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COCONUT TISSUE CULTURE IN INDIA: STATUS AND PROSPECTS

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ABSTRACT

The first report of successful induction of somatic embryoids and their subsequent organisation into clonal plantlets was from CPCRI in 1984. Using tender leaf segments from one to two-year old WCT seedlings, somatic embryoids were induced in 16 weeks on a modified Y-3 medium supplemented with high levels of auxin (NAA), inositol and casein hydrolysate. After a process of hardening, four of these plantlets have been successfully field planted in January 1989 and are growing well. More recently, with a view to reproducing the above success with mature palm tissues, a non-destructive method of tissue extraction has been standardised. Rachilla explants have so far given only shoot-like outgrowths, but no true leaves yet, whereas anther culture gave only multi-celled pollen embryoids.

INTRODUCTION

A survey conducted by CPCRI scientists has brought to light several elite disease-free coconut palms showing unusually high yields (over 470 nuts/year), although many of these are growing in root (wilt) disease-affected 'hot spots' of Kerala (Iyer *et al.*, 1979). If such rare single super palms could be propagated clonally through *in vitro* approaches, this would certainly open up tremendous possibilities of breaking the yield barrier in coconut. With this objective in view, attempts have been made in different laboratories to establish *in vitro* cultures of vegetative explants from seedling as well as adult palm tissues, including floral tissues.

Besides clonal propagation, work has also been done on standardising an embryo-culture technique with twin objectives: (i) to obtain viable rooted plants which could be used for further experiments on clonal multiplication using these aseptically produced seedlings, and (ii) to collect embryos and retrieve coconut germplasm in field expeditions, so as to obviate the need for whole nut collection now becoming problematic in view of the bulky nature of seed which restricts the size of each accession, quarantine difficulties, and the high cost of air transportation of whole nuts.

Work Done in Different Laboratories in India

A survey of published literature on coconut tissue culture has revealed that nine centres have been engaged in this work, in different university as well as national laboratories. These are dealt with here in chronological order of their reporting in publications.

1. Department of Botany, Kerala University, Trivandrum

Abraham and Thomas (1962) have the credit of publishing the earliest paper on coconut embryo-culture. They scooped out the embryo along with surrounding endosperm, from the large eye of a ripe coconut, and the embryo measuring 7.5 to 10 mm long and 5 mm wide was then separated and transferred to a modified White's medium (White, 1943), supplemented with Nitsch's minor elements (Nitsch, 1951), cobalt chloride (25 ppm), White's vitamins (two and a half times that of White, 1954) and 50 ppm Ca-pantothenate, ferric citrate (10 mg/l), 3-IAA (2 mg/l), casein hydrolysate (200 mg/l), agar (0.8 per cent) and sucrose (20 g/l). Coconut water extracted from tender nut by sterile syringe was added to autoclaved medium cooled to 40°C, in 2 : 1 ratio. The cultures were kept at room temperature (15° to 28°C). In two months, the coleoptile emerged at the blunt end of the embryo, the first leaf appeared in four months, and was completely developed in six months. However, the root growth was rudimentary. No further progress was reported by these authors.

2. Central Plantation Crops Research Institute, Kasaragod

As a sequel to the recommendations arising out of the First International Symposium on Coconut Research and Development (ISOCRAD-I) and the special study groups held in December, 1976 at CPCRI, Kasaragod (Iyer *et al.*, 1977), a tissue culture facility was established by the author in 1977.

Work on Rachilla Tissues

Coconut rachillae explants were cultured *in vitro* to explore the possibility of converting the floral primordia into vegetative shoots as indicated in the paper presented by Prof. Schwabe (1983) at the ISOCRAD-I. Although bulbil formation is a known phenomenon in certain freak mutants of coconut (Iyengar, 1922; Davis, 1948), these bulbils do not strike roots in nature. However, Davis *et al.* (1981) have succeeded in air layering these bulbils, and raised these clonal palms in soil, but they again produced only bulbils instead of normal flowers. Nevertheless, this was the first demonstration of clonal propagation of coconut *via* the naturally occurring bulbils (Sudasrip *et al.*, 1978).

