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Embryo and Tissue Culture for Crop Improvement, Especially of Perennials, Germplasm Conservation and Exchange — Relevance to Developing Countries*

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ABSTRACT

Plant tissue and organ culture techniques are increasingly applied for the improvement of economically important crops in the developing countries. Work done at this Institute on coconut spathe tissue and embryo culture, as well as turmeric tissue culture for rapid multiplication of superior clones, is briefly described. The application of tissue and organ culture in other crops, especially the perennials, like agricultural plantation crops, forest trees and horticultural plants is briefly reviewed with regard to (a) the conservation and international exchange of germplasm, especially of bulky seeded materials like coconut and vegetatively propagated ones like tapioca, potato and certain fruit trees; (b) clonal multiplication of elite materials; (c) induction of haploids; (d) induction of mutations and rare recombinations and (e) embryo culture. The rapid advances made in tissue, cell and organ culture hold great promise for their immediate application to problems in the developing countries where bulk of the farm holdings for most crops fall below one hectare. Hence, any advance towards improving the yield or disease resistance of the planting materials, made through the use of tissue culture techniques is sure to benefit the farmers.

INTRODUCTION

It is customary to begin every discussion on plant tissue and organ culture with Haberlandt (1902) who was the first to conceive of the totipotency concept for plant cells (see Krikorian and Berquam, 1969) and Hannig (1904) who first demonstrated the embryo culture technique. Clonal multiplication has been achieved in several higher plants, where tissue and organ culture has got established as a vital tool of biotechnological research as well as applied horticulture and agriculture. One of the main areas of vital importance where tissue and organ culture can be useful is in the conservation of germplasm, particularly of the endangered species, the native flora, and wild relatives of crop plants, which are facing elimination owing to expanding urbanisation and the rapid advent of improved crop varieties and hybrids. In the monotypic coconut (*Cocos nucifera* L.), massive replanting programmes are threatening to destroy native genetic resources in Philippines, Indonesia, India and Malaysia, and responsible scientists like Carlos (1979) have cautioned the Governments and over-enthusiastic scientists, to go slow on replanting large areas with the same source of hybrids which certain business-minded agencies are trying to push into every country. Embryo culture technique for transport of such bulky materials like coconut would also greatly aid in germplasm collection and conservation in a limited space, free from quarantine problems. Screening for disease and pest reaction using tissue and embryo cultures would also become feasible with advnaces in technology and with more knowledge on the toxins released by the pathogen/pest. This would facilitate evaluation of germplasm in early stages without having to raise a plantation which would require a lot of space, time and manpower.

The other application of embryo culture for overcoming the barrier of incompatible crosses in crop plants is quite well recognised, and has been documented in several reviews. Some of the recent work will be dealt with here briefly. Work on coconut and other perennial plantation crops carried out at the Central Plantation Crops Research Institute (CPCRI) and elsewhere, is also described in this paper, which is of direct relevance to the economic uplift of the developing countries.

MATERIALS AND METHODS

Coconut: At the CPCRI, we have the largest collection of world germplasm in coconut consisting of 62 exotics and 32 indigenous cultivars. The most common local Tall variety, namely, West Coast Tall has been used for the tissue culture studies. Meristematic tissues excised from one year old seedlings as well as adult palms were used for standardizing the techniques of callus induction and morphogenesis. For embryo culture, both mature, 12-month old nuts, as well as immature (7–10 months) ones were selected. The fibrous husk at the 'eye' region was carefully chopped off and the embryo along with surrounding endosperm tissue was scooped out of the soft eye with the help of a double-edged sharp knife. This was then surface-sterilized in 20% chlorine water for 10 minutes and after thorough rinsing with water placed in a mixture of ascorbic acid and citric acid (150 ppm each) to prevent oxidation and release of polyphenols before transfer to the culture medium.

From one year old seedlings, the apical bud region was carefully dissected out and surface-sterilized as described. So also, the immature spathe excised from the first or second open leaf axils of an adult palm, was surface-sterilized and the rachilla explants were cultured. In an earlier experiment, it was found that older spathes were unsuitable, as they gave rise to only normal flower morphogenesis. The stage of the spathe (preferably from first open leaf axil after the central spindle) was critical for effecting the conversion of floral primordia into vegetative shoot-primordia. Two expert climbers were trained to excise the first spathe, without damaging the single shoot bud of an adult palm. After giving a swab with 70% ethyl alcohol the spathes were dissected inside a Laminar-flow chamber. The rachilla explants measuring 1–1.5 cm were excised and cultured either on MS (Murashige & Skoog, 1962) basal medium supplemented with Nitsch's (1969) vitamins and 4–8% sucrose, or on Eeuwen's (1976, 1978) Y-3 medium containing 8% sucrose. Initially the correct stage of spathe was determined by

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* This paper is humbly dedicated to the memory of the later Dr Emerita V. de Guzman of the Philippines, for her pioneering contributions to coconut embryo and tissue culture.

planting the entire range of developing spathes taken from trees that were either felled for other reasons or had fallen due to wind. As a result of the screening studies, it was found that the spathe subtended in the axil of the first open leaf after the central spindle, was the ideal stage for effecting the conversion of floral primordia into vegetative shoot-like out-growths.

