IN VITRO RESPONSE AND CAUSES OF RECALCITRANCY IN COCONUT (*Cocos nucifera* L.)

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled "***In vitro* response and causes of recalcitrancy in coconut (*Cocos nucifera* L.)**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship, associateship or other similar title, of any other university or society.

Vellanikkara
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CERTIFICATE

Certified that the thesis entitled "***In vitro* response and causes of recalcitrancy in coconut (*Cocos nucifera* L.)**" is a record of the research work done independently by **Ms.K. Chandralekha**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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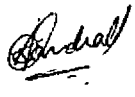

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CONTENTS

LIST OF TABLES

ABBREVIATIONS

BAP	- Benzyl Amino Purine
°C	- degree celsius
cm	- centimeter
cw	- coconut water
2,4-D	- 2,4-Dichloro phenoxy acetic acid
GA	- Gibberellic acid
$HgCl_2$	- Mercuric chloride
IAA	- Indole-3-acetic acid
IBA	- Indole-3-butyric acid
2iP	- 2-isopentenyl adenine
M	- Molar
mg	- milligram(s)
mg l^{-1}	- milligram(s) per litre
ml	- millilitre
mm	- millimeter
mM	- milli Molar
μg	- microgram
N	- Normality
NAA	- naphthalene acetic acid
nm	- nanometer
pH	- hydrogen ion concentration
psi	- pounds per square inch
pvp	- polyvinyl pyrrolidone
rpm	- revolutions per minute
v/v	- volume in volume
w/v	- weight in volume

LIST OF PLATES

INTRODUCTION

The coconut palm (*Cocos nucifera* L.) is well known as 'Kalpavriksha' because, each and every part is useful to mankind in a number of ways right from birth to death. The palm supplies not only food, invigourating water and oil for cooking but also valuable source of timber, fibre for ropes and mats, leaves for thatching roofs, shells that can be used as utensils and ornaments and the sweet sap of the inflorescence from which sugar and alcohol are made. Therefore, the coconut industry is intimately connected with the economic and domestic life of the inhabitants of the coconut growing countries.

In India, coconut is estimated to sustain ten million farm families for their livelyhood besides contributing six per cent towards the edible oil output in the country. Coconut is grown in more than 86 countries located in the tropical belt of the world and among them, India ranks second in area and production with 1.69 million hectares and 13,231 million nuts, respectively (George, 1996). Eventhough, cultivation is mainly confined to the coastal belt, demand for coconut and its products is wide-spread in the country.

India is one of the important coconut producing countries in the world. Nevertheless, the productivity of coconut in India is very low. The existence of heterozygous, genetically variable population due to the allogamous mode of pollination could be one of the reasons for low productivity. As coconut is a monocot, incapable of branching and devoid of cambium, the perpetuation of varieties through traditional methods of vegetative propagation is impossible. The palms bestowed with regular bearing habit and yielding not less than 80 nuts/annum have been

selected as mother palms for collecting seed nuts for achieving higher production potential per unit area. The occurrence of a super palm which produce upto 470 nuts per palm per year has also been identified. Clonal multiplication through tissue culture techniques may alleviate the dearth of propagules of such elite palms which could increase the yield many fold.

The *in vitro* clonal multiplication is the most advanced method of propagation in date and oil palm (Buffard-Morel *et al.*, 1992). Hitherto coconut palm was believed to be recalcitrant (Ammirato, 1983) even after three decades of tissue culture work. Although some studies evidenced with successful regeneration process have been reported, reliable regeneration protocol with certitude is scanty. The ensnaring problem of recalcitrancy in this palm species would become more elucidative if sufficient attention is paid to molecular and biochemical events lurking in the various explant sources.

The chemical compounds responsible for biochemical suppression of growth in plants are primarily secondary substances including phenolics, alkaloids, flavanoids and terpenoids. The phenolic inhibitors act as germination inhibitors by inhibiting the transport of amino acids and the formation of proteins. Misirly *et al.* (1995) reported that there were significant differences in the phenolic (tannin) contents of different plant parts in some apricot varieties.

Keeping all these in view, the objectives of the present study are to find out:

i) the response of various explant sources for somatic embryogenesis and organogenesis

ii) the possible reasons of *in vitro* recalcitrancy in coconut.

REVIEW OF LITERATURE

The coconut growers in India are faced by several problems such as the long juvenile phase of the crop, heterozygous nature, difficulty in vegetative propagation, the constraints in the availability of quality planting materials, the root (wilt) and Thanjavore wilt diseases etc. Eventhough the average productivity of coconut in India is only 35 nuts per palm per year, individual palms which produce upto 470 nuts per year have already been reported (Iyer *et al.*, 1979). If such rare single super palms could be propagated clonally, this would certainly break the yield barrier in coconut.

2.1 Propagation of coconut

Coconut can be propagated naturally only from seed. Since the palms do not breed true to type, the offsprings differ greatly in vigour, productivity and resistance to diseases. A few spontaneous vegetative propagation phenomena have sometimes been observed, though these are exceptional. They include aerial branching (Ridley, 1907; Davis, 1950 and 1956), suckering (Davis, 1956a), formation of bulbils (Davis, 1969); which can sometimes root (Sudasrip *et al.*, 1978) but conventional horticultural techniques cannot be used for routine multiplication.

Tissue culture, which is very effective for cloning a number of species (Murashige, 1974) including certain palms like date (Tisserat and De-Manson, 1980; Veramendi and Navarro, 1996) and oil palm (Corley *et al.*, 1977; Pannetier *et al.*, 1981) is the only channel opened for intensive vegetative propagation of coconut (Verdeil *et al.*, 1989).

Various explants such as zygotic embryos (Abraham and Thomas, 1962; De Guzman *et al.*, 1978; Bhalla-Sarin *et al.*, 1986; Thanh-Tuyen and Dionzon (1986), stem (Apavatjrut and Blake, 1977), root (Fulford *et al.*, 1981), leaf (Pannetier and Buffard-Morel, 1982; Raju *et al.*, 1984), inflorescence (Eeuwens, 1976; Verdeil *et al.*, 1994) have been used for coconut tissue culture for the last three decades.

2.2 General aspects on plant tissue culture

The cell theory postulated by Schleiden (1838) and Schwann (1839), which reveals the totipotent nature of plant cells is the basis of cell, tissue and organ culture. Haberlandt (1902) reported that isolated cells are capable of resuming uninterrupted growth. Widespread success with plant tissue culture were reported after the discovery of auxins and cytokinins by Skoog and Miller (1957). They putforth the concept of hormonal control of organ formation and showed that the differentiation of roots and shoots in tobacco pith tissue cultures was a function of the auxin cytokinin ratio and that organ differentiation could be regulated by changing the relative concentration of these two substances in the medium. The early development of plant tissue culture technique was based on the efforts of many pioneering investigators including White (1934), Gautheret (1939), Miller *et al.* (1956), Reinert (1958), Steward *et al.* (1958), Bergmann (1960), Vasil and Hildebrandt (1965). It was Murashige and Skoog (1962), who developed a completely defined nutrient medium for plant tissue culture.

In the recent years, the most widely used application of tissue culture technology in horticulture is the use of *in vitro* techniques for clonal propagation (Thorpe, 1990).