Our attempts at CPCRI to culture immature rachillae explants excised from the first to the third leaf axil after the unopen spindle, resulted in formation of shoot-like structures, some of which turned green and even formed roots on Y3 medium (Eeuwens, 1978) supplemented with cytokinins 6-BAP and 2-ip (2 mg/l each). This transformation was noticed in over 50 per cent of the floral primordia. It was possible to excise a responding spadix without damaging the shoot meristem of the palm. Browning of explants was severe in older spathes, and addition of 5 of 10 g/l activated charcoal reduced this considerably. However, so far no viable plantlet could be obtained from floral primordia *in vitro* (Kuruvinashetti and Iyer, 1980; Iyer, 1982).

Leaf Tissue Culture

Work on tender leaf tissue culture was initiated in 1982 using one to two-year old WCT seedlings from the nursery. Seedling tissues from apices, tender leaves, leaf bases and leaf sheath were cultured initially on Y3 basal medium supplemented with various combinations of auxins (NAA and 2,4-D) and cytokinins (Kinetin, BAP and 2-ip). The cut surfaces of tender leaf bases of one-year-old seedlings showed profuse callusing on 2,4-D (10 mg/l) and Kinetin (0.5 mg/l) media whereas tender leaf lamina segments showed only limited callusing (Raju *et al.*, 1982). Subsequently, these leaf cultures produced rooting profusely. In order to induce somatic embryogenesis these leaf cultures were subjected to a wide range modified Y3 media (Anon. 1985) in a broad spectrum experiment running into 2,430 combinations involving varying concentrations of the components. This resulted in the induction of somatic embryoids after 16 weeks in 12 per cent of the explants, which could be shortened to three weeks by adjusting Mg : K ratio, and optimising the hormone level, avoiding the use of 2,4-D. A single leaf explant could give up to 48 somatic embryoids which after 45 to 60 days of maturation became detached from parent leaf tissue. Anatomical studies indicated that these somatic embryoids probably arose from the phloem cells of the vascular bundles of leaf vein. The individual embryoid had a tripolar structure with root and shoot poles and an incipient haustorial bulge. Initially, the shoot growth was promoted by cutting away the root pole and part of the haustorium. However, in subsequent experiments, reducing auxin to 75 per cent and increasing cytokinin 125 per cent has resulted in successful sprouting of the embryoids to give a normal green shoot with scale leaves and sheathing leaf base, a primary root with root cap, and an incipient haustorium (Raju *et al.*, 1984).

A technique for the extraction of the tender spindle tissues without damaging the single meristem of the mature palm, has been standardised. The central column of the spindle is exposed by removing two whorls of leaves outside the spindle, and cutting away the spindle 15 to 20 cm above the shoot meristem. The exposed tissues of the palm are then treated with insecticide (endosulfan) and fungicide (Bavistin), and covered by polybag to prevent infection. The severed spindle column is then brought to the laboratory in a polybag, the outer sheaths removed and swabbed with alcohol for surface sterilisation. The tender leaf tissues are then quickly dissected and bulk-inoculated into liquid media initially. Subsequently, each of these bulk-inoculated tissues are further subcultured as 1 cm segments containing a minimum of three folds of the unopen leaf. In this manner, from each palm nearly 2000 to 2,500 cultures could be established.

Constraints Experienced in Achieving Repeatable Results

One of the constraints in working with coconut seedlings or mature palms is the plant to plant differences in genotypic response, since each time we sample a different seedling or adult palm. Apart from this, there is also the possible variation in physiological maturity of different regions of the spindle leaf tissues. There is seasonal variation also in response of tissues *in vitro*. Those sampled in June, July and August gave a higher rate of somatic embryoids than those sampled at other times.