Root tissues both from primary as well as secondary laterals, excised from adult palms, as well as young seedlings, and from embryo cultures, were grown both on agar as well as liquid media after surface-sterilization with chlorine water. Leaf bases as well as tender leaf primordia around the apical meristem of seedlings were also cultured in a similar way. All cultures were incubated initially in darkness at 28+1°C in a BOD incubator at 70% relative humidity, for 4–6 weeks. The addition of activated carbon (5–10 g/l) was beneficial in arresting browning of coconut explants on agar media. Among hormones, NAA (0.5–1 mg/l) or 2,4-D (0.2–2 mg/l) was used individually in conjunction with a cytokinin like Kinetin (0.1 mg/l), 6 Benzylaminopurine (BAP) 2–10 mg/l, and 6 (y-y-dimethyl allylamino) purine riboside (PR 2–10 mg/l) particularly for floral tissues.

For anther cultures spathes on the 6th to 7th leaf-axil had suitable stage of uninucleate microspores. The spathes were opened after wiping with ethanol and the anthers were surface-sterilized with mild chlorine water and inoculated both on agar and liquid media of Y-3, B-5 (Gamborg *et al.*, 1968) and N-6 (Chu *et al.*, 1975) compositions, and the cultures were incubated in dark. Anthers were periodically examined in acetocarmine squashes for pollen development into embryoids. Responding anthers were transferred to fresh media.

Turmeric: Sprouting buds from storage rhizomes of a high-yielding, high curcumin clone 15B, obtained from the All India coordinated Spices and Cashewnut Improvement Project, were used for culture. After surface-sterilisation in chlorine water, individual buds measuring 1 cm were cultured on MS agar medium containing 4% sucrose, Kinetin (0.2 mg/l), BAP (0.4 mg/l) and GA_3 (0.01 mg/l) and incubated at 28°C+1°C under fluorescent light (alternating 12 hrs light and dark period) of 3000 lux (4300°K). After the production of multiple shoots, individual shoots along with some roots were separated carefully and transferred to fresh media with or without auxin and GA_3. This operation was repeated several times in order to obtain a high rate of multiplication. Multiple shoot production and rate of growth of shoots and roots was considerably enhanced in liquid cultures kept on a platform flask shaker. Rooted plants were then transferred to sterilized soil in poly-bags and kept in a glasshouse for further growth. When the plants had grown fully in about eight months, and leaves started drying, the rhizomes were harvested and weighed separately for each individual plant.

RESULTS

Coconut: Rachilla explants taken from spathe primordia at the 1st leaf axil (Fig. 2) showed the maximum response in the conversion of floral primordia into vegetative shoot-like outgrowths (Fig. 3). As the explants became older, the frequency of such conversions decreased and after the 3rd leaf axil, the tissues of rachillae gave rise to normal or abnormal floral primordia, which showed either (a) the male flower with a trilobed pistillode that developed to a flattened column or (b) a trifid structure or (c) an indeterminate columnar structure full of scale leaves, which could be subcultured individually (Fig. 4). So far, only one of them has rooted but no true leaves are discernible yet. However, nearly 50% of the floral primorida developed into shoot-like structures on Y3 medium of Eeuwens (1978) supplemented with 6-BAP (2 mg/l) and 6 (Y, Y-dimethyl-albylamino) purine riboside (2 mg/l) with 80 g/l sucrose.

Embryos from mature coconuts grew well on MS medium without any growth adjuncts. Addition of activated carbon was essential to prevent rapid browning of tissues which otherwise arrested further growth. The haustorium (= cotyledon) which in nature enlarges into a spongy, sweet, tissue drawing nourishment from the endosperm, for the growing embryo, degenerated in culture and turned dark brown (Fig. 5). When the seedling (Fig. 6) was transplanted to soil, the haustorium portion was infected, resulting in high mortality of the plants. Root growth *in vitro* was stimulated by decapitating the first large root that emerged out of the sprouted embryo with development of many laterals and sublaterals (Fig. 6). Liquid cultures, with filter paper platforms for supporting the embryos, showed better rooting response, with less problems of tissue browning.

Anthers cultured at uninucleate microspore stage, after cold pretreatment at 4°C for 48 hrs, on Y3 medium containing 6 per cent sucrose, 10 mg/l 2,4-D, 1 mg/l Kinetin and 1% activated carbon, in dark at 28°+1°C, showed multi-celled pro-embryoids within the exine (Fig. 7). Responding anthers have been subcultured on fresh media.

Meristematic tissues excised from one year old seedlings grew rapidly especially on liquid media placed in darkness, in petridishes in a thin layer and from the cut ends a slow growing, friable callus was observed. Leaf primordia also grew and expanded rapidly, turning green on exposure to light.

Root tip segments excised from sprouted embryos, and from seedlings or germinated nuts, cultured on MS medium supplemented with 40 g/l sucrose, 0.5 mg/l NAA, 0.2 mg/l 2,4-D, and 5–10 g/l activated charcoal, grew rapidly producing fresh growth in 4–6 weeks. However, without root tip, the segments showed no noticeable growth. The laterals and sublaterals showed elongation, and those on the surface of medium produced typical pneumatophores, containing loose, granular and spongy tissue, growing upwards, apogeotropically (Fig. 8). Roots excised from embryo cultures grew in better way and several fresh roots arose from the base of the embryonal tissue which had callused on 2,4-D medium (Fig. 9). On the other hand, roots excised from adult palms have not responded so far in culture, due to rapid browning.