2.3 Factors influencing success of *in vitro* propagation

The success of *in vitro* propagation is directly or indirectly affected by various factors which include genotype, size, age and type of explant, surface sterilization, media used, hormones and their concentration, presence or absence of other additives, nitrogen source and concentrations, physical condition of the medium, pH, light, temperature, relative humidity etc. (Brown and Thorpe, 1986).

2.3.1 Genotype and age of the donor plant

Monfort (1985) obtained embryoids from anthers of Malayan Yellow Dwarf x West African Tall (PB 121) and West African Tall x Rennell Tall. Bhalla-Sarin *et al.* (1986) cultured immature embryos of West Coast Tall and they got white compact nodular callus and later plantlets. Zygotic embryo culture in coconut is successfully reported in various genotypes like PB 121 (Assy-Bah *et al.*, 1989), Hybrid Tall x Gangabondam (Kalamani and Rangasamy, 1990 and Ghanainan Yellow Dwarf (Assy-Bah and Engelmann, 1993). Reproducible obtention of callogenesis and whole somatic embryos from immature leaf or inflorescence fragments in 20-25 years old PB 121 hybrid palms was reported by Verdeil *et al.* (1992).

One of the constraints in tissue culture with coconut seedlings or mature palms is the plant to plant differences in genotypic response (Iyer, 1993). The pertinacious difference in callogenesis responses of the three genotypes PB 121 (Malayan Yellow Dwarf x West African Tall), PB 111 (West African Tall x Malayan Yellow Dwarf) and Malayan Yellow Dwarf was also reported by Verdeil *et al.* (1994). Hybrids PB 121 and PB 111 gave the best results.

Callogenesis percentage in leaves and inflorescence tissues varied according to the age of the palm from which samples were taken (Verdeil *et al.*, 1989). Anthers taken from mature palms of 15-20 years old produced proembryoids while those from young palms did not respond (D'Souza and Mallya, 1993).

2.3.2 Age, size and type of the explant

Thanh-Tuyen and De Guzman (1983) observed that microspores at the stage just before, during or immediately after the first pollen mitosis was the most responsive to embryogenesis in coconut. Karunaratne and Periyapperuma (1989) found that the age of the zygotic embryo was an important factor determining callus proliferation and subsequent embryogenesis and immature embryos responded better than mature embryos. Size of the embryo at culture was an important factor for obtaining higher percentage of plants and the germination was maximum when the size of the embryo was 7 x 4 mm (Gupta *et al.*, 1984).

Young inflorescence from coconut and other palms with their abundance of flower meristem may serve as a suitable source of explant for vegetative propagation as reported by Eeuwens (1978). Sugimura and Salvana (1989) reported that one mm thick slices gave 32 per cent browning while 0.5 mm thick slices gave 11.0 per cent only when immature inflorescence was used as explant. Orense *et al.* (1993) reported that immature inflorescence was found to be the most reponsive to callus induction due to the presence of numerous meristematic points. Inflorescence with external spathe length 10 and 25 cm gave the best callusing (Verdeil *et al.*, 1994).

Leaf tissues from 12-24 months old seedlings were embryogenic but the potential was quickly lost with the onset of juvenility (Karunaratne *et al.*, 1991). The developmental stage of the leaf and immature embryo is extremely important in coconut somatic embryogenesis (Karunaratne, 1993).

2.3.3 Surface sterilization

Different surface sterilization techniques were carried out for coconut explants to remove all the micro organisms present on them. Thanh-Tuyen and De Guzman (1983) rapidly immersed the male flower buds in 70 per cent ethanol and rinsed with sterile distilled water and the anthers were excised aseptically. Immature embryos with the endosperm plugs were sterilised with 0.1 per cent $HgCl_2$ for 20 minutes and leaf and rachillae were sterilized effectively with 0.05 per cent $HgCl_2$ for 10 minutes (Gupta *et al.*, 1984). Kumar *et al.* (1985) swabbed the surface of the dehusked nuts with 90 per cent ethanol and scooped out the embryo aseptically whereas, Ashburner *et al.* (1991) gave a detailed sterilization technique to endosperm plugs using 70 per cent ethanol, 4.0 per cent sodium hypochlorite and 2.0 per cent cetrimide and finally to the embryo using 2.0 per cent sodium hypochlorite and 1.0 per cent cetrimide for 20 minutes and rinsed with sterile distilled water. Verdeil *et al.* (1994) sterilized the inner spathe of inflorescence for 10 minutes in a hypochlorite solution and then removed it under aseptic condition.

2.3.4 Exudation from the explant

Browning of the tissues and adjacent medium is assumed to be due to the oxidation of polyphenols and formation of quinones which are highly reactive and toxic to the tissues (Maier and Metzlier, 1965). This browning response might be

minimized by selecting a suitable stage of explant cultured under defined conditions (Sugimura and Salvana, 1989).

The prominent problem of browning has been noticed in coconut tissue culture by Apavatjrut and Blake (1977), Fisher and Tsai (1978), Tisserat (1984), Sugimura and Salvana (1989) and Areza *et al.* (1993). Sharma *et al.* (1980) and Zaid (1987) reported that *in vitro* browning of tissues was a great problem in date palm. Establishment of *in vitro* cultures of several woody species was hampered by the polyphenol interference (Lloyd and McCown, 1980, Mathew *et al.*, 1987; D'Silva and D'Souza, 1993).

2.3.4.1 Methods to overcome polyphenol interference under *in vitro* conditions

2.3.4.1.1 Collection and preparation of explant

Coarse slices of non uniform explant size exhibited browning regardless of the age of the coconut inflorescence and by reducing the thickness of the transverse slices of rachillae, the browning was minimised (Sugimura and Salvana, 1989). Iyer (1993) reported that browning of explant was severe in old spathes of coconut.

Unwounded tissues as whole leaves or embryos do not exude phenol and grow well (Reuveni and Kipinis, 1974). According to Ripley and Preece (1986), lesser wounding or cutting with a sharp blade reduces the exudation.

2.3.4.1.2 Pretreatments given to explants

Sugimura and Salvana (1989) reported that slicing the coconut rachilae in Y_3 liquid formulation supplemented with half the strength of vitamin and 3.0 per

cent sucrose reduced the browning considerably. Soaking the inflorescence tissues in ascorbic acid, citric acid and ascorbic acid or PVP for 20 minutes may lessen the browning, but inflorescenes with outer spathe lengths of approximately 20 cm need not be soaked in any antioxidant prior to slicing (Areza *et al.*, 1993). Browning incidence can be minimized by cutting the rachillae in auxin free liquid preculture medium not exceeding three days (Orense *et al.*, 1993).

Pretreatments like soaking explants in adsorbants like PVP or in water (Gupta *et al.*, 1980) or in antioxidant solutions like ascorbic or citric acid (Gupta *et al.*, 1980; Zaid and Tisserat, 1983; D'Silva and D'Souza, 1993) reduce polyphenol exudation and its oxidation.

2.3.4.1.3 Selection of basal medium

Apavatjrut and Blake (1977) suggested that browning in coconut tissues could be eliminated by a nutritionally balanced medium.

2.3.4.1.4 Rapid transfer of explants into fresh medium

Browning of tissues was eliminated after three to four subcultures at an interval of 10 days in date palm as reported by Sharma *et al.* (1980). Zaid and Tisserat (1983) reproted that reculturing the date palm tissues to fresh medium after a short period of incubation decreased the browning.

