuing *Agrobacterium*-mediated transformation as the choice gene delivery system. Tea regeneration from both shoot tips and cotyledonary callus-derived somatic embryos has been reported from Japan (Nakamura, 1987). Breeding for resistance/tolerance to blister blight fungus (*Exobasidium vexans*) would be facilitated if the *in vitro* system is employed for evaluating and screening fungicides, as also the resistance of the tissues to various races of the pathogen (Agnihothrudu & Chandra Mouli, 1991).

COFFEE:

Coffee is considered as a model *in vitro* system for crop improvement *via* tissue culture and genetic transformation. At the Coffee Research Institute of Indian Coffee Board at Balehonnur, protocols have been developed for obtaining direct as well as indirect somatic embryogenesis, which is being exploited for large scale commercial level multiplication of superior selections. Immature embryo cultures have been established for plantlet regeneration through embryo-rescue in hybrids. Synthetic seed technology for encapsulating embryos in sodium alginate has been developed. Anther culture technique has been successfully employed for callus induction and plantlet regeneration in C x R inter-specific hybrid, for rapid fixation of heterosis. This programme was carried out in collaboration with SPIC Science Foundation, Madras, where protocols for isolation of embryogenic cultures, using leaf and nodal explants were developed. Work has been initiated to develop the *Agrobacterium*-mediated transformation system using a leaf-disc procedure.

At the DNA Plant Technology Corporation, in Cinnaminson, New Jersey, USA, Maro Sondahl and colleagues have developed a system of high frequency somatic embryogenesis from leaf explants of *Arabica* Coffee, and have traced their precise mode of origin using EM scanning (Sondahl & Sharp, 1977; Sondahl *et al.*, 1979). They have also developed bioreactor scale of micropropagation using embryogenic cell suspensions of *Robusta* coffee in flask cultures, estimated to yield 460000 somatic embryos after 7 weeks of culture, which was dependent on cell density. For a 3-litre capacity bioreactor charged with 3g fresh weight of embryogenic cells, approximately 600000 somatic embryos could be realized every two months of culture (Zamarippa *et al.*, 1991). Thus, coffee micropropagation *via* somatic embryogenesis promises to be an efficient method of propagating elite individual trees from a segregating population, which will be competitive in time and cost.

Somaclonal variation in coffee would be an excellent way of shortening breeding programmes, since it provides access to genetic variability within existing cultivars, carrying few alterations, while preserving the genetic integrity of commercial varieties. This variation is either naturally occurring or induced *in vitro* during plant regeneration from callus, due to chromosome breaks, translocations, deletions, aneuploidy, polyploidy, gene amplification, transposons, somatic crossing over, or point mutations (Evans and Sharp, 1983).

In *C. arabica*, some 40 mutants have been identified which are controlled by single genes with expression of dominance, recessiveness, or partial dominance, over the alleles of *Typica* variety. Appearance of mutants with monogenic inheritance indicates the partially diploidized nature of *Arabica* coffee (Carvalho *et al.*, 1969).

Out of a total of 185000 somatic embryos isolated, 54000 (29%) completed maturation and germination phases. About 20000 plantlets were transferred to nursery and over 16000 somaclones and controls were established in the field. To date, 12000 (78%) of these coffee somaclones have been screened after 2 to 4 years under field conditions, and 1196 variants (10%) recorded. Another 1686 somaclones that did not survive in field could have carried lethal mutations.

Out of 7772 somaclones evaluated, 452 (12%) were derived from high frequency pathway and 415 (10.4%) from low frequency pathway of somatic embryogenesis. Thus, there is no evidence to say that increased variability depends on origin of somatic embryos. The successful recovery of plants via somatic embryogenesis in several wild coffee species, five *C. arabica* cultivars and two interspecific hybrids demonstrates the potential of using *in vitro* methods for coffee improvement. (Söndahl & Lauritis, 1992).

Transient transformation was demonstrated in callus derived from *Arabica* coffee protoplasts co-cultivated with *Agrobacterium* carrying NPT II and 'beta' glucuronidase marker genes under control of Ca MV 355 promoter. Considering the repeatability of protoplast regeneration systems, the utilization of useful genes for coffee improvement is now a near to mid-term possibility with a high degree of success. However, embryo rescue and anther culture techniques need further refinements. Thus biotech breeding in coffee can lead to improvements in agronomy by reducing farming costs, superior beverage quality and stable prices.

