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TISSUE CULTURE OF COCONUT - A PERSPECTIVE

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ABSTRACT

Coconut biotechnology started with embryo culture during 1960s. Several laboratories in India started research work on coconut tissue culture during late 70s. Coconut being a very difficult crop to manipulate *in vitro*, several problems were encounted during tissue culture of coconut. Different aspects of tissue culture including leaf tissues *in vitro*, inflorescence culture, plumule culture, ovary culture zygotic embryo culture has been elaborated in the article. Other aspects of coconut biotechnology like *in vitro* conservation, explant response with reference to somatic figures and excised embryos are also discussed.

INTRODUCTION:

The interest in coconut biotechnology was sparked of when embryo culture work was started in early 1960s in the university of Philippines, for multiplying the Elite Makapuno coconut which would not germinate due to defective endosperm. Subsequently, research activities began in other coconut varieties also. The asceptic culture of palms particularly of coconut is of special significance because it is still one of the most difficult and recalcitrant species in plant kingdom. In the nineties work on molecular markers was initiated in India.

Considering the long gestation period, and prevalence of diseases which are yet to be conquered, the preservation of germplasm would depend on the developments in coconut biotechnology. The potential value of coconut tissue culture is well recognized for a host of activities associated with various specialized fields in breeding, production and management of coconut plantations. Other spin of benefits are sure to emerge as more experience is gained. For instance, a simple replacement of activated charcoal in the tissue culture media would reveal the nutritive and metabolic status in growing tissues in culture. Jacobsen (personal communication 1997) has developed a medium for coconut tissue culture devoid of activated charcoal. Many laboratories in India, *viz.* Tamil Nadu Agricultural University, Madurai Kamaraj University, Jawaharlal Nehru University, National Chemical Laboratory, Pune, and Hindustan Lever Research Centre, Mumbai, besides CPCRI, entered into bio-technology research on coconut in late 1970s. The problems and difficulties encountered, made all these laboratories except CPCRI to abandon the research on coconut biotechnology. Research at CPCRI is continuing on the following two aspects: (1) Callus induction, somatic embryogenesis and plant regeneration from various explants sources. (2) Development of embryo culture techniques for germplasm exchange and transportation.

Using embryo culture techniques, germplasm of coconut has been collected from different Indian Ocean Island countries. This is the first successful attempt to use embryo culture technique for germplasm collection in coconut. The protocols developed is given in Table 1. Coconut being a very bulky fruit with fibrous mesocarp, its bulk and weight is a disadvantage in transportation. Besides, the mesocarp harbours pathogens which are difficult to eliminate; hence, embryoculture technique is the most feasible accepted method of germplasm collection, free from quarantine risks.

TISSUE CULTURE:

Coconut is a difficult crop to manipulate *in vitro*. However, after Eeuwens' (1976) initial

standardization of media and successful report of callus induction from various explant sources like stem, leaf, and inflorescence, a few laboratories around the world initiated intensive research. The most commonly used basal medium at present is the Y3 formulation (Eeuwens, 1976). However, del Rosario (1984) found no difference between Murashige & Skoog's (1962) and Y3 media. Her work indicated that Glucose was better for callus growth than sucrose. The major problem in coconut tissue culture has been the browning of tissue and its consequent death. To offset this problem, the antioxidant used is activated charcoal (AC), which adsorbs even auxins and cytokinins, such as 2,4-D and Benzyl Amino Purine, to the tune of 99.4% and and 97.8% respectively after 5 days of culture media preparation (Ebert *et al.*, 1993). This kind of inactivation of supplementation results in excess use of auxins and cytokinins. Oropeza and Taylor (1994) used radiolabelled 2,4-D to study the uptake by coconut inflorescence explants. Most of the radioactivity was taken up by the tissue within 24 hours. At this time, the volume of the explant was only about one tenth of that of the external medium and the uptake of 2,4 -D occurred against a concentration gradient. Thus, uptake of radio labelled 2,4 -D by coconut inflorescence cannot be explained by simple diffusion. Alternatively, 2,4-D may be taken up by facilitated diffusion. They concluded the importance of pH for 2,4 -D uptake by coconut explants. Other auxins used were 2,4,5 -T which led to the formation of nodular calli on inflorescence explants (Buffard - Morel *et al.*, 1988), NAA and IAA resulting in direct embryogenesis in leaf explants (Raju *et al.* 1984).