2.3.4.1.5 Use of media additives

Siqueira and Inoue (1991) reported that the addition of citric acid and ascorbic acid for young leaves and PVP for adult leaves and inflorescence as media adjuvants were effective to control the *in vitro* browning of explants in coconut.

Addition of 0.1 per cent PVP in the culture medium prevents the phenolic exudation in coffee (Sreenath *et al.*, 1995) and in rubber (Seneviratne and Wijesekara, 1996).

Activated charcoal has the ability to adsorb toxic metabolites released into the culture medium. Use of activated charcoal is preferred to L-cystein HCl and other reducing agents such as ascorbic acid, citric acid and glutathione (George and Sherrington, 1984) because the latter are often toxic to the plant tissues at higher concentrations. Tisserat (1979) reported that activated charcoal 0.1-3.0 per cent enhanced survival and organogenesis especially in date palm. Incorporation of activated charcoal in the medium plays a key role in minimizing browning of coconut explants (Sugimura and Salvana, 1989; Areza *et al.*, 1993).

Inclusion of charcoal reduces the availability of growth hormones and therefore, addition of an abnormally high concentration of auxin is recommended (Zaid and Tisserat, 1983; Sugimura and Salvana, 1989; Ebert and Taylor, 1990).

2.3.4.1.6 Selection of growth regulators and carbon sources

Execessive levels of 2,4-D are known to cause browning and growth inhibition of inflorescence tissues and BAP alone causes higher incidence of browning than when BAP and 2iP were combined, in coconut tissue culture (Ebert, 1993). Cytokinins are known to stimulate the synthesis of polyphenols (Bergmann, 1964).

Sucrose at 3.0 per cent in the culture medium was the most effective in preventing phenolic browning of explant in coconut (Sugimura and Salvana, 1989).

2.3.4.1.7 Selection of culture conditions

Reduction of light intensity was reported to be an effective method for

reducing oxidation (Forrest, 1969; Ziv and Halevy, 1983). Monaco *et al.* (1977) reported that high temperature inside the transfer chamber and cultures incubated under light condition exhibited increased browning in coffee.

The cultures of coconut kept in darkness inside an incubator at $28\pm2^{\circ}C$ produced well developed callus without browning (Sugimura and Salvana, 1989).

2.3.5 Culture medium

Callus formation from coconut occurred on nutrient media containing high salt concentration similar to that found in MS medium with additional iodine. Growth of all tissues, irrespective of their origin was considerably greater on Y_3 mineral medium and when MS macro-elements were combined with Y_3 micro elements, growth equal to that on the complete Y_3 minerals was obtained by Eeuwen (1976). Tahardi and Warga-Dalem (1982) reported that White's basal medium was the best for promoting the growth of excised coconut embryos. Thanh-Tuyen and De Guzman (1983) reported that Blaydes and Keller medium were suitable for coconut anther culture. Bhalla-Sarin *et al.* (1986) obtained white nodular callus from coconut embryos in B_5 medium. Kalamani and Rangasamy (1990) reported that Y_3 mineral medium was found to be the best for growth and development of coconut embryos.

2.3.6 Growth regulators

Coconut inflorescence explants produced shoots in response to high cytokinin levels and roots in response to high auxin levels (Eeuwens and Blake, 1977). High concentration of 2,4-D combined with activated charcoal plays an important role in the growth of coconut explants (Gupta *et al.*, 1984) and 2,4-D is essential for callus induction in coconut embryos (Tahardi, 1987).

2.3.7 Carbon and energy sources

Eeuwens (1978) observed that growth of coconut tissue on fructose exceeded than that obtained with sucrose. Increasing the sucrose concentration to 6 to 9 per cent made it possible to obtain pollen embryoids (Thanh-Tuyen and De Guzman, 1983) and sturdy plantlets from zygotic embryos (Assy-Bah *et al.*, 1989).

2.3.8 Other media additives

Activated charcoal is an essential medium additive for the germination and development of coconut embryos (De Guzman *et al.*, 1971; Karunaratne *et al.*, 1985; Assy-Bah *et al.*, 1989; Rillo and Paloma, 1990). Casein hydrolysate or amino acid mixture containing six aminoacids added to the medium stimulated growth of inflorescence *in vitro* significantly (Eeuwens, 1976). Blake and Eeuwens (1982) reported that addition of coconut water gave an improvement of culture growth. Addition of phloroglucinol promoted the culture growth in *Cinchona ledgeriana* (Hunter, 1979). Role of these complex organic compounds are usually unpredictable and repeatability is also poor, therefore it has been recommended to avoid their use as far as possible (Gamborg and Shyluk, 1981). Addition of silver nitrate 0.5 and 1.0 per cent induced green friable calli with green roots in *Kaempferia galanga* (Joseph, 1997).

2.3.9 Nitrogen source and concentration

The presence of reduced nitrogen in addition to nitrate appears to be essential for satisfactory growth of coconut tissues and optimum concentration of

ammonium and nitrate (NH_4Cl 10 mM, KNO_3 20 mM) resulted in healthy cultures (Eeuwens, 1976).

2.3.10 Culture conditions

The culture conditions such as physical form of medium, pH, light, temperature and relative humidity are very important in *in vitro* growth and differentiation. Number of shootlets from inflorescence was increased by growing on a static liquid medium obtained by Blake and Eeuwens (1982). Assy-Bah (1986) obtained plantlets from embryos only in the presence of agar and charcoal but Ashburner *et al.* (1991) reported that greater enlargement and faster germination were obtained in liquid medium.

Basu *et al.* (1988) observed that cell division in suspension obtained from leaf cells of coconut was influenced by the pH of the medium and a three fold increase in cell number was obtained at pH 7.0 compared with pH 5.0.

Blake and Eeuwens (1982) found that growth of coconut inflorescence cultures in the dark was better than in the light and a temperature of 30-32°C was optimal. Assy-Bah *et al.* (1987) kept the zygotic embryo cultures in the dark at 27 ± 1°C until the gemmule appeared, then they were received light for 12 hour a day and Karunaartne and Periyapperuma (1989) reported that exposure of embryo cultures to stronger light suppressed embryogenesis.

2.4 Organogenesis

Gupta *et al.* (1984) observed globular callus from young stem explants of mature coconut palms but no organogenesis was obtained. Indirect organogenesis in

mature embryos of Bali x Nias hybrid (KINA II) was successfully reported by Tahardi (1987).

Bhalla-Sarin *et al.* (1986) obtained a few plantlets via indirect organogenesis from immature zygotic embryos using IAA-conjugates in B_5 medium.

No repeatable reports are available in the differentiation of shoot buds either directly or through callus in coconut tissue culture (Rao and Ganapathi, 1993).

2.5 Somatic embryogenesis

Karunaratne and Periyapperuma (1989) obtained embryogenic callus from 6-7 months old zygotic embryos in the presence of 12-20 μM 2,4-D and embryoids in the presence of 8 μM 2,4-D. The embryoids germinated when BAP and Kinetin were supplemented to the medium.

When immature rachillae tissues were used as explant source, they were developed into leaf and (or) root like growths via. embryogenesis (Branton and Blake, 1983; Gupta *et al.*, 1984). Blake (1990) reported that high 2,4-D levels and activated charcoal were found necessary for callus induction and gradual lowering of 2,4-D level was associated with embryo production in immature inflorescence. Reproducible *in vitro* system of coconut regeneration through indirect somatic embryogenesis was reported by Verdeil *et al.* (1994).