Turmeric: On a simple MS medium devoid of growth regulators, the cultured buds sprouted only single plantlets. However, when supplemented with Kinetin (0.2 mg/l), BAP (0.4 mg/l) and GA (0.01 mg/l), the buds gave rise to a large number of adventitious multiple shoots (Fig. 10), with or without a preceding callus phase (Fig. 11). When the shoots attained sufficient height and rooting, individual shoots were separated and cultured on a medium with or without IAA and GA_3. At the end of 60 days a large number of rooted plantlets were produced from a single initial bud, the highest being 8 per culture tube on a medium containing Kinetin (1.0 mg/l) and BAP (2.0 mg/l). Thus, at the rate of 8 plantlets/tube/60 days, we could provide 384 plants in a year from a single bud. The multiplication was even more rapid with better root growth, in liquid media in flasks kept on a mild shaker (Fig. 12). When the plants attained a reasonable height and vigour they were planted individually in soil mixed with farmyard compost contained in polybags (Fig. 13) and transferred to a glasshouse (Fig. 14), and irrigated regularly. The plants grew as vigorously as in open field and bulked good amount of rhizomes, ranging from 10 gm to 136 g/plant (Fig. 15). A closer examination of the tissue-culture derived plants revealed a small percentage of chlorophyll chimeras (Fig. 13) and some dwarfish types. These are being studied separately for understanding the


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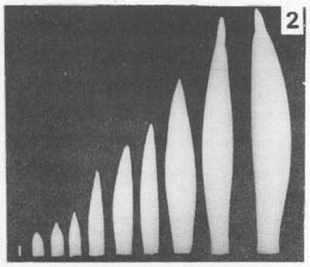
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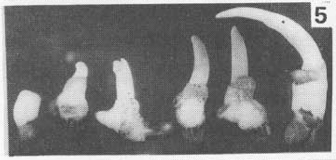
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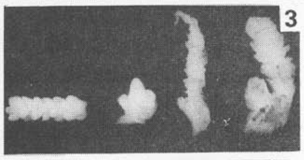
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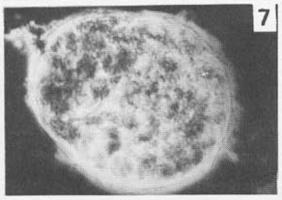
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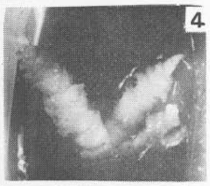
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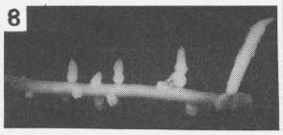
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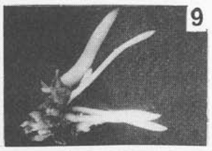
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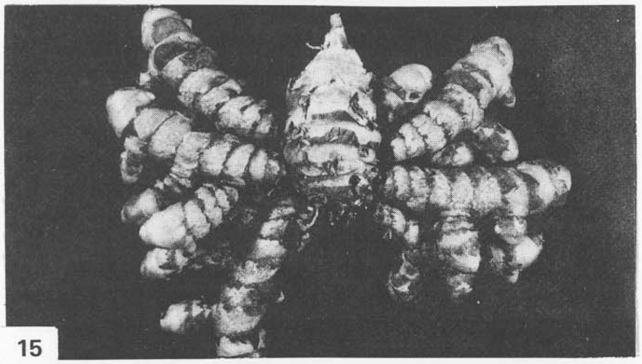
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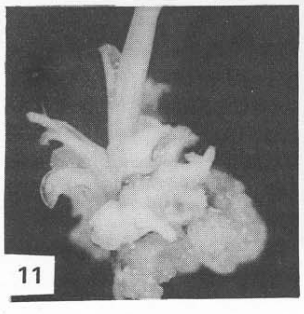
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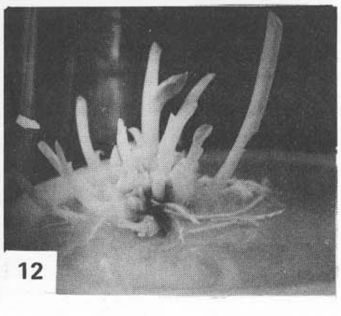
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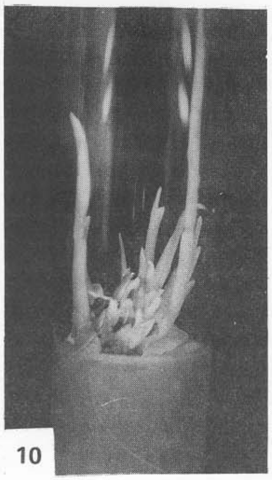
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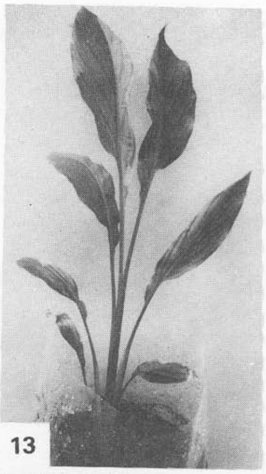
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Figs. 1—15: Coconut tissue and organ cultures. Fig. 1. A high-yielding coconut palm producing 400 nuts/year and free from disease-symptoms, although growing in a root-wilt affected garden of South Kerala, a prospective candidate for clonal propagation through tissue culture. Fig. 2. Spathes removed from 1st to 9th axils of an adult palm. Responding floral primordia are usually from 1st spathe and 7th being suitable stage for anther culture. Fig. 3. Left to right: Coconut rachilla explant (1—2 cm) from 1st spathe with 25—30 primordia at the time of excision and culture. Extension of pistillode after 4 weeks in vitro; indeterminate shoot-like development of the male flower primordium, after 8 weeks growth; the last one showing a second bud developing, all on Y3 medium + BAP (2 mg/l) + 6 PR (2 mg/l). Fig. 4. 12-week old, same medium. Fig. 5. Left to right: Mature embryo at inoculation, 2 months, 3 months, 3½ months, 4 months on Y3 basal medium containing 1% activated carbon and 40 g/l sucrose. Fig. 6. 4½-month old seedling on Y3 medium supplemented with 0.5 mg/l IBA. Fig. 7. Coconut anther cultured on Y3 medium + 2,4-D 10 mg/l + Kinetin 1 mg/l and sucrose 6%. Fig. 8. 6-week old root culture on MS medium with 40 g/l sucrose + 0.5 mg/l NAA + 0.2 mg/l 2,4-D and 5 g/l activated carbon. Fig. 9. 8-week old culture on same medium. Figs. 10—15. Turmeric tissue culture. Fig. 10. 4-week old culture on MS medium supplemented with Kinetin (1.0 mg/l) + BAP (2.0 mg/l). Fig. 11. 2-week old subculture on same medium. Fig. 12. 4-week old subculture on above medium. Fig. 13. A vigorously growing turmeric plant transferred to soil mixture in polybag. Note chimeral leaf with asymmetrical lamina and discoloured stripes. Fig. 14. 8-month old plants in glasshouse. Fig. 15. The first crop of rhizome clump (136 gm) harvested from a single clonal plant raised in tissue culture.