Leaf Tissues in vitro: Plantlet development was first achieved at CPCRI, Kasaragod from tender leaf tissue explants taken from 1-2 year old WCT seedlings (Raju *et al.*, 1984). However, it was not reproducible in subsequent trials. Profuse callus induction was achieved from immature zygotic embryos. Regeneration of somatic embryos from the embryogenic callus has been achieved but plantlet differentiation is not regular. Several experiments in this direction are in progress. Somatic embryogenesis is usually indirect in coconut and has to pass through the callogenesis stage. Raju *et al.* (1984) reported direct embryogenesis and somatic embryoids were reported to arise directly from the peri-vascular tissue, but Blake (1989) reported that this is an unusual phenomenon, as this area gives rise to root primordia, normally. Karunaratne and Periyaperuma (1989) found that the embryogenic capacity of leaf explants was found to be related to their physiological maturity in young palms of coconut. Leaf tissues from 12 to 24 month old palms were embryogenic but the potential was quickly lost with the onset of juvenility. Even in young palms, explants of tender leaves responded differently according to their maturity. Only a particular leaf in a particular state produces embryogenic cells and only a portion of this leaf yielded embryogenic explants (Karunaratne *et al.*, 1991). This may be one of the reasons why the experiments of Raju *et al.* (1984) proved difficult to reproduce (Iyer and Parthasarathy,2000). Sporadic reports of success were reported using leaf explants by other workers also (Blake & Eeuwens, 1982; Shirke *et al.* 1993; de Siqueira & Inoue, 1992; and Verdeil *et al.* 1993, 1994).

Inflorescence Tissue Culture: Tissue culture work with other explants such as zygotic embryos, leaf base, apical meristem and endosperm were also tried. Calli initiated from embryos, leaves, leaf bases, and apical meristem could not be regenerated (Neera Bhalla Sarin et al 1986). Calli induction from anthers and rachilla did not give repeatable response (Neera Bhalla - Sarin and Suman Bagga, 1988). However, root explants (Jones, 1983), and sub apical and leaf explants due to their limited embryogenic potential (Karunaratne *et al.*, 1991) have proved of limited value. Immature inflorescence and immature embryos have been found to be of promise. Blake and Eeuwens (1978) reported initial success using inflorescence tissue for callus production. They used immature rachillae on Y3 medium (Eeuwens, 1976) supplemented with 0.5µM NAA. Branton and Blake (1986) produced plantlets in 9 months from immature rachilla explants through somatic embryogenesis of nodular callus by reducing 2,4-D on Y3 medium to 100µM 2,4-D, with 5µM each of 2ip and BAP, and 0.25% AC. Areza *et al.*, (1993) soaked the

inflorescence tissue in antioxidants viz., Citric acid (50mg/l) and Ascorbic acid (100mg/l), prior to slicing and culturing in Y3 medium supplemented with activated charcoal, which resulted in less browning. Verdeil *et al.,* (1994) reported successful embryo maturation through somatic embryogenesis from inflorescence explants, which further regenerated plantlets. They cultured immature inflorescences of coconut belonging to three different genotypes (PB-121, PB-111 & MYD), on an agar medium supplemented with activated charcoal (0.2%) and a range of 2,4-D (0.15 to 0.35 mM). Globular white callus emerged from immature floral meristems, depending on inflorescence age and 2,4-D levels. The use of immature inflorescences has been most successful among the different explants tried for plantlet regeneration, even though their transfer to nursery has yet to be achieved.