COCOA:

The present holding of 106 accessions of cocoa germplasm at CPCRI represents a narrow variability, acquired mainly from Nigeria. We need to enrich this by collecting large bean and pleasant aroma types from Amazon, Ghana, Brazil and North and Central Americas, for evaluation. Cocoa seeds being recalcitrant with short viability, budwoods are the only means of introduction, with all the attendant problems of contamination, and transit losses. Hence, the collection of embryos and *in vitro* shoots under aseptic condition needs to be perfected for future collection expeditions. We need to select and screen available germplasm for resistance to canker, and *Phytophthora*- incited black pod diseases, combined with high yield and large bean size.

Drought tolerance is a vital character to be desired in our cocoa breeding programmes, and screening has been done for drought tolerance using physiological parameters. The productivity of present cocoa gardens is only about 400 Kg/ha whereas the potential is more than 800 Kg/ha. Drought, disease and pest losses are the chief depressants of yield. Hence, there is good scope for selection of cultivars and hybrids possessing higher yields and tolerance to biotic and abiotic stresses. These studies will be greatly helped by the use of RAPD for DNA finger printing of cocoa clones to select desirable cultivars.

The Technical Group on Cocoa convened in 1986 in the Ministry of Agriculture, Govt. of India had estimated a grinding capacity of cocoa-based industries, of 20,000 tonnes by 1990 and a production target of 1.0 lakh tonnes by 2000 AD. However, the present grinding capacity is about 16,000 tonnes only, and the production of cocoa beans is 7000 tonnes which has not been fully utilized due to lack of demand for finished and semi-finished products. Export prospects of dry beans is also not bright due to our high cost of production. Considering these trends, the internal demand for cocoa

beans is not likely to go above 12000 tonnes per annum, which can be fixed as our production target for the terminal year of 8th Plan.

Role of biotechnology in cocoa improvement

Although conventional methods of clonal propagation such as budding, side grafting and rooting of cuttings are possible in cocoa, they are relatively expensive and provide only a limited number of propagules. Hence, there is good scope for exploitation of tissue culture for rapid clonal propagation and crop improvement in cocoa, as a supplement to conventional propagation techniques. Cocoa being recalcitrant in tissue cultures using conventional protocols, scientists working in Purdue University, have found that a high CO_2 environment promoted shoot and root development in cotyledon-derived somatic embryos. Depletion of CO_2 level in tissue cultures during the day, was limiting photosynthesis. Shoot elongation and leaf development increased asymptomatically after 30 days, with CO_2 levels from ambient (470ppm) to 28,000ppm (Figueira, Whipkey and Janick, 1991).

Mallika *et al.* (1992) have used nodal segments excised from field grown cocoa plants, and axenic seedlings as explants. Fungal contaminants were warded off by fungicidal spray (Bavistin 0.2% & Dithane M-45 - 0.3%) of the field grown plants every 3 days. Aseptic seedlings raised by mature embryo culture, were also used wherever mature tree explants gave poor response. Woody Plant Medium (Lloyd & McCown, 1980) containing the cytokinin 2-ip (1 ppm) + IAA (0.02 ppm), promoted bud sprouting and leaf expansion. Same medium was suitable for continued growth of shoots, but supplements of aminoacids, ascorbic acid, peptone and phloroglucinol were essential for sustained growth of proliferated shoots from field explants. Profuse callusing without differentiation could be suppressed by $AgNO_3$ (5ppm) addition to the medium. Rooting was induced by a pulse treatment of IBA in ethanol, and the rooted plantlets were gradually hardened in pots kept at high humidity and then transferred to the field.

At CPCRI, Kasaragod, Philomina Abraham *et al.* (1992) have standardized *in vitro* induction and growth of somatic embryoids from immature cotyledon segments cultured on Murashige & Skoog's (1962) medium supplemented with NAA and BAP (0.5 mg/l each) and coconut water (15%). Indulekha & Iyer (1993) have observed direct somatic embryogenesis from immature cotyledon explants, at a 20% frequency, on MS medium + NAA and BAP (0.5 mg/l each). These somatic embryoids on subsequent transfer to MS medium containing abscisic acid (0.026 mg/l), underwent maturation and greening process. Plantlet formation occurred on transfer to MS + BAP (0.5 mg/l).