nature of these somatic variants — both for curcumin content and yield. Preliminary work on other plantation crops like cashew, cardamom and ginger has revealed good possibilities. Shoot meristems of cashew excised from adult trees, cultured on B-5 agar medium containing 10 per cent coconut water and 40 g/l sucrose grew rapidly and produced a soft light green callus, particularly with 2,4-D and NAA (2—10 ppm) added to the medium. The calli were transferred to liquid media and shaken gently in a flask shaker, to obtain rapid multiplication and embryogenesis in the cell aggregates which were formed. In the case of cardamom and ginger shoot buds cultured in same manner as turmeric gave raise to multiple shoots and this work is hence being intensified.

DISCUSSION AND OUTLOOK

In the light of our experience with some of the perennial, plantation crops, it would be worthwhile to examine the results in terms of future possibilities for the application of tissue and organ culture for (a) germplasm collection, conservation and even evaluation for resistance to pests/pathogens, (b) rapid clonal multiplication of elite genotypes (c) induction of haploids and production of isogenic lines, (d) induction of mutations and new recombinants.

(a) *(Germplasm conservation, evaluation and exchange:* Based on the reports of successful transportation of meristem cultures of certain tuber crops like cassava (CIAT Rep., 1980, 1981), potato, sweet potato and the yams (Kartha and Gamborg, 1979), as well as *Citrus* clones (Button, 1977), one is inclined to believe that such a technique would indeed be a boon to bulky-seeded plants like the coconut palm. In a recent germplasm collecting expedition in the Pacific Ocean Islands, conducted by this Institute, the bulk of the cost was taken up by transportation of seednuts, nearly amounting to US$10.00 per nut. The large size and weight of coconuts also greatly restricted the size of sample that could be collected and transported from the smaller islands where only small aircraft operated. Also the quarantine problems imposed further restrictions on import of seednuts from areas known to have seedborne diseases or pests (Rao and Koshy, 1981). It is in this context that meristem tissue or embryocultures could prove to be an effective means of transporting disease-free samples of coconut germplasm across continents at much less expense and greater elegance, free from the cumbersome quarantine procedures. Embryo and tissue cultures would occupy much less space, so that larger numbers of samples can be collected to ensure a reasonably higher recovery of germplasm accessions at the receiving end. This technique combined with freeze preservation of cells and tissues, if not embryos, should prove useful in conserving the vanishing native reserves of germplasm, in addition to the exotic collections, which are being threatened due to the massive replanting programmes now in progress in the major coconut growing countries of the developing world.

Closely related to the above is the question of conserving endangered and rare plant species which are facing destruction due to modern developmental activities such as construction of dams and hydroelectric projects. One such area in Kerala is the famous 'Silent Valley' in Palghat District, which is the sole surviving, unique tropical rainforest in India harbouring some rare species of plants as well as animals. This is facing extinction owing to the proposed hydro-electric project which threatens to submerge vast areas of this Valley causing permanent destruction of some rare medicinal plants, orchids, as well as timber species, and drastically changing the microenvironment of this area. Thanks to the hue and cry from well-meaning men of science and conservationists, the Government of India has heeded to their call for 'development without destruction' and intervened to save this and other last remnants of Nature's gift in that part of India (Swaminathan, 1979; St. Barbe Baker, 1980; Bahuguna, 1980). Before many of these species are lost to posterity, we have the opportunity of saving and conserving them in tissue cultures and to rapidly multiply them for planting in other areas or at least preserve them in Botanical Gardens. The Botanical Survey of India has already listed the last survivors of plant species in the Silent Valley of Kerala, such as *Salacia chinensis, Antistrophe serratifolia, Toxocarpus palghatensis, Cryptocarya wightiana, Didymoplexis pallens, Aphyllorchis prainii* and several others which are in the endangered list, and call for immediate conservation. This Institute (CPCRI) has also surveyed the Valley for wild relatives of spices such as *Piper galeatum, P. trichostachyon, P. hookeri, P. schmidtii* and so on, besides species of *Mysistica* and *Cinnamomum.* Plant tissue culture offers tremendous scope for immediate conservation of these rare specimens for use by the breeders.