Iyer (1982) was able to obtain multicelled proembryoids within the exine of coconut microspores in cultured anthers. Thanh-Tuyen and De Guzman (1983) and Monfort (1985) reported formation of pollen embryos from cultured anthers. Direct embryogenesis seems to be the principal pathway of haploid formation in

coconut (Thanh-Tuyen, 1990). Hegde *et al.* (1994) reported that somatic embryos were emerged out of the dehiscent anther segment through a prolonged culture of anthers for 8 months in MS medium supplemented with L-Glutamine, high amount of sucrose and BAP.

Gupta *et al.* (1984) reported that root like structures and globular embryo like structures were developed directly from young leaves. Direct differentiation of embryos from leaf was reported by Raju *et al.* (1984). Bhaskaran (1985) obtained somatic embryos from induced callus and also directly from mid-rib region of leaf explant.

It has been observed that coconut tissue cultures do show a tendency towards somatic embryogenesis (Rao and Ganapathi, 1993).

2.6 Biochemical attributes

2.6.1 Phenol and polyphenol oxidase activity

In the natural state phenols play an important role in plants, the hormone balance. compartmentalization, disease resistance and protection of injured tissues from infection (Crompton and Preece, 1986). Total phenol and orthohydroxy phenols are more in coconut palms affected by Thanjavore wilt (Suriachandraselvan *et al.*, 1993) and high content of phenols in varieties susceptible to root (wilt) (Joseph and Jayasankar, 1974; CPCRI, 1975). Misirly *et al.* (1995) reported that there was a significant difference in the phenolic (tannin) contents of different plant parts in some apricot varieties.

Upon wounding, the phenols are converted to quinones through the action of copper containing enzymes like polyphenol oxidase and tyrosinase (Lerch, 1981). Quinones are highly toxic to the tissues due to their reversible hydrogen

bonding to protein (George and Sherrington, 1984). Shivashankar (1988) observed increased activity of polyphenol oxidase with increasing level of water stress. Chempakam *et al.* (1993) reported that coconut cultivars tolerant to root (wilt) showed higher polyphenol oxidase activity.

2.6.2 Protein pattern by electrophoresis

Application of an electric field to a buffered homogeneous protein results in the differential migration of proteins (Malik and Singh, 1980). Coleman and Ernst (1991) reported that in *Populus deltoides*, protein of molecular weight 32000 and 35000 Daltons were determined for shoot regeneration and callus growth respectively. Baaziz *et al.* (1994) obtained differential pattern of acid soluble proteins in explants, germinated embryos and calli of two date palm cultivars.

2.7 *In vitro* rooting

Generally auxins favour rhizogenesis. Shoots obtained from coconut zygotic embryos were transferred to Y_3 medium supplemented with NAA 1.5 mg l^{-1} and activated charcoal 0.1 per cent to induce root formation as reported by Kalamani and Rangasamy (1990). Activated charcoal has profound influence on rooting of shoots *in vitro*. It may adsorb residual cytokinin from the shoot and shades *in vitro* roots from light.

2.8 Hardening and planting out

Tisserat (1982) reported that there was direct relationship between the size of the initial plantlet and the survival in soil and direct transfer to green house conditions without an intermediate high humidity stage was not feasible.

Gupta *et al.* (1984) transferred the seedlings obtained from zygotic embryos to polybags containing mixture of soil:sand:compost (3:3:1) and incubated at 25°C with 16 hours photoperiod for four weeks and later to glass houses.

MATERIALS AND METHODS

Studies on *in vitro* response and causes of recalcitrancy in coconut (*Cocos nucifera* L.) were carried out at the Tissue Culture Laboratory of the Department of Plantation Crops and Spices and Biochemistry Laboratory, College of Horticulture, Vellanikkara during 1995-'97. The materials used and the methodology adopted for the study are described in the following sections.

3.1 Materials

3.1.1 Chemicals

Chemicals used for the preparation of various media were procured from SISCO Research Laboratory (SRL), British Drug House (BDH), Qualigens, Merck or Sigma.

3.1.2 Glasswares

Borosilicate glasswares of Corning or Borosil brand were used for the experiment. They were cleaned by initially boiling for half an hour, after cooling they were thoroughly washed with the detergent solution, then rinsed with potassium dichromate solution in sulphuric acid, washed using tap water and finally rinsed with double distilled water. They were dried in hot air oven at 100°C for 24 hours and were stored away from dust and contaminants.

3.1.3 Collection and preparation of explant

The explants used for the study included zygotic embryos from mature and immature nuts, immature spadices, anthers and tender leaves of approximately

17 years old pre-potent palms of D x T parentage grown in the coconut plantation under the Department of Plantation Crops and Spices, Collge of Horticulture, Vellanikkara.

3.1.3.1 Zygotic embryos

Zygotic embryos along with the surrounding endosperm (endosperm cylinders) were scooped out from the large eye of mature (11-12 months old) and immature (7-8 months old) dehusked nuts using a cork borer without causing any injury to the embryos.

3.1.3.2 Immature inflorescences

Immature spadices of average external spathe length 40 cm corresponding to the fourth frond from the spindle leaves were selected. The inflorescence rachillae bits from these immature spadices were used for inoculation.

3.1.3.3 Anthers

Closed spadices were collected at about four-five weeks before splitting. It is often the unopened spadix next to the newly opened inflorescence. Immature male flowers from the top one third portion of the spikelets were used for the excision of anthers.

3.1.3.4 Tender leaves

Tender non-chlorophyllous spindle leaves were cut into pieces and were used.

hood of a clean laminar air flow chamber. The working table of the laminar air flow chamber was first surface sterilized with absolute alcohol and then by putting on the ultraviolet light for 30 minutes. The steam sterilized petridishes and inoculation accessories were flame sterilized before each inoculation. The hands were also scrubbed with absolute alcohol before inoculation.

3.1.5 Culture media

MS (Murashige and Skoog, 1962), Blaydes (Blaydes, 1966), B_5 (Gamborg *et al.*, 1968), Y_3 elements (Eeuwens, 1976) with Morel and Wetmore vitamins (Morel and Wetmore, 1951) except Biotin and MS macro and Y_3 micro elements (MS + Y_3) with Morel and Wetmore vitamins were used as basal media. Composition of these media are given in Table 1.

3.2 Preparation of media

Standard procedures (Gamborg and Shyluk,1981) were followed for the preparation of media. Stock solutions of major and minor nutrients were prepared first by dissolving the required quantity of chemicals in distilled water and stored under refrigerated conditions in amber coloured bottles. Stock solutions for the major and minor nutrients were prepared fresh every three months and vitamin stock solutions were prepared fresh after every six to eight weeks. The stock solutions for various phytohormones were prepared and were stored in refrigerated condition.