Browning of tissues is another constraint which has been tackled by: (a) incorporating activated carbon in the medium (1 g to 3 g/l) to absorb the polyphenolic exudates, (b) the use of antioxidants such as ascorbic acid (100 to 300 ppm) and cystein-HCl both in the medium and for dipping the explant prior to transfer to medium, (c) using liquid medium with filter paper bridges, and frequent transfers to fresh medium in the induction phase of culture, and (d) avoiding the use of phenolic auxins such as 2,4-D or to use them in very low concentrations, and reducing the duration and concentration in each passage.

Preponderance of root formation at the cost of shoot was seen in leaf tissue cultures. This could be overcome by aseptically severing off the root pole along with a portion of the haustorial bulge of the tripolar somatic embryoid.

Embryo Culture for Germplasm Collection, Transport and Conservation

The collection of coconut germplasm and their transport to one's own country has now become not only too expensive but is wrought with quarantine risks.

Hence, techniques for aseptic collection of embryos under the palm itself, their storage, transport and retrieval had to be standardised (Raju and Bavappa, 1987). Using a simple portable equipment weighing about 4 kg and measuring 40 × 30 × 8 cm, up to 100 embryos could be collected in the field aseptically in a culture tube measuring 25 × 150 mm, and on subsequent transfer to fresh media gave 95 per cent germination (Anon. 1990). In an earlier trial zygotic embryos excised from immature coconuts and cultured in January, 1987 were found to remain viable even after two years of storage without any environmental control and periodic transfers (Anon. 1989). Presently, a systematic trial is underway using embryos extracted in the field from eight, 10, 11 and 12 months old nuts and brought to culture, to see the relative recovery percentage of viable plants.

Field Planting of Clonal and Embryo Culture Derived Plants

During January 1989, four tissue culture raised clonal plants, derived from tender leaf explants of one year old WCT seedlings, and two embryo culture derived seedlings of WCT cultivar which had been maintained in pot culture in a green house, were transplanted in the field. The pit for planting was filled with a mixture of coir dust + farmyard manure + sand. All these plants have established and are growing vigorously (Anon. 1989).

Endosperm Culture

A fast growing friable callus was obtained from coconut endosperm which was in contact with zygotic embryo, and maintained by repeated subcultures on Eeuwens Y-3 medium containing 2,4-D (50 mg/l` Kinetin (2 mg/l) and 1 g/l activated charcoal and with lowered auxin levels. Callus showed high aneuploidy and abundant oil globules when maintained for over eight weeks without subculturing. Hence, this system could be used for extraction of edible oil and other metabolites like cytokinins and amino-acids which are known to occur in the coconut endosperm tissue (Prakash Kumar *et al.,* 1985).

Future Thrust

a) While future emphasis will be on the use of mature palm tissues for clonal propagation, seedling tissues would also be used in a limited way for maximising response of somatic embryogenesis, their maturation and sprouting to give viable clonal plantlets. Clonal plantlets, if any, derived from adult palm tissues could also be subjected to further subculturing to produce somatic embryoids from juvenile leaf tissues.

b) The use of lower concentration of auxins, avoiding 2,4-D, and also of conjugated auxins like IAA-aspartate, IAA-alanine etc. would be emphasised especially when activated charcoal is being used in media, so as to ensure that the auxins are available in a stable form for the cultured tissues to respond at the induction phase of somatic embryogenesis (Neera *et al.,* 1986; Neera and Bagga, 1988).

c) High CO_2 concentration of the order of 20,000 ppm in the culture environment is reported to enhance sprouting of somatic embryos in cacao as well as oil palm (Figueira *et al.,* 1991). Hence, suitable chambers would be devised for subjecting the coconut leaf tissue cultures to high levels of CO_2 to achieve higher frequency of response.