Plumule culture: The use of plumular tissues from germinating embryos has been another source from where success has been forthcoming, because of the juvenile nature of the tissue (Hornung, 1996). Buffard - Morel *et al.,* (1995) used young non chlorophyllous leaves and immature inflorescence in Eeuwens (1976) inorganic nutrients supplemented with Morel and Wetmore vitamins, 30g/l sucrose, 2 g/l activated charcoal and 40 to 60 mg/l 2,4 - D. They observed calli after 6 - 8 months after culture initiation. They observed a multicellular pathway, which led to the formation of meristematic and epidermal structures with low 2,4 - D (40 to 60 g/l). The first stage of development of these structures was characterized by the fragmentation of the cambium like zone and the formation of complex meristematic structures followed by their epidermization. They observed a unicellular pathway, which led to the appearance and individualization of embryogenic cells isolated by thick wall, with dense cytoplasm, a high nucleo-cytoplamsic ratio, and single large nucleolus and starch and protein reserves. This pathway was the result of the presence of a high 2,4 -D concentration (80 - 120 g/l). Chan *et al.,* (1998) developed a protocol using plumules of zygotic embryos. They used Y3 medium supplemented with 0.1mM of 2,4-D, 2.5-g/l activated charcoal, and solidified with 3g/l gelrite. The cultures were incubated for 3 months in darkness at 27° C. The calli bearing embryogenic structures were cultured in same medium with 1µM 2,4-D and 50µM BAP at a photoperiod of 12-hour light at 27° C and subcultured every three months. Plantlets were produced after 6 to 9 months.

Ovary Culture: Griffis and Litz (1997) used anthers and filaments, unfertilized ovaries and immature leaf pieces. Both callus initiation and direct initiation of somatic proembryos were stimulated by addition of 2,4 -D to the culture media. In a few cases, somatic embryos arose directly on filaments attached to immature anthers after several months in culture. Unfertilized ovaries cultured in media supplemented with 2,4 -D and diethylstilbestrol (DES) monitored for 24 months indicated substantial fresh weight gains and numerous unusual morphogenic changes in ovaries cultured on Y3 medium supplemented with 5 or 15 mg/l DES, 25 or 50mg/l 2,4 -D and 3 mg/l 2iP. Several unfertilized ovaries formed callus and adventitious roots but not somatic embryos. On similar media, some immature seedling leaf tissues formed calluses on the cut edges while other formed roots or numerous somatic proembryos directly. Some pro embryos also developed haustoria like tissues or roots and obvious bipolarity, but further shoot apical development did not appear except in one case.

Zygotic Embryo Culture: Abscisic acid is also reported to induce somatic embryogenesis in coconut. Recently Fernando and Gamage (2000) induced nodular callus from 7-9 months old immature zygotic embryos in BM72 medium supplemented with 24 µM 2,4-D. This callus was subcultured on to a medium supplemented with 2.5 - 7.5 µM abscisic acid for 3 - 7 weeks, and subsequently subcultured at 5 weekly intervals on media containing gradually reduced concentrations of 2,4 -D. They found that incorporation of ABA enhanced the production of somatic embryos. These embryos formed normal plants.

Immature zygotic embryo explants were found to be more likely to undergo somatic embryogenesis than mature ones. Samosir *et al.* (1998) used longitudinally sliced mature zygotic embryo explants and cultured in medium

supplemented with 125-µM 2,4-D and 2.5 g/l activated charcoal. Plantlets were successfully produced by the application of NAA (10µM) and allowed for normal seedling growth to occur.

Control of ethylene and polyamines have been found to improve somatic embryogenesis in coconut. Adkins *et al.* (1998) used cotyledonary slices from coconut embryos and cultured in medium with additives like aminoethoxyvinylglycine (AVG) and silver