Table 1. Composition of various basal media tried for *in vitro* culture of coconut

Ingredients mg l^{-1}	MS	Blaydes	B_5	Y_3
Macro elements				
NH_4NO_3	1650	1000	-	-
KNO_3	1900	1000	2500	2020
KCl	-	65	-	1492
KH_2PO_4	170	300	-	-
$MgSO_4.7H_2O$	370	35	250	247
$Ca(NO_3)_2.4H_2O$	-	347	-	-
NH_4Cl	-	-	-	535
$NaH_2PO_4.2H_2O$	-	-	150	312
$(NH_4)_2SO_4$	-	-	134	-
$CaCl_2.2H_2O$	440	-	150	294
Micro elements				
KI	0.83	0.8	0.75	8.3
H_3BO_3	6.2	1.6	3.0	3.1
$MnSO_4.4H_2O$	22.3	-	-	11.2
$MnSO_4.H_2O$	-	4.4	10	-
$ZnSO_4.7H_2O$	8.6	1.5	2.0	7.2
$Na_2MoO_4.2H_2O$	0.25	-	0.25	0.24
$CuSO_4.5H_2O$	0.025	-	0.025	0.16
$CoCl_2.6H_2O$	0.025	-	0.025	0.24
$Na_2EDTA.2H_2O$	37.25	32	37.3	37.25
$FeSO_4.7H_2O$	27.85	-	27.8	13.90
$NiCl_2.6H_2O$	-	-	-	0.024
Additive and vitamins				
Myo-inositol	100	100	100	100
Nicotinic acid	0.5	0.5	1.0	1.0
Pyridoxine HCl	0.5	0.1	1.0	1.0
Glycine	2.0	2.0	-	-
Thiamine HCl	0.1	0.1	10.0	1.0
Calcium-d-Pantothenate	-	-	-	1.0

Known volumes of different stock solutions were pipetted out into a clean vessel, which was rinsed with distilled water. Sucrose and inositol were added fresh and dissolved, and the volume was made upto the required level using distilled water. The pH of the solution was adjusted between 5.6 and 5.8 using 0.1 N HCl or 1.0 N NaOH.

In order to prepare the semisolid media, good quality agar was added (0.75 w/v) and the solution was heated for melting the agar. In the case of liquid medium, agar was avoided and filter paper bridges of 'M' shape were provided to support the explants. The bulk media was poured into culture tubes and were plugged with cotton plugs. Sterilization of the media was done in an autoclave by applying 15 psi pressure at 121 °C temperature for 20 minutes. After sterilization, the media were allowed to cool to room temperature and were stored in an air conditioned culture room until further use.

3.3 Culture establishment

Different sterilization treatments tried are listed in Table 2. The explants after surface sterilization were rinsed five times with sterile distilled water to remove traces of the sterilant from the surface.

3.3.1 Standardisation of pre-treatments to overcome polyphenol interference

Coconut explants usually turn brown due to the leaching of polyphenols into the medium. Since they were found to interfere with the growth of *in vitro* cultures, response of explants in the five different basal media such as MS, Blaydes, B_5, Y_3 and MS + Y_3 medium was assessed. The best basal medium was selected

Table 2. Standardisation of surface sterilization treatments for the explants of coconut (*Cocos nucifera* L.)

Treatment No.	Explant	Sterilant	Concentration (percentage)	Duration in minutes
1	Soaking the endosperm cylinder	$HgCl_2$	0.1	10 20 30 45
2	Soaking the male flowers	$HgCl_2$	0.1	7 5 2 1
3	Soaking the tender leaves	$HgCl_2$	0.1	5, 7
4	Alcohol wiping - soaking the tender leaves	$HgCl_2$	70, 0.1	1, 2
5	Soaking the immature inflorescence with spathe intact	$HgCl_2$	0.1	30
6	Soaking the inflorescence with spathe intact +	$HgCl_2$	0.1	20
	Soaking the rachillae	$HgCl_2$	0.1	1, 3, 7
7	Soaking the inflorescence with spathe intact +	$HgCl_2$	0.1	20
	Soaking the rachillae	Chlorine water	3.5	1, 3

and various treatments were tried to overcome the browning of immature rachillae. Details are given in Table 3. All the treatments were kept both under dark and light conditions to study the effect of light on polyphenol interference.

Numerical scores were given to quantify the polyphenol exudation (Mathew, 1994). Cultures in which the medium was clear, 15 days after incubation were given 'zero' score. Those cultures in which half the volume of the media turned dark brown within 15 days were scored as 'four'. The others were scored in between 'zero' and 'four' according to the extent of discolouration. In culture medium supplemented with activated charcoal, percentage of survival and intensity of browning of explants after one month were recorded.

3.3.2 Culture conditions

The cultures were incubated at 26 $\pm$ 2°C in an air conditioned culture room under dark unless, otherwise mentioned in separate experiments where 16 hours photoperiod (3000 lux) was provided. Humidity in the culture room varied between 60 and 80 per cent according to the climate prevailing.

3.4 Direct organogenesis

3.4.1 Explant source

Immature and mature zygotic embryos were dissected out from the surface sterilized endosperm cylinders and were inoculated aseptically into the medium. The cultures were kept under dark until germination and later on they were put under light condition.

Table 3. Standardisation of treatments for preventing polyphenol oxidation and browning of coconut inflorescence explants

A. Soaking the surface sterilized sliced explants and shaking in an orbital shaker at 100 rpm for 24 hours in sterile

a. Ascorbic acid	0.01%
b. PVP	0.1%
c. Coconut water	15%
d. Activated charcoal	0.5%
e. Sucrose	4%
f. Sterile distilled water	-

B. Media additives

a. Ascorbic acid	0.01%
b. PVP	0.1%
c. Coconut water	15%
d. Activated charcoal	0.25%
e. Control	

The male flower bearing rachillae from the surface sterilized immature inflorescence were sliced transversely (1-1.5 mm long) and were used for culturing in the media. Anthers were taken carefully from the surface sterilized male flowers and the connectives were removed and were inoculated. The surface sterilized tender leaves were cut into small pieces of 0.8 cm x 0.5 cm size and were inoculated.

3.4.2 Basal media

Solid and liquid phases of MS, Blaydes, B_5, Y_3 and MS + Y_3 media were used for organogenesis. The solid media were supplemented with 0.25 per cent activated charcoal.

3.4.3 Growth regulators

Growth regulators like auxins (NAA) and cytokinins (BAP, kinetin) were incorporated into the basal media. The details of various combinations tried are given in Table 4.

3.4.4 Carbon source

Sucrose at three per cent concentration was used as carbon source.

3.5 Indirect organogenesis

3.5.1 Callus induction

Immature zygotic embryos, immature rachillae, anthers and tender leaves were used for callus induction in different basal media like MS, Blaydes, B_5, Y_3 and MS + Y_3.

Table 4. Standardisation of media supplements for direct organogenesis in coconut explants

Basal medium	Growth regulator
MS, Blaydes, B_5, Y_3, MS + Y_3 (liquid and solid phase)	Kinetin (0.1, 0.5, 2.0 mg l^{-1})
	BAP (1, 2, 3, 5, 7 mg l^{-1})
	NAA (0.1, 0.2, 0.5 mg l^{-1}) + BAP 5 mg l^{-1}
	NAA 1.5 + Kinetin 1 mg l^{-1}
	NAA 2 + Kinetin 1 mg l^{-1}
	NAA 2 + Kinetin 2 mg l^{-1}

Explants include mature and immature zygotic embryos, immature rachillae, anthers and leaves.

3.5.2 Growth regulators

Different basal media were supplemented with different levels of growth regulators such as auxins (2,4-D, NAA, IAA), cytokinins (BAP, kinetin, 2iP) and gibberellin.