Coming back to coconut, it is also our aim to preserve some of the rare high yielding single palms which also are disease-free, although some are growing in root wilt affected gardens. The root wilt of coconut is the single major cause for losses of yield in Kerala amounting to over 300 million nuts annually, and hence, any strategy that can prevent this loss will greatly boost the economy of the State. Whereas the average yield of a coconut palm in this State is only 35 nuts/year some of these elite single palms (Fig. 1) have shown potentials of over 400 nuts/year (Iyer, Rao and Govindankutty, 1979). Since field screening of palm for disease-reaction is not an easy task, we wish to develop tissue culture screening methods, as has been shown to be possible in certain annual crops like tobacco (Helgeson, Kemp, Heberlach, and Maxwell, 1972) and maize (Gegenbach, Green and Donovan, 1979; Earle, York and Gracen, 1979). In future, it should also be possible to screen tissue cultures of germplasm against resistance to toxins released by pests such as the tea-mosquito in cashew which blackens the entire inflorescence resulting in total loss of yield. Another malady that can be analysed *in vitro* is the mango malformation syndrome, in view of its complexity. Thus, tissue culture offers an elegant method of evaluating germplasm collections for resistance, without having to raise a plantation, which would require enormous land area.

(b) *Clonal multiplication:* As already indicated, tissue culture offers the only possibility of multiplying elite coconut palms as has already been achieved in the oilpalm (Corley, Wooi and Wong, 1979; Wooi, Wong and Corley, this Symposium), using leaf and root tissues of adult palms of proven potential. In coconut, we have attempted the conversion of floral primordia into vegetative shoots, since this is a phenomenon already recorded as a freak occurrence in certain natural, sterile mutants which normally do not set seeds but only produce bulbils (Iyengar, 1922, Davis, 1967). Although they do not strike roots in nature, recently these bulbil shoots have been rooted by air layering and planted in Indonesia (Sudasrip, Kaat and Davis, 1978). However, it would be a breakthrough if bulbil shoots could be induced in normal high yielding trees, either *in situ* or by *in vitro* culture of floral tissues. So far both in UK (Blake and Eeuwens, this Symposium), Philippines (Guzman and del Rosario, 1979) and our laboratory (Kuruvinashetti and Iyer, 1980) only shoot-like structures with scale leaves and occasional rooting have resulted, but viable plants are yet to materialise. More intensive research on the culture of floral tissues holds great promise since this is only a duplication of the phenomenon already occurring in Nature.

Leaf tissues have already been successfully employed for callus induction and somatic embryogenesis in the oilpalm (Rabechault and Martin, 1976), and it is only a matter of time and more effort for this approach to succeed in coconut too. Tender leaf bases excised from seedling tissues of coconut have shown callussing at the cut ends. In general, the use of liquid medium and dark incubation in initial stages of culture appear to be favourable for callus induction and further growth.

With regard to root tissue, the oilpalm workers at Unilever UK (Corley *et al.*, 1976) have been more successful than the coconut tissue culturists, in eliciting favourable response. The coconut roots present problems of acute tannin accumulation, particularly in thicker primary roots taken from adult palms, but thinner secondary laterals of seedling roots as well as those from embryo cultures seem to be more suitable for experimentation. At the East Malling Research Station, U.K., coconut roots have been used successfully both to induce callus and also to maintain the mycoplasma of lethal yellowing disease for basic studies (Fulford and Justin, 1978). Secondary roots were found to be more responsive than the thicker primary roots in these studies. However, for clonal propagation to be a reality in coconut, we must standardize a method using root tissue from adult palms of proven potency. Both the Unilever and French (IRHO) scientists have followed the callus approach for oilpalm propagation *in vitro*, but for ensuring the stability of yield performance in the clones produced, it may be more desirable to attempt "direct differentiation of organs" from leaf and root segments, to obviate any somatic variation arising from callus, especially when a high level of 2,4-D is incorporated in the medium. However, if the callus phase is not too prolonged, it would be possible to keep in check any somatic changes in ploidy levels. It is encouraging to hear reports that the 1500 clonal palms in a 10 ha. orchard of oilpalm planted at Kluang in Johore, Malaysia by Unilever, have proved superior in yield (by at least 30%) and stability of performance, to the seedling progenies (Anon., 1981b), which has helped to offset the higher initial cost (M$4.00 each) of tissue cultured clonal plants compared to that of true seedlings (40 cents each).

Another palm of vital importance to developing countries especially in the arid zone, where extensive tissue culture for clonal multiplication of elite female trees is being done, is the date palm (*Phoenix dactylifera* L.), in Israel (Reuveni, 1979; Reuveni and Lilien – Kipnis, 1974), USA (Tisserat, 1979a, b; 1980; Reynolds and Murashige, 1979); and in India (Sharma *et al.*, 1980).