Table 1. Summary of results on coconut tissue/embryo culture

Explant	Response	Reference
Somatic tissue		
Stem explants	Proliferation but no callus subculture	Apavatjrut and Blake (1977)
Root explants	Callus formation and subculture	Fulford et. al. (1981)
Leaf explants	Callus formation and embryoids neoformation	Pannetier and Buffard Morel (1982a, b)
Seedling leaf explants of WCT	Direct somatic embryos and plantlet formation	Raju et al. (1984), Iyer (1995)
Inflorescence explants	Surface callusing callus formation and multiplication by subculture	Eeuwens (1976) Blake and Eeuwens (1982)
Endosperm explants	Callus formation and subculture	Fisher and Tsai (1978) Prakash Kumar et al. (1988), Iyer (1993)
Excised seedling apices	Growth of the vegetative apex and plantlet formation	Blake and Eeuwens (1982)
Immature leaf (1 cm long young non-chlorophyllous tissue)	(Y3+ 80 mg 2, 4-D/1) callus after 4 months	Verdeil et. al. (1992)
Haploid tisue		
Anthers	Torpedo embryos	Thanh Tuyen and de Guzman (1993), Monfort (1985)
	Multicellular pollen and embryo-like nodules	Iyer (1982)
Immature inflorescence (MYD x WAT, WAT x MYD and MYD)	Eeuwen's medium+2% activated charcoal + 1.5-3.5 mM 2,4-d induced callus after 8 months	Verdiel et. al. (1994)
Immature inflorescence	Y3+20-30 ppm 2, 4-D callus induced	Sugimura and Salvana (1989)
Excised embryos		
Macapuno variety	Complete seedlings	De Guzman *et. al.* (1971) de Rosario and Guzman (1976)
Immature inflorescence	Y3+10^{-5}M 2, 4-D + 10^{-6}M 2 ip	Cueto *et al.* (1997)
West Coast Tall variety	Complete seedlings	Karun *et al.* (1996)
Immature embryos (6-7 months)	Callus after 4 weeks	Karunaratne and Periyapperuma (1990)

thiosulphate (STS) which could reduce ethylene production or polyamines such as spermine, putrescine and spermidine. Somatic embryogenesis was promoted by supplementation with AVG (2μM) or STS (3μM) or by the addition of putrescine (7.5μM) and spermine (1μM). STS also aided somatic embryo proliferation, maturation, and germination.

Use of zygotic embryo culture for germplasm collection, storage and retrieval has been standardized and put in practice into India (Anitha Karun *et al* 1993; Anitha Karun *et al.*, 1996). Koshy and Kumaran (1997) collected 15 accessions from the Indian Ocean Islands of Mauritius, Madagascar and Seychelles (Anon, 1998). Santamaria *et al.* (1999) suggested that sucrose might be important in early stages of coconut embryo cultures, to keep high chlorophyll concentrations and high number of chloroplasts. The continuous growth of the resulting plantlets in sucrose containing medium, however, will affect the development of photoautotrophy and in turn affect their performance when transferred to soil.

A brief summary of the developments in coconut tissue culture is presented in Table-1.

IN VITRO CONSERVATION:

Coconut is a recalcitrant species and the nuts do not undergo maturation drying, and are shed at relatively high moisture content (Chin and Pritchard, 1988; Parthasarathy, 1999). Chin *et al.*, (1989) reported one of the earliest works on cryopreservation of coconut embryos. They found that embryos cryoprotected with 10% DMSO showed the highest percentage survival after cryopreservation followed by 10% glycerol. Earlier Bajaj (1984) reported only elongation of whole embryos or proliferation of the cut ends of transverse halves of the embryos after cryopreservation and he did not observe normal development. He used 7% DMSO and 4% sucrose as cryoprotectants and the percentage of survival was low (17 - 25%). Assy-Bah and Engelmann (1992 a) found that immature embryos of coconut (7 to 8 months after pollination) could withstand rapid freezing in liquid nitrogen after 4 hours of pre-growth on a semi-solid medium containing 600g/l (60%) glucose and 10 to 15% glycerol or sorbitol. In these conditions, survival ranged from 10 to 43% and one embryo developed into a rooted plantlet, 2.5 months after freezing. While in a later study Assy-Bah and Engelmann (1992b) observed that mature embryos (10 - 12 months after pollination) of four varieties of coconut could withstand cryopreservation in liquid nitrogen and develop into plants. Pretreatment consisted of a 4-hour desiccation in the air current of a laminar flow cabinet followed by a 11 to 20 hour culture on a medium containing 600g/l glucose and 15% glycerol. They carried out freezing and thawing with recovery rates between 33 and 93% of frozen embryos, depending on the variety. The same workers (Assy-Bah and Engelmann, 1993) subsequently developed optimal conditions for the medium-term conservation of zygotic embryos. After 6 months of storage on a medium devoid of sucrose and containing 2g activated charcoal/l, 100% of the embryos developed into whole plantlets within 5 months after transfer to the recovery medium. After a 12-month storage period on medium containing 15g/l sucrose and devoid of activated charcoal, 51% of the embryos germinated within 2 months after transfer to the recovery medium. Engelmann *et al.* (1995) studied the factors affecting the cryopreservation of coconut embryos. They found that embryos should be used only when they are in an optimal physiological state as regards notably their maturity and metabolic status. Modifications of recovery conditions can greatly increase the survival rate of zygotic embryos. Use of sodium-algimate encapsulatim of immature zygotic embryos for cryo-storage needs to be studied critically.