3.5.3 Media additives

Additives like casein hydrolysate, silver nitrate, amino acids, phloroglucinol, coconut water and sucrose were used for callus induction and proliferation. The details are given in Table 5 and 6.

Cultures were kept both under dark and light conditions for the first time. In order to reduce the oxidation of polyphenols and to favour the callus induction, later on, all the cultures were incubated under dark conditions. Observations were recorded for callus induction, growth rate and morphology of callus at fortnight interval. Callus index (CI) was worked out as follows.

$$CI = P \times G$$

Where P is the percentage of callus initiation and G is the growth score. Scoring was based on the spread of the calli and a maximum score of four was given for those that have occupied the whole surface of the media within 60 and 120 days of culture period in the case of inflorescence parts and immature zygotic embryos, respectively.

3.5.4 Callus mediated organogenesis/somatic embryogenesis

Callus obtained from immature zygotic embryos, rachillae and anthers

Table 5. Standardisation of media supplements for indirect organogenesis/somatic embryogenesis in coconut

*Basal medium	Growth regulator
1. Immature zygotic embryos	
MS, Blaydes, B_5, Y_3	NAA (0.1, 2.0, 4.0 mg l^{-1})
	2,4-D (5, 10, 20, 30 mg l^{-1})
Y_3	2,4-D (33, 44, 55, 66, 77, 80, 100 mg l^{-1})
	2,4-D 33 mg l^{-1} + casein hydrolysate (50, 200 mg l^{-1})
	2,4-D 33 mg l^{-1} + $AgNO_3$ (0.1, 0.2%)
2. Immature inflorescence rachillae	
MS, Blaydes, B_5, Y_3, MS + Y_3	NAA (0.1, 2.0, 4.0 mg l^{-1})
	2,4-D (5, 10, 20, 30 mg l^{-1})
	2,4-D 1 + Kinetin 1 mg l^{-1}
	2,4-D 1 + Kinetin 2 mg l^{-1}
	2,4-D 2 + Kinetin 2 mg l^{-1}
Y_3	2,4-D (11, 22, 30, 33, 40, 44, 55, 66, 77 mg l^{-1})
	IAA (0.1, 0.5, 2.0 mg l^{-1})
	2iP (0.1, 0.5, 2.0 mg l^{-1})
Additives Y_3	2,4-D (22, 33, 44 mg l^{-1}) + $AgNO_3$ 0.1%
	2,4-D 33 mg l^{-1} + casein hydrolysate 200 mg l^{-1}
	2,4-D 33 mg l^{-1} + L-glutamine 100 mg l^{-1}
	2,4-D (33, 55 mg l^{-1}) + L-glutamine 100 mg l^{-1} + L-asparagine 100 mg l^{-1} + L-aspartate 100 mg l^{-1}
	2,4-D (10, 33 mg l^{-1}) + CW 15%
	2,4-D (33 mg l^{-1}) + CW 15% + phloroglucinol 200 mg l^{-1}
	GA (1, 2, 5 mg l^{-1})
	GA (1, 2, 5 mg l^{-1}) + CW 15% + phloroglucinol 200 mg l^{-1}
3. Tender leaves	
Y_3	2,4-D (1, 5, 10, 20, 30 mg l^{-1})
	NAA (1, 2, 4 mg l^{-1})

*Incorporated with 0.25 per cent activated charcoal and 3.0 per cent sucrose

Table 6. Standardisation of media supplements for anther culture in coconut

Basal medium	Growth regulator combinations
a) Solid phase	
MS, Blaydes, B_5, Y_3	NAA (0.1, 2.0 mg l^{-1})
	2,4-D (2, 5, 10, 30, 40 mg l^{-1})
	NAA 0.1 + 2,4-D 0.1 mg l^{-1}
	NAA 0.2 + 2,4-D 0.2 mg l^{-1}
	2,4-D 1 + Kinetin 1 mg l^{-1}
	2,4-D 1 + Kinetin 2 mg l^{-1}
	2,4-D 2 + Kinetin 2 mg l^{-1}
MS + Y_3	NAA 1 + Kinetin (1, 2 mg l^{-1})
	NAA 1 + BA (1, 2, 5 mg l^{-1})
Blaydes	2,4-D 0.25 mg l^{-1} + NAA (1,2,3 mg l^{-1}) + Sucrose 6%
	2,4-D 0.5 mg l^{-1} + NAA (1,2,3 mg l^{-1}) + Sucrose 6%
	2,4-D 1.0 mg l^{-1} + NAA (1,2,3 mg l^{-1}) + Sucrose 6%
	2,4-D 2.0 mg l^{-1} + NAA (1,2,3 mg l^{-1}) + Sucrose 6%
	2,4-D 0.25 mg l^{-1} + NAA (1,2,3 mg l^{-1}) + Sucrose 6% + CW 15%
	2,4-D 0.5 mg l^{-1} + NAA (1,2,3 mg l^{-1}) + Sucrose 6% + CW 15%
	2,4-D 1 mg l^{-1} + NAA (1,2,3 mg l^{-1}) + Sucrose 6% + CW 15%
	2,4-D 2 mg l^{-1} + NAA (1,2,3 mg l^{-1}) + Sucrose 6% + CW 15%

Table 6. Continued

Basal medium	Growth regulator combinations
	2,4-D 0.25 mg l^{-1} + NAA (1,2,3 mg l^{-1}) + Sucrose 9% + CW 15%
	2,4-D 0.5 mg l^{-1} + NAA (1,2,3 mg l^{-1}) + Sucrose 9% + CW 15%
	2,4-D 1.0 mg l^{-1} + NAA (1,2,3 mg l^{-1}) + Sucrose 9% + CW 15%
	2,4-D 2.0 mg l^{-1} + NAA (1,2,3 mg l^{-1}) + Sucrose 9% + CW 15%
b) Liquid phase	
Blaydes	NAA (1,2,3,5 mg l^{-1}) + Sucrose 6% + L-glutamine 500 mg l^{-1} + CW 15%
	2,4-D + Sucrose 6% + L-glutamine 500 mg l^{-1} + CW 15%
	NAA (1,2,3,5 mg l^{-1}) + Sucrose 9% + L-glutamine 500 mg l^{-1} + CW 15%
	2,4-D + Sucrose 9% + L-glutamine 500 mg l^{-1} + CW 15%
MS	BA (10, 50, 100, 200 mg l^{-1}) + Sucrose 5% + L-glutamine 500 mg l^{-1}
	BA (10, 50, 100, 200 mg l^{-1}) + Sucrose 5% + L-glutamine 250 mg l^{-1}
	BA (10, 50, 100, 200 mg l^{-1}) + Sucrose 9% + L-glutamine 500 mg l^{-1}
	BA (10, 50, 100, 200 mg l^{-1}) + Sucrose 9% + L-glutamine 250 mg l^{-1}

were subcultured to the very same medium and to different combinations of auxins, cytokinins and gibberellins at three to four weeks intervals for the induction of organoids or embryoids. Response of the callus was observed and recorded fortnightly.

3.6 Hardening and planting out

Plantlets germinated from zygotic embryos were taken out of the culture tubes, medium was washed off and the plantlets were planted in small mud pots filled with sterilized sand. The mud pots were kept under controlled conditions.

3.7 Biochemical attributes

Biochemical analysis was carried out for both the explants and *in vitro* cultures.