d) Partial dehydration and water stress induced by osmo-regulants like PEG, is also reported to favour sprouting of somatic embryoids and their encapsulation. This method would also be tried on coconut somatic embryoids.

e) Use of translation inhibitors and antiauxins for temporary arrest of root growth in somatic embryos and to promote shoot elongation at a predictable frequency would be worth attempting (Sethi and Guha-Mukherjee, 1990).

f) Immature floral tissues will continue to be used to realise the transformation of the floral primordia into vegetative shoots and their subsequent rooting.

g) Induction of androgenic haploids through anther culture would also be tried more vigorously in view of some preliminary success obtained in inducing multi-celled pollen and early stages of embryogenesis (Iyer, 1982; Thanh-Tuyen and Guzman, 1983; Thanh-Tuyen, 1985).

h) Another area which has potential and deserves future attention is the isolation and culture of protoplasts in coconut as a tool for clonal propagation and genetic engineering studies. Haibou and Kovoor (1982) have reported the successful isolation and culture of protoplasts from tender tissues of rachilla extracted without any damage to the palm. Raphide damage to protoplasts was reduced to a minimum by avoiding agitation in enzyme mixture and prompt removal of raphides upon centrifugation in a percoll gradient. These protoplasts regenerated cell walls within 24 hours, and cell division has led to the formation of micro-calli on agar media although at a low frequency.

3. Plant Research Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi

Under a five-year cess fund supported scheme financed by ICAR, entitled "Vegetative propagation of coconut palm through tissue culture", work was carried out by a 10-member team under Prof. Sipra Guha-Mukherjee, as Principal Investigator, during 1979–84 (Guha-Mukherjee, 1984). Much of the work was done on zygotic embryo cultures raised from seed-nuts sent from CPCRI. The major findings of this project are summarised below:

a) Callus was raised from various explants such as young (eight to 10-month old) zygotic embryos, on B5 medium with IAA-amino-acid conjugates (IAA-aspartate and IAA-alanine 2 mg/l).

b) Shoots and roots differentiated by transferring calli to B-5 + IAA-asp (2 mg/l) + Kinetin (2 mg/l) or NAA (2 mg/l).

c) Embryoids were formed on roots originating from callus as well as from sections of an embryo.

d) Complete plantlets were obtained on B-5 medium + NAA (0.5 mg/l) + BAP (2 mg/l) + PVP (1.0 mg/l). Three plantlets were obtained which could not grow further for transfer to soil due to poor root growth (Neera Bhalla-Sarin *et al.,* 1986).

e) Callus initiated from leaves, leaf bases, and apical meristems could not be subcultured or regenerated.

f) Other tissues tested, such as, endosperm (semi-solid and solid), apical meristem, leaf and leaf base, male flowers, anthers and rachilla, did not give repeatable response on the media tested (Neera Bhalla-Sarin and Suman Bagga, 1988).

g) Viable protoplasts were isolated after plasmolysing young leaves in 0.6 M mannitol, cutting into small bits and incubating in enzyme mixture of 1.5 per cent cellulase + 1 per cent macerozyme + 0.6 M mannitol at 5.8 pH for 16 hours followed by washing in B-5 + 0.6 M mannitol. Few divisions occurred on B5 with conjugated auxins.

4. Laboratory of Applied Biology, St. Aloysius College, Mangalore

Mature embryos of 'Tiptur Tall' and 'West Coast Tall' cultivars were cultured on modified Y3 medium (Eeuwens, 1976), to study the effect of sugar, coconut water, N and Cl levels on germination and growth of embryos — NH_4Cl was used at 803 mg/l, with sucrose (68.4 g/l) myo-inositol (102 mg/l), Thiamine (10 mg/l) and CW (120 ml/l). Cultures were kept at 26° ± 2°C under 14 hours illumination (D'Souza, 1980).