Success with other forest trees and woody perennials is limited largely to angiosperms, so far as clonal multiplication of mature trees through somatic tissue culture is concerned (Sommer and Brown, 1979). There are only two striking examples of *Populus* (Whitehead and Giles, 1976) among the temperate forest trees and teak *(Tectona grandis)* among the tropical ones where tissue culture has shown promise of large-scale application in afforestation programmes (Gupta *et al.*, 1980), using clonal progenies developed from tissue cultures of mature trees. Among other woody perennials, *Eucalyptus* (Goncalves *et al.*, 1979; Lakshmi Sita and Vaidyanathan, 1980; Gupta and Mascarenhas, 1981; Gupta *et al.*, 1981), sandalwood (Rao and Bapat, 1978, 1980; Lakshmi Sita *et al.*, 1980) and *Citrus* (Chaturvedi and Mitra, 1974) have been successfully propagated clonally, using somatic tissues from adult trees, as also from leaf tissue of *Rauwolfia serpentina* (Mitra and Chaturvedi, 1970). Attempts have also been made to standardize tissue culture propagation of certain tropical trees such as *Bauhinia, Cassia, Eugenia, Pterocarpus, Swietenia* and *Cinnamomum* (Lee and Rao, 1980).

Mention may also be made of the extensive work in progress on the temperate fruit trees such as apple and pear in UK (Jones *et al.*, 1978) and in India (Mehra and Sachdeva, 1980; Mehra and Jaidka, 1980) as well as in almond (Mehra and Mehra, 1974). The UK group at East Malling has also used *in vitro* shoot cultures of dwarf cherry rootstocks to induce tetraploids by treating them with colchicine, which was not possible to achieve with intact trees (Gayner *et al.*, 1978). Mass propagation of clonal, virus-free strawberries has been achieved through meristem as well as anther culture both in Japan (Nishi and Oosawa, 1973) and in Belgium (Boxus *et al.*, 1977). Among other woody perennials, tissue culture for clonal propagation has been attempted with limited success in tea (Wu, 1976; Wu *et al.*, this Symposium), coffee (Herman and Hass, 1975; Monaco *et al.*, 1977; Sondahl and Sharp, 1979), cacao (Lee and Rao, this Symposium), cashew (Philip and Unni, 1979) and *Bougainvillea* (Sharma *et al.*, 1981).

Accelerated clonal multiplication has also been achieved in certain non-woody perennials such as turmeric (Nadgauda *et al.*, 1978; Kuruvinashetti *et al.*, 1980), ginger (Hosoki and Sagawa, 1977; Nadgauda *et al.*, 1980), potato (Upadhya and Ramesh Chandra, 1977), papaya (Litz and Conover, 1977), *Dioscorea floribunda* (Chaturvedi and Sinha, 1979), and other tuber crops like *Xanthosoma* (Strauss and Arditti, 1980) and sweet potato (Nielson, 1960).

(c) *Induction of haploids:* Although anther culture was first demonstrated in 1964–66 (Guha and Maheshwari, 1964, 1966) in India, a vast field has been opened up both in basic as well as applied fields of haploidy with immense possibilities for rapid agricultural advance in all developing, countries (Iyer, 1977). In recent times, the Chinese scientists with their vast human resource have achieved remarkable success in mass-producing haploids through anther culture in the cereals, particularly wheat and rice (Hu Han, 1978; Hu Han and Shui, 1980a, b; Chen Ying and Li Liang-tsai, 1978), besides maize (Ku Ming – Kuang *et al.*, 1978; Hu Han and Hao Shui, 1980a, b), where, recently Brettel *et al.*, (1981) have also reported haploid formation at a low frequency. The Chinese workers have also succeeded in getting haploids in the rubber tree, *Hevea brasiliensis* (Chen *et al.*, 1978, 1979; Chen Zhenghua *et al.*, 1981) through anther culture. This success is commendable, not only for the large-scale massive effort that has gone in, but also for their critical analysis of ammonium nitrogen in their N6 medium (Chu *et al.*, 1975) in triggering pollen embryogenesis, and the stimulatory effects of potato extract

(Chuang *et al.,* 1978). At the IARI, New Delhi, following initial basic studies on rice anther culture, on pathways of pollen embryogenesis (Iyer and Raina, 1972), and on variations in genotype response (Guha *et al.,* 1970; Guha-Mukherjee, 1973), the technique is now being applied extensively in rice improvement for grain quality, yield stability, earliness and plant type (Raina, 1981). Following these encouraging developments in rice anther culture both in India and China, the International Rice Research Institute in Philippines has launched a massive programme on anther and pollen culture since 1979 and 85 rice varieties have been screened for high, medium or low pollen callus production. They have developed two improved media (modified Gamborg *et al.,* 1968) where 53% green plant regeneration has been achieved in high-callus producing rice varieties, as against 3–10% normally possible (Anon. IRRI Reporter, 1981, Zapata *et al.,* 1981).