3.7.1 Total phenol

Total phenol content in various samples were determined by the method suggested by Sadasivam and Manikam (1992) and it is expressed as percentage. The details are given in Annexure I.

3.7.2 Polyphenol oxidase enzyme activity

Polyphenol oxidase enzyme assay was done by the method suggested by Malik and Singh (1980). The details are given in Annexure II. The enzyme activity and specific activity were recorded.

3.7.3 Estimation of Protein

Protein content in various samples were estimated by Lowry's (Lowry *et al.*, 1951) method and expressed in percentage. Details are given in Annexure III.

3.7.4 Protein pattern by electrophoresis

Poly Acrylamide Gel Electrophoresis (PAGE) using vertical slab gel analysis was carried out for determining the electrophoretic pattern of protein. Details are given in Annexure IV.

RESULTS

The results of the investigations on *in vitro* response and causes of recalcitrancy in coconut (*Cocos nucifera* L.) conducted during 1995-97 in the Tissue Culture Laboratory of the Department of Plantation Crops and Spices and Biochemistry Laboratory of College of Horticulture, Vellanikkara are presented in this chapter.

4.1 Culture establishment

4.1.1 Surface sterilization

Since the explants harboured a lot of micro-organisms, various surface sterilants were used to remove them. Their effects are presented in Table 7a and 7b. The inflorescence rachillae explants were effectively surface sterilized by soaking the inflorescence with the spathe intact in 0.1 per cent $HgCl_2$ for 20 minutes followed by treating the rachillae in 3.5 per cent chlorine water for 1 minute aseptically. This treatment resulted in 100 per cent survival of rachillae explants after two weeks of inoculation. In contrast, the extension of chlorine water treatment upto 3 minutes exhibited surface browning of the explants.

Sterilization was effective for endosperm cylinders and male flowers by treating them with 0.1 per cent $HgCl_2$ for 30 minutes and 2 minutes, respectively with minimum damage to the explants. Wiping the tender leaves with 70 per cent alcohol followed by soaking in 0.1 per cent $HgCl_2$ for 2 minutes was effective in avoiding contamination. Higher concentrations of sterilants caused browning of the explants eventhough cent per cent contamination was avoided.

Table 7a. Effect of surface sterilization on culture establishment of coconut inflorescence rachillae explants

Sterilant	Duration of treatment (minutes)		Cultures established 2 weeks after incubation		
	Rachillae with spathe intact (nonaseptically)	Rachillae (aseptically)	Contaminated cultures(%)	Uncontaminated cultures (%)	
				Living	Dried
$HgCl_2$	30		2	98	-
	20	7	-	--	100
	20	3	-	30	70
	20	1	-	65	35
$HgCl_2$ 0.1% + Chlorine water 3.5%	20	1	-	100	-
	20	3	-	100	-

Treatment with $HgCl_2$ nonaseptically and with chlorine water aseptically

Table 7b. Effect of surface sterilization on culture establishment of various explants of coconut

Explant	Sterilant	Duration of treatment (minutes)	Cultures established 2 weeks after incubation		
			Contaminated cultures(%)	Uncontaminated cultures (%)	
				Living	Dried
Endosperm cylinders	$HgCl_2$ 0.1%	45	--	90	10
		30	--	100	--
		20	25	75	--
		10	35	65	--
Male flowers	$HgCl_2$ 0.1%	7	--	100	--
		5	--	100	--
		2	--	100	--
		1	6	94	--
Tender leaves	Alcohol wiping (70%) - $HgCl_2$ 0.1%	2	--	100	--
		1	18	82	--
	Soaking in $HgCl_2$ 0.1%	7	--	95	5
		5	--	100	--

4.1.2 Standardisation of treatments to overcome polyphenol interference

When the explants were inoculated, it was found that the exudation of polyphenols from the explants adversely affected their survival.

4.1.2.1 Effect of pretreatments in reducing polyphenol interference in immature rachillae explants

Results of the pretreatments tried are given in Table 8. Soaking and shaking the explants with 0.5 per cent activated charcoal for 24 hours was found to be the most effective pretreatment for reducing the browning of the explants with a survival of 63.63 percentage followed by pretreatment with ascorbic acid or PVP (27.27%). Soaking and shaking with coconut water or sucrose slightly improved the survival of explants compared to control.

4.1.2.2 Effect of basal media and light on reducing polyphenol interference

Five different basal media were tested for finding out their effect on polyphenol exudation and browning. All the treatments were kept under light and dark conditions to find out the effect of light on exudation. It was found that Y_3 medium supported the lowest polyphenol exudation under dark condition with a score of 0.26 followed by MS (0.33), MS macro and Y_3 micro elements (MS + Y_3) and Blaydes (0.4) and finally B_5 (0.86). The intensity of polyphenol exudation decreased in all the cases under dark condition (Table 9a).

Enlargement of the explant tissues was studied both under light and dark conditions in different basal media supplemented with and without activated charcoal. The results are shown in Table 9b. In medium supplemented with activated

Table 8. Effect of pretreatments on reducing polyphenol interference in inflorescence rachillae explants

Basal medium	Treatment	Concentration (%)	Survival percentage of cultures	
			2 weeks after incubation	4 weeks after incubation
Y_3	Ascorbic acid	0.01	54.4	27.27
	PVP	0.1	45.4	27.27
	Coconut water	15.0	54.4	18.18
	Activated charcoal	0.5	81.8	63.63
	Sucrose	4.0	24.2	14.20
	Control		21.4	9.09

Table 9a. Effect of basal media and light in reducing polyphenol exudation from immature inflorescence rachillae explants

Medium	*Intensity of exudation (2 weeks after incubation)	
	Light	Dark
MS	0.53	0.33
Blaydes	1.00	0.40
B_5	1.33	0.86
Y_3	0.33	0.26
MS + Y_3	0.40	0.40

*Based on the score

Table 9b. Effect of activated charcoal and light in reducing polyphenol exudation from immature inflorescence rachillae explants

Medium without activated charcoal	Enlargement of explants		Medium with activated charcoal	Enlargement of explants	
	Light	Dark		Light	Dark
MS	2.33	2.4	MS	2.06	2.2
Blaydes	1.73	1.73	Blaydes	1.33	1.53
B_5	1.06	0.8	B_5	0.8	0.8
Y_3	2.53	2.66	Y_3	2.2	2.4
MS + Y_3	2.66	2.66	MS + Y_3	2.23	2.31

charcoal and under light condition, the enlargement of explant tissues was decreased. Among the various basal media tried, the tissue enlargement was almost similar in Y_3, MS + Y_3 and MS media. It was found that B_5 medium was unsuitable for growth of the rachillae tissues.

4.1.2.3 Effect of different media supplements in reducing polyphenol interference

Among the four different media supplements tried, activated charcoal was found to be the most effective in nullifying the polyphenol interference with 98.0 per cent explant survival (Table 10) followed by PVP. Addition of coconut water did not improve the survival of the tissues.

Later, all the treatments supplemented with 0.25 per cent activated charcoal were incubated under dark condition to reduce the polyphenol interference.

4.2 Direct organogenesis

Mature and immature zygotic embryos, immature rachillae and anthers were utilized to analyse their potential for organogenesis in various basal media.The mature embryos were germinated when auxins and cytokinins were supplemented to the medium and were grown into plantlets with well developed root and shoot system (Plate 1). But the immature embryos produced only shoots.