RUBBER

Rubber cultivation is just a century old, and in fact large-scale plantations began only 80 years ago. Although the genus *Hevea* comprises nine species, *H. brasiliensis* Muell. Arg. is the main cultivated species, which has adapted to a wide range of countries in the South and S. East Asia, Central and W. Africa, China, Papua New Guinea, besides its home in Brazil. All the trees in S. E. Asia covering millions of hectares are said to have been derived only from a few trees, thus showing a narrow genetic base. Being a perennial out-crossing tree, taking 5-7 years from seed to bloom, the present varieties are highly heterozygous, and inbreeding gives only a few seeds or none at all, which has slowed down the pace of rubber breeding. Hence, if haploids could be generated in rubber through anther culture or unpollinated ovule culture, it would prove a valuable tool for improving rubber through biotech breeding, resulting in superior genotypes and new recombinants stabilized through diploidized haploids. (Zhenghua, 1990).

The pure lines (isogenic lines) thus btained can be used in heterosis breeding y propagating self-rooting plants or artificial seeds' from trees of good combinations by issue culture propagation, which could eplace budding. Induction frequency in nther culture was cultivar dependent, PB-86 being more responsive than RRIM-600 n terms of obtaining pollen embryoids. Flower buds measuring 3.0 - 3.5 mm length with green corolla, contain mostly uninuclear nicrospore stage which is appropriate for nther culture. Embryoids began to appear n 25th day and multicelled masses emerged, nd by 45-50th day these calli proliferated nd were transferred to differentiation nedium (Rubber tree or RT medium) containing Kinetin (1 mg/1) + 2, 4-D (1 ng/1) + NAA (1 mg/1) + Coconut water 5% + Sucrose 7% + agar 0.6% at pH 5.8. After 90 days in this medium with 2-3 fresh ransfers, embryoids could be transferred to he plantlet forming medium. Pollen plantlets ack a strong root system and hence the main and verticillate lateral roots have to be well developed, after which the plantlets can be exposed to sun for 2 days.

Roots are removed after raising in a mixture of NAA and IBA (25-35 ppm each) for 15-30 minutes, and the plantlets are transplanted quickly in the soil: sand (1:1) mixture in bamboo baskets kept at 25-30°C under high humidity in a culture room. They are exposed to sun for 3 hrs in the morning and 2 hrs. in the afternoon for 15 days, before field planting. Cytological examination of embryoids and pollen plantlets revealed chromosome numbers in multiples of 9, ie. 27, 18, showing that they were of pollen origin. *Seven pollen-derived rubber trees* showed 18 to 27 chromosomes mostly, and 18% had 36 chromosomes. Analysis of latex and dry rubber yield of pollen tree No. 1 and donor clone revealed that the pollen tree yield was significantly higher than that of the donor clone (22% and 29% respectively).

Unpollinated ovule culture yielded four plantlets in 1984 arising from haploid cells of the embryosac. Around 90 days growth was necessary for the embryosac-derived embryoids to grow on differentiation medium.

Thus, haploids have good prospects in rubber breeding and research.

Micropropagation through culture of mature tissues of rubber trees and through callogenesis from inner integuments of immature seed has resulted in several embryos and plantlets (El Hadrami *et al.*, 1991). They have observed that excess of auxin and cytokinin in the medium, even of a weak auxin like 3,4-D, and prolonged exposure to these hormones suppressed embryogenic potential of the callus. Thus, instead of 9 M each of 3, 4-D and BAP in the control medium they used only 4.5, 2.25 or 0.45 M each of 3, 4-D and BAP. After 40 days they were subcultured on a medium containing 0.45 M each of NOA and BAP. Germination of somatic embryos was obtained on woody plant medium of Lloyd and McCown (1980) supplemented with GA_3 (2.9 μM). High hormonal level present during induction of callus, previously thought necessary, are thus shown to be responsible for arresting somatic embryogenesis in recently divided cells. This effect may explain the reputation of rubber as a recalcitrant embryogenic species. Genotypic differences were also seen between the two clones in the accumulation and degradation of these hormones, which in turn explains the genotype variation in embryogenic potential. Asokan *et al.* (1988) have reported successful *in vitro* multiplication and field planting of Clone-1 in media supplemented with 1.5 –3mg/l 1AA and 0.5-1.5 mg/l kinetin.

With regard to the problem of browning due to phenolic exudates it was found that water soluble PVP (polyvinyl polypyrrolidone) at 100 ppm was most effective, followed by $HgCl_2$ treatment instead of NaOCl. Shoot tip explants had higher phenolics than nodal explants. Activated charcoal had no effect in liquid media, but it delayed browning when incorporated into semi-solid media.

Frequent transfers to fresh media did help in reducing casualties due to browning. There was also clonal variation in the extent of browning, RRIC-110 exhibiting the highest and PB-86 the least extent of browning.