APPLICATIONS OF BIOTECHNOLOGY:

Cloning would provide uniform planting materials of elite selections and hybrids with higher productivity.

Disease tolerant materials can be multiplied which could be true to type.

Using embryo culture we can transport disease free materials easily and safely instead of carrying nuts and also to multiply certain varieties.

If somatic embryogenesis and plant

regeneration is standardized, Recombinant DNA techniques could be applied to transfer genes of economic importance.

Using molecular markers, resistance genes can be tagged for traits such as root (wilt), drought etc.

CONCLUSION

The above review would amply show the effort that has been put into the coconut biotechnology. Unfortunately, in India coconut biotechnologyhasbeencarriedoutonlyatCPCRI atpresent. If one goes by the history, there were nearly a dozen laboratories involved in coconut biotechnogyineighties. Whenitwasdiscovered that coconut biotechnology is a difficult one, many laboratories stopped working on coconut biotechnology.Harries(1998)rightlystatedthus " A devil's advocate, asked to say if clonal coconuts really do have any use, would have to admit that the rate of progress has been disappointingly slow. The early aims, to clone high yielding individual palms as farm plating material, are now seen to be naïve. Academic studies may have generated higher degrees for research scientists but they have not spawned the financially successful industries enjoyed by some crops". But , recent developments may prove Harries wrong- may be that is our holy intention.

EXPLANT RESPONSE REFERENCE SOMATIC TISSUE

Stem explants Proliferation but no callus subculture Apavatjrut and Blake (1977)Root explantsCallus formation and subcultureFulford *et. al.* (1981)Leaf explants Callus formation and embryoids neoformationPannetier and Buffard Morel (1982a,b)Seedling leaf explants of WCTDirect somatic embryos and plantlet formation. Raju *et al.* (1984),Iyer (1995) Inflorescence explantsSurface callusing callus formation and multiplication by subculture, Eeuwens (1976), Blake and Eeuwens (1982) Endosperm explants Callus formation and subculture Fisher and Tsai (1978), Prakash Kumar *et al.* (1998), Iyer (1993) Excised seedling apicesGrowth of the vegetative apex and plantlet formationBlake and Eeuwens (1982), Embryos in vitro culture Calluses Nodular protocorm like proliferation with occasional scale leaves D'Souza (1982), Iyer (1982) Immature leaf (1cm long young non-chlorophyllous tissue) (Y3 + 80 mg 2,4-D/1) callus after 4 months Verdeil *et. al.* (1992). **Haploid tissue** Anthers,Torpedo embryosThanh Tuyen and de Guzman (1983), Monfort (1985) Multicellular pollen and embryo-like noduleslyer (1982), Immature inflorescence (MYD x WAT, WAT x MYD and MYD) Eeuwen's medium +2% activated charcoal + 1.5-3.5 mM 2,4-D induced callus after 8 monthsVerdiel *et. al.* (1994), Immature inflorescenceY3 + 20-30 ppm 2,4-D callus inducedSugimura and Salvana (1989).

EXPLANT RESPONSE REFERENCE EXCISED EMBRYOS

Macapuno variety Complete seedlings De Guzman *et. al.* (1971) de Rosario and Guzman (1976), Immature inflorescenceY3 +10-5M 2,4-D +10-6M 2 ip, Cueto *et al.* (1997), West Coast Tall variety Complete seedlings Karun *et. al.* (1996), Immature embryos (6-7 months) Callus after 4 weeks Karunaratne and Periyapperuma (1990)

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