On basal medium without NAA, embryos germinated to produce coleoptile and roots. On transfer to NAA-medium before the onset of differentiation, growth was stunted at lower NAA-levels (1 to 3 mg/l) whereas at higher levels (5 to 7.5 mg/l) and low chloride and high N-levels the cotyledonary sheath enlarged to form a callus consisting of 'protocorm' and bud-like structures, which on subculture to low (1 to 2 mg/l) NAA medium, produced roots with pneumatophores. The embryos developed better in liquid rather than on semi-solid media. Rooting was inhibited without high sugar levels. Haustorium was underdeveloped and darkened (D'Souza, 1980; 1982; D'Souza *et al.*, 1988).

The seedlings were first transferred to a sand-vermiculite mixture and kept under a plastic hood in a culture room. Roots did not develop further and the haustorium started decaying causing infection to the plant. If the haustorium was cut away the cut surface became infected. The above work was carried out by Dr. Leo D'Souza, Principal Investigator and his assistants under an ICAR Cess Fund Scheme during 1980 to 1983.

5. Department of Plant Morphogenesis, School of Biological Sciences, Madurai Kamaraj University, Madurai

Embryo-culture work was carried out under a C.S.I.R. financed scheme on "Experimental cloning of palms", using mature nuts of the West Coast Tall cultivar. Embryos isolated from mature seed-nuts after surface sterilization with 50 per cent alcohol, were planted on Eeuwens (1976) agar medium containing BAP, auxins (NAA) and GA_3, with ascorbic acid (AA) as antioxidant, and cultures were kept at 85 to 90 per cent RH, 30° ± 2°C in diffuse light. A balanced growth of root and shoot was achieved on a new medium named YNBG (Padmanabhan, 1982) consisting of Y-3 basic + NAA (1 mg/l) + BAP (0.5 mg/l) + GA_3 (0.5 mg/l) + AA (100 mg/l), which totally suppressed the release of polyphenols into the medium. Three types of roots were observed—the primary root, shoot and brownish adventitious roots induced in three weeks, and the ageing adventitious roots, white, wiry and pale ones formed in large numbers. The shoots formed eophylls in the order seen in germinating nuts. The culture-raised embryos were hardy and took five to seven weeks to form seedlings that could withstand transplantation to soil whereas in nature they took 12 weeks (Jagadeesan and Padmanabhan, 1982b).

In another experiment, Jagadeesan and Padmanabhan (1982a) dissected out explants of 10 × 5 mm size from cotyledonary, radicle and plumule regions of 20-day old WCT seedlings from aseptic embryo-cultures, and planted them on MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972), and Y-3 (Eeuwens, 1976) basal media supplemented with NAA (0.5 mg/l) + BAP (1 mg/l).

The haustorial tissue showed more rapid callus formation and regeneration after initial enlargement, utilising the fatty acids. Being rich in nutrients and edible, haustorial culture might become a means of food production (Padmanabhan, 1988).

6. Tissue Culture Laboratory, Hindustan Lever Research Centre, Bombay

Work on tissue culture of coconut was initiated in 1977 using immature leaf explants for induction of callus and somatic embryogenesis (Bhaskaran, 1985). By modifying culture media it was possible to get somatic embryos both from induced callus and also directly from mid-rib region of leaf explant. Calli were induced from immature leaves obtained from coconut seedlings of five varieties and hybrids on media containing high auxin and low cytokinin. Somatic embryogenesis could be induced in calli by lowering the auxin level gradually.

Direct embryogenesis from the mid-rib region of leaf explants was also observed, the number of embryoids per 1 cm explant varying from 10 to 40. Histological studies showed that the embryoids originated from the phloem cells surrounding the vascular bundle without undergoing dedifferentiation to form a callus. Both direct and indirect (via callus) somatic embryogenesis occurred in similar culture conditions. However, the embryos arising from callus showed shoot and root apices as well as a well-developed haustorium like that in zygotic embryo, but those arising directly from leaf explant bypassing callus phase, were bipolar with shoot and root apices, with little evidence of the presence of a haustorium.