SPICES

Among the biotechnological tools, tissue and organ culture can be successfully exploited in a number of spice crops for (a) rapid clonal multiplication of elite, disease free planting material, (b) embryo rescue, (c) generation of somaclonal variation, (d) *in vitro* screening for resistance/tolerance to pathogens, (e) germplasm conservation and exchange *in vitro* and, (f) *in-vitro* production of active components of flavours and essences.

Protocols for micropropagation are now available for a number of spice crops and in some it has been even commercialized, as in Cardamom and Vanilla. Cardamom clones have been produced both from vegetative buds (Nadgauda *et al.*, 1983) as well as immature panicles (Kumar *et al.*, 1985). Today a number of commercial Biotech Companies like A. V. Thomas Co., Hindustan Lever, Indo- American Hybrid Seeds etc. are producing and selling lakhs of cardamom clones using the above methods. Vanilla tissue culture technology has been standardized by Philip and colleagues at Calicut University, Botany Department, using both shoot (Philip & Nainar, 1986), and root meristems (Philip & Nainar, 1988). Field evaluation of micropropagated cardamom has demonstrated their superiority in yield and better tiller production (Lukose *et al.*, 1993). Plant regeneration from callus of seedling, vegetative bud or rhizome explants gave 20-50 plantlets per culture which is now being used for large scale production of somaclones and their field evaluation.

Both *ginger* and *turmeric* have also been successfully multiplied by tissue culture for raising disease-free planting material of ginger as also to multiply high curcumin types of turmeric (Nadgauda *et al.*, 1980; Kuruvinashetti & Iyer, 1981; Kuruvinashetti *et al.*, 1982). In ginger, Nirmal Babu *et al.*, (1993) have developed a technology of producing somatic embryoids and their encapsulation in sodium alginate to make artificial seeds, which will be useful in germplasm exhange and conservation. Nirmal Babu *et al.* (1992a) have also obtained direct regeneration of ginger plantlets from immature inflorescences. Kulkarni *et al.* (1987) have attempted to screen ginger calli for resistance to culture filtrates of *Pythium* sp. This requires to be reconfirmed by field testing the plants raised from resistant/ tolerant calli.

In vitro clonal propagation has also been achieved in Black Pepper using mature shoot tip explants (Nirmal Babu *et al.*, 1993; Nazeem *et al.* 1992; Philip *et al.*, 1992), from seedling tissue (Mathews & Rao, 1984), and from stem and leaf callus (Nazeem *et al.*, 1993). These regeneration systems have been used in studies on *Agrobacterium*-mediated transformation for transfer of resistance (to *Phytophthora capsici* causing foot-rot and *Radopholus similis* nematode causing slow wilt) from *Piper colubrinum* of S. America to *P. barberi* at NRC Spices (Nirmal Babu *et al.* 1993).

Tree spices have proved more recalcitrant in tissue cultures, particularly explants from mature trees. Micropropagation of *clove* from seedling explants has been achieved (Jagadish Chandra & Rai, 1986; Superman & Blake, 1990; Mathew and Hariharan, 1990, 1992). However, Nirmal Babu *et al.*, (1992b), have successfully multiplied the mace tissue of *Nutmeg*, obtaining a 10-fold increase in fresh weight in 2 weeks, and retaining the deep red colour and its characteristic flavour intact. If scaled up, this can be a commercial proposition for *in vitro* production of myristicin and myristic acid.

Thus, the future strategy of Biotech-breeding for Spice crops will focus attention on the following:

1. Extensive screening of somaclones and induced mutations may be done for resistance/tolerance to *Phytophthora capsici* in black pepper (Shylaja *et al.*, 1994), *Pythium*-incited rhizome rot and *Pseudomonas* - incited bacterial wilt in ginger besides higher yield, quality and drought tolerance; and heat tolerant cardamom clones for the South Indian plains.
2. Production of haploids through Anther/Pollen culture, and homozygous diploids in cardamom, pepper and ginger and their utilization in crop improvement.
3. Bio-reactor technology for large-scale production of biochemicals, essential oils, alkaloids, and flavours.
4. Use of artificial seeds in germplasm conservation and retrieval, after short-term as well as long-term storage (Ramanatha Rao & Riley, 1994).
5. Use of cell and protoplast culture and regeneration for genetic transformation through electro-fusion, and microprojectile bombardment for direct incorporation of DNA. RFLP/RAPD analysis will help in determining clonal fidelity, and identifying the transformants for the major spice crops.

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