No attempt is made here to review all the work on haploids since several comprehensive reviews have appeared from time to time (Sunderland, 1971; Iyer, 1977; Acharya and Ramji, 1977; Reinert and Bajaj, 1977; Vasil, 1980; Maheshwari and Tyagi, 1981; Guha-Mukherjee, 1981). However, the achievements of anther culture in woody prrennials would be of greater significance to crop breeders in view of their long life-cycle and the time it takes to get inbred lines by conventional methods. It is to these difficult crops that our future efforts must be concentrated if we wish to fully exploit this valuable technique. Already quite a few encouraging attempts have been reported in tree crops like poplars (Sato, 1974), cacao (Dublin, 1974), tea (Raina and Iyer, 1974, 1981), coffee (Monaco *et al.,* 1977), *Vitis vinifera* (Gresshoff and Doy, 1974), rubber (Satchuthananthavale, 1974; Chen *et al.,* 1978, 1979; Chen Zhenghua *et al.,* 1981) and a few forest trees (Bonga, 1977). Much more needs to be done in these and other tree crops, particularly in palms like coconut and oilpalm, and other plantation crops like cashew, the tree spices like nutmeg and cloves, where haploidy would be of great applied value, for developing countries. In coconut, if doubled haploids can be produced it can immediately be fixed for high yield especially in prepotent palms showing a high rate of transmission of parental traits (Herland, 1957). Another vegetatively propagated crop where haploidy through anther culture has found maximum application is the potato, particularly for its propagation as true seed which would render it safe from virus infection (Dunwell and Sunderland, 1973; Sopory *et al.,* 1978; Jacobsen and Sopory, 1978; Sopory, 1979; Wenzel, 1979).

(d) *Induction of Mutations and rare recombinants:* Plant tissue, cell, and protoplast culture, particularly those at the haploid level, offer the most potent and elegant biotechnological tools to the geneticist, which has prompted Melchers (1974) to christen it as the 'microbiological method' of plant breeding (Adams *et al.,* 1975). Some workers have even developed for higher plant cells, a 'replica-plating' technique of isolating auxotrophic mutants (Guha-Mukherjee and Keller, 1972; Bhalla *et al.,* 1974). With these tools, tissue and cell culture research has undergone quantum jumps across interdisciplinary areas of molecular biology and genetic engineering, making it possible for the plant breeder to effect altogether unusual recombinations of higher plants, such as between potato-tomato (Melchers *et al.,* 1978), between *Vicia* and *Petunia* (Binding and Nehls, 1978) and between prokaryotes and eukaryotes through transgenosis (Doy *et al.,* 1973; Malhotra and Maheshwari, 1980; Maheshwari *et al.,* 1981). The technique of transgenosis holds great promise for the plant breeder to realise his dream of making non-legumes to fix atmospheric nitrogen by evolving a *'Rhizobium cerealis'* (by 1990 according to Borlaug, 1971) through plasmid engineering (Nuti *et al.,* 1979) and bacteriophages (Smith *et al.,* 1975).

Another aspect of variability arising from tissue culture is the generation of chromosomal variants through callus which has been commercially exploited in vegetatively propagated crops like sugarcane, where Fiji disease resistant calli-clones (Krishnamurthi and Tlaskal, 1974), and in India, a high yielding sub-clone showing resistance to leaf-drying symptom (Sreenivasan and Jalaja, 1981). Being a high polyploid, sugarcane is amenable to induced chromosomal variations which get fixed through vegetative propagation as agriculturally superior varieties (Liu and Chen, 1976, 1978; Skirvin, 1978). Similar variations have also been identified for resistance to black shank in tobacco (Hegelson *et al.,* 1972, 1976). By screening mutagenised haploid cells or protoplasts against host specific pathotoxins or their analogues, resistant mutants have been isolated in tobacco for wild-fire disease caused by *Pseudomonas tabaci* (Carlson, 1973), and in maize for *Helminthosporium maydis* race-T (Gegenbach *et al.,* 1979; Earle *et al.,* 1979). In future it may become possible to select cell lines possessing resistance to specific herbicides, and pollutants (Riley *et al.,* 1979).

Tissue and cell culture has also been employed for selecting mutants for tolerance to drought (Holder and Kirkham, 1981) salinity (Dix and Street, 1975, Kochba *et al.,* 1980) and low temperatures (Dix and Street, 1976), all of which are of vital importance to developing countries in the arid and alkaline-saline zones. In the oilseed crops, like *Brassica campestris, B. nigra, B. oleracea, B. juncea* and *B. napus,* a series of haploids, diploids and few polyploids have been generated from anther cultures, and from F1 hybrids of *B. campestris* recombinants with low erucic acid have been identified (Keller *et al.,* 1981). In the palms such as coconut and oil palm there is need to exploit cell culture and haploidy to generate new variability for fatty acid composition so that the relative proportions of saturated and unsaturated fatty acids can be altered to suit the oil property for specific end uses, such as for edible purposes and for soap manufacture or other industrial uses. Already the oilpalm scientists have studied the naturally occurring variations in fatty acid composition (Ng *et al.,* 1976) and indicated possibilities for improving the composition in hybrids and cultivars (Noiret and Wuidart, 1977). There is also some evidence for the existence of genetically controlled variation in photosynthetic rates of oil palm seedlings (Corley *et al.,* 1973; Berlyn *et al.,* 1978). Now that clonal propagation of oilpalm is fairly well established (Noiret, 1981), both through leaf callus (Pannetier *et al.,* 1981) and somatic embryogenesis (Ahee *et al.,* 1981) it is now possible to attempt induction of variability for fatty acid composition and other quality characteristics. Similar effort is called for in coconut since it is essentially going to benefit the small farmers who depend on it for their livelihood.