The somatic embryos arising *via* callus, on transfer to high-cytokinin media, germinated to give plantlets, whereas the bipolar are yet to be stimulated to give rise to clonal plantlets (Bhaskaran, 1985). According to Bhaskaran and Prabhudesai (1986), the propagules should be produced from a wide range of mother palm selections to avoid risk of monoculture, and to ensure interpopulation variation.

7. Division of Plant Tissue Culture, National Chemical Laboratory, Pune

Initial studies on WCT embryos of 5 to 10 mm size grown on Y-3 medium (Eeuwens, 1978) supplemented with auxins, cytokinins and activated charcoal, gave rise to whole plants, but attempts to transplant these to the field were unsuccessful (Mascarenhas *et al.,* 1988). In a detailed study, Gupta *et al.* (1984), showed that neither MS (liquid or solid) nor Y3 agar media supported growth of coconut embryos, but only Y3 liquid medium + CW (10 per cent) + BA (4.44 mg/l) + NAA (0.27 mg/l) gave plantlets in two to three weeks, without any antioxidant use. However, the initial size of embryo at culture was seen to be important, the larger sized ones (7 × 4 mm) being more suitable for getting complete plantlets. The cultures were kept at 27°C under 16 hours light regime of 1500 lux, and at 25°C in darkness for eight hours. Plantlets were transferred to polybags containing a 3 : 3 : 1 mixture of soil : sand : compost, and kept at 25°C for four weeks in 16-hour photoperiod. When transferred to a glasshouse after emergence of new leaves, the plants survived for four weeks and later withered and died. From mature trees, explants taken from young stem, leaf and rachilla tissues of young inflorescence, were cultured on Y3 liquid media without success. Best response was obtained on Y3 + 2,4-D (4.52×10^2 μM) + Ca pantothenate (2.1 μM) + biotin (4.10 μM) and activated charcoal (0.25 per cent), in which all tissues swelled and turned green in four to five weeks. Stem explants gave globular callus in 10 weeks but no organogenesis occurred even after subculture to fresh medium.

8. Tissue Culture Laboratory, Punjab Agricultural University, Ludhiana

Bajaj (1984) has studied the survival of frozen WCT zygotic embryos of 1 to 1.5 cm size. Following storage for one month, the embryos were partially dehydrated and then cut into two transverse halves. After pretreatment with cryoprotectant (7 per cent DMSO + 7 per cent sugar in MS liquid), the embryos were blotted dry, wrapped in sterile aluminium foils, and frozen by gradually lowering into liquid nitrogen for five minutes. They were thawed in warm water (35° to 40°C), washed, and cultured on MS + 2,4-D (0.2 mg/l) + NAA (0.5 mg/l) + Kinetin (0.1 mg/l).

The retrieved coconut embryos and their segments showed a lag period of four months without any sign of growth. This indicated the possibility of long-term conservation of coconut germplasm.

9. Biotechnology Unit, School of Genetics, Tamil Nadu Agricultural University, Coimbatore

Four different media (White's, Nitsch's, MS and Y3) were tried for coconut embryo culture using mature seednuts of Tall × Gangabondam hybrid, of which Y3 proved better than others. Frequent subculturing in differential medium promoted high shoot differentiation and leaf formation. These on transfer to Y3 + NAA (1.5 mg/l) and charcoal (0.1 per cent) produced roots (Kalamani and Sree Rangaswamy, 1990).

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DISCUSSION

I.S. Dua: Have you tried different ratios of cytokinins/auxins in the media and which cytokinins have you used?

R.D. Iyer: Auxin-cytokinin ratios were tried at several thousand combinations and at different intervals. The cytokinins tried were Kinetin, BAP, 2ip and zeatin riboside in various ratios with auxins, NAA, 2,4-D and IAA.