(e) *Embryo culture:* Although it is not intended here to review all work on utilisation of embryo culture for crop improvement, some of the more recent applications which are relevant to the economy of developing countries will be considered. Particularly, the dedicated and pioneering work of the late Dr. Guzman of Philippines in the last two decades on coconut (makapuno) embryo culture is very significant to the developing world in view of its great potential. Thus, we are exploring the possibilities of using the embryo cultures for (a) large-scale transportation of cocnut germplasm as well as their conservation and storage, (b) rapid propogation of prepotent high yielding palms for replanting programmes and (c) induction of multiple shoots for accelerating the rate of multiplication of such elite palms (see, Fisher and Tsai, 1978; D' Souza, 1980).

Among other classical uses of embryo culture, is the work on successful raising of interspecific hybrids in papaya to transfer mosaic resistance from wild *Carica cauliflora* to cultivated *C. papaya* at National Chemical Laboratory, Poona (Hendre *et al.,* 1977; Khuspe *et al.,* 1980). Embryo culture has also been used to riase interspecific hybrids in pasture legumes (Williams *et al.,* 1979) using it along with 'nurse' endosperm; and in the genus *Phaseolus* (Alvarez *et al.,* 1981), to raise hybrid tomato plants (Gunay and Rao, 1980), using hypocotyl and cotyledon explants. Sharma *et al.,* (1980) have raised interspecific hybrids between *Solanum melongena* and *S. khasianum* through embryo culture. All these developments are of tremendous value to plant breeding researchers in the developing world (Jensen, 1981; Yeung *et al.,* 1981). Another novel use made of embryo culture is for the induction of mutations in rice and for root type inheritance studies (Bairagi, 1968; Bahduri and Shom, 1969; Bhaduri and Brahmachari, 1976). Even so, there are some recalcitrant species like cotton which offer problems for morphogenetic studies (Sethi and Rangaswamy, 1976), but here again ovule culture, even of unfertilized ones, has been successfully used for basic studies on hormonal control of fibre development, cell-wall biogenesis, and embryo development (Beasely, 1977).

CONCLUSIONS

In this brief review an attempt has been made to highlight some significant advances made in the application of tissue, cell and organ culture, particularly in woody perennial, plantation and certain field crops, which are of immediate relevance to the different developing countries. Since the bulk of the farm holdings of agricultural plantation crops like coconut, arecanut and spices fall below one hectare size in many countries, the application of rapid tissue culture propagation of elite, disease-free genotypes, as well as other applications such as haploidy induction, for accelerated plant breeding researches on these long-duration crops, would have a tremendous impact on the economy of the small farmer. The dramatic increases witnessed during last 25 years in crop production from small farms of India, whereby annual wheat production has quadrupled from 8 to 35 million tons and rice has doubled from 40 to 80 million tons, are the result of sustained application of plant breeding tools, fertilizers, pesticides and rapid transfer of technology from laboratory to the farm, and not due to increase in area of land farmed. The small farms can afford the cost of improved seed which is the main crux of sustained increases in production and profits. In perennial oilseed crops such as oilpalm, tissue-culture produced high yielding clones planted in Malaysia, have already shown stability of performance which offsets the higher initial investment on planting material. Keeping in view the dangers of genetic uniformity, polyclonal orchards representative of a wide genetic base and possessing multiple pest and disease resistance will have to be thought of as a future safeguard against possible epidemics (Russell, 1981). Another striking example of yield increase reported is the quadrupling of per hectare yield of *Pyrethrum*, accomplished by planting clones of a selected high yielding, disease resistant type (Strohl, 1981).

Consequent upon the progressive shrinkage of forests, intensive agricultural lands and the replanting of older plantations with new hybrids and other improved materials, we are witnessing a worldwide phenomenon of gene erosion of native germplasm resources. The situation is particularly alarming in developing countries where the rare botanical wealth of tropical rainforests are facing permanent destruction owing to pressures of various kinds (Raven, 1980; Nair *et al.,* 1979). Rapid conservation of the threatened plant species of these invaluable sanctuaries (Jain and Sastry, 1980) can be achieved through the application of the modern tools of tissue and cell culture. Even cryobiological methods of long-term or short-term storage of valuable genes for use by posterity can be attempted (Bajaj, 1979; 1981), although it would be equally important to examine carefully the technical as well as genetical aspects of tissue culture storage for such gene banks (Henshaw, 1973; D'Amato, 1973).

Considering the vital importance of both basic and applied researches on the use of biotechnological tools, the Government of India has recently constituted a National Committee to be headed by Dr. M. S. Swaminathan, an eminent geneticist and agricultural scientist, and comprising of all the heads of the various Research Councils of agriculture (ICAR), Scientific Research (CSIR), Medical Research (ICMR) and the Department of Science & Technology. This Committee will formulate national priorities of research on biotechnology, and the main areas identified are, Tissue Culture, biological Nitrogen Fixation where also tissue and protoplast culture are to be applied (Giles and Vasil, 1980) besides genetic engineering, immunology and related research applications. With this national awareness of the great potentials of tissue and cell culture, there is little doubt that more rapid advances will be made which would help the geneticists and plant breeders to tackle the more challenging problems particularly in the perennial, plantation crops, so as to realise the fruits of research endeavours in the near future.

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