4.2.1 Effect of cytokinins

Low levels of Kinetin (0.1, 0.5 and 2.0 mg l^{-1}) were tried for direct organogenesis in various explants. But none of the treatments altered the response in zygotic embryos.

Table 10. Effect of media additives on reducing polyphenol interference in coconut inflorescence rachillae explants

Basal medium	Additives	*Intensity of browining	Survival percentage of cultures	
			2 weeks after incubation	4 weeks after incubation
Y_3 + 5 mg l^{-1} 2,4-D	Acscorbic acid 0.01%	1.93	75.0	62.0
	PVP 0.1%	1.46	84.0	72.0
	Coconut water 15%	2.86	15.0	4.0
	Activated charcoal 0.25%	-	100.0	98.0
	Control	3.73	6	Nil

- Extent of browning unable to be determined
* Based on the score

The rachillae explants remained cream coloured with the enlarged and opened flowers at lower levels of Kinetin (0.1 and 0.5 mg l^{-1}) whereas, at 2.0 mg l^{-1}, yellowish opened flowers were produced.

Anthers exhibited browning after one month of incubation.

Among the various levels of BAP tried, mature zygotic embryos produced shoots in MS medium supplemented with 1.0 and 5.0 mg l^{-1}. In Y_3 medium supplemented with 1.0 mg l^{-1} BAP, 20 per cent of the mature embryos produced seedlings with well developed roots and shoots. Maximum percentage of success was evidenced for shoot development from immature embryos at BAP 1.0 mg l^{-1}. They produced shoots in all media tried except in Blaydes medium. In Y_3 medium supplemented with 2.0, 5.0 and 7.0 mg l^{-1} BAP shoots were produced. Twenty per cent of the immature embryos produced shoots in MS medium supplemented with 5.0 mg l^{-1} BAP. BAP at 1.0 or 2.0 mg l^{-1} was found to be superior for the production of shoots eventhough it took about 75-110 days for germination.

At lower levels of BAP (1.0, 2.0 or 3.0 mg l^{-1}), the male flower buds present on the immature rachillae pieces were enlarged, thickened and opened with cream colour. Above 5.0 mg l^{-1} level, BAP exhibited severe browning and death of the explants in all the basal media.

Anthers turned brown and later dried one month after incubation in all the media tried.

4.2.2 Effect of combinations of auxins and cytokinins

Different concentrations and combinations of auxin (NAA) and cytokinins (BAP and Kinetin) were attempted for direct organogenesis in different basal media.

When NAA and BAP were combined at 0.1 and 5.0 mg l^{-1}, seedlings were produced from mature zygotic embryos in MS and Y_3 medium. Mature embryos produced seedlings in Y_3 medium (NAA 0.2 and BAP 5.0 mg l^{-1}) and in B_5 medium (NAA 0.5 and and BAP 5.0 mg l^{-1}). Immature zygotic embryos produced shoots in MS medium.

When 1.5 or 2.0 mg l^{-1} NAA and 1.0 mg l^{-1} Kinetin were added onto five different basal media, mature zygotic embryos germinated and produced *in vitro* seedlings in all the basal media. Maximum germination occurred when Y_3 medium was supplemented with NAA 2.0 and Kinetin 1.0 mg l^{-1} (Plate 2). When NAA 2.0 mg l^{-1} and Kinetin 2.0 mg l^{-1} were added, the embryo did not germinate. In all the cases the germination occurred within 50 days of incubation.

The minute, male flower buds present on the immature rachillae enlarged several times in size, opened with thick tepals in all the combinations of auxin and cytokinin tried (Plate 3).

Anthers remained fresh without any change for a period of 30 days after incubation and later they turned brown.

It was found that liquid medium was not suitable for culturing various explants of coconut. Germination of zygotic embryo was very poor when liquid

Table 11. Effect of various treatments on direct organogenesis in coconut explants

Growth regulator ($mg\ l^{-1}$)	Type of the explant	Basal medium (solid phase)				
		MS	Blaydes	B_5	Y_3	MS+Y_3
Kinetin 0.1	A	-	-	-	-	-
	B	-	-	-	-	-
	C	-	-	-	-	-
	D	-	-	-	-	-
Kinetin 0.5	A	-	-	-	-	-
	B	-	-	-	-	-
	C	-	-	-	-	-
	D	-	-	-	-	-
Kinetin 2.0	A	-	-	-	-	-
	B	-	-	-	-	-
	C	-	-	-	-	-
	D	-	-	-	-	-
BAP 1.0	A	10%(S)	-	-	20%(S+R)	-
	B	50%(S)	-	20%(S)	50%(S)	20%(S)
	C	-	-	-	-	-
	D	-	-	-	-	-
BAP 2.0	A	-	-	-	-	-
	B	-	-	50%(S)	-	-
	C	-	-	-	-	-
	D	-	-	-	-	-
BAP 3.0	A	-	-	-	-	-
	B	-	-	-	-	-
	C	-	-	-	-	-
	D	-	-	-	-	-
BAP 5.0	A	20%(S)	-	-	-	-
	B	20%(S)	-	-	10%(S)	-
	C	-	-	-	-	-
	D	-	-	-	-	-
BAP 7.0	A	-	-	-	10%(S)	-
	B	-	-	-	-	-
	C	-	-	-	-	-
	D	-	-	-	-	-

Contd.

Table 11. Continued

Growth regulator (mg l^{-1})	Type of the explant	Basal medium (solid phase)				
		MS	Blaydes	B_5	Y_3	MS+Y_3
NAA 0.1 + BAP 5.0	A	10%(S+R)	-	-	10%(S+R)	-
	B	-	-	-	-	-
	C	-	-	-	-	-
	D	-	-	-	-	-
NAA 0.2 + BAP 5.0	A	-	-	-	10%(S+R)	-
	B	-	-	-	-	-
	C	-	-	-	-	-
	D	-	-	-	-	-
NAA 0.5 + BAP 5.0	A	-	-	30%(S+R)	-	-
	B	40%(S)	-	-	-	-
	C	-	-	-	-	-
	D	-	-	-	-	-
NAA 1.5 + Kinetin 1	A	40%(S+R)	10%(S+R)	10%(S+R)	50%(S+R)	10%(S+R)
	B	-	10%(S)	-	20%(S)	-
	C	-	-	-	-	-
	D	-	-	-	-	-
NAA 2 + Kinetin 1	A	40%(S+R)	10%(S+R)	20%(S+R)	60%(S+R)	30%(S+R)
	B	-	-	-	-	-
	C	-	-	-	-	-
	D	-	-	-	-	-
NAA 2 + Kinetin 2	A	-	-	-	-	-
	B	-	-	-	-	-
	C	-	-	-	-	-
	D	-	-	-	-	-

A - Mature zygotic embryos, B - Immature zygotic embryos, C - Immature inflorescence, D - Anther, S - Shoot, R - Root, S+R - Seedling with shoot and root
- no response

Plate 1. *In vitro* seedling germinated from mature zygotic embryo in Y_3 medium

Plate 2. Complete plantlet derived from mature zygotic embryo

Plate 3. Enlargement of minute male flowers in Y_3 medium + NAA 0.1 mg l^{-1} + BAP 5.0 mg l^{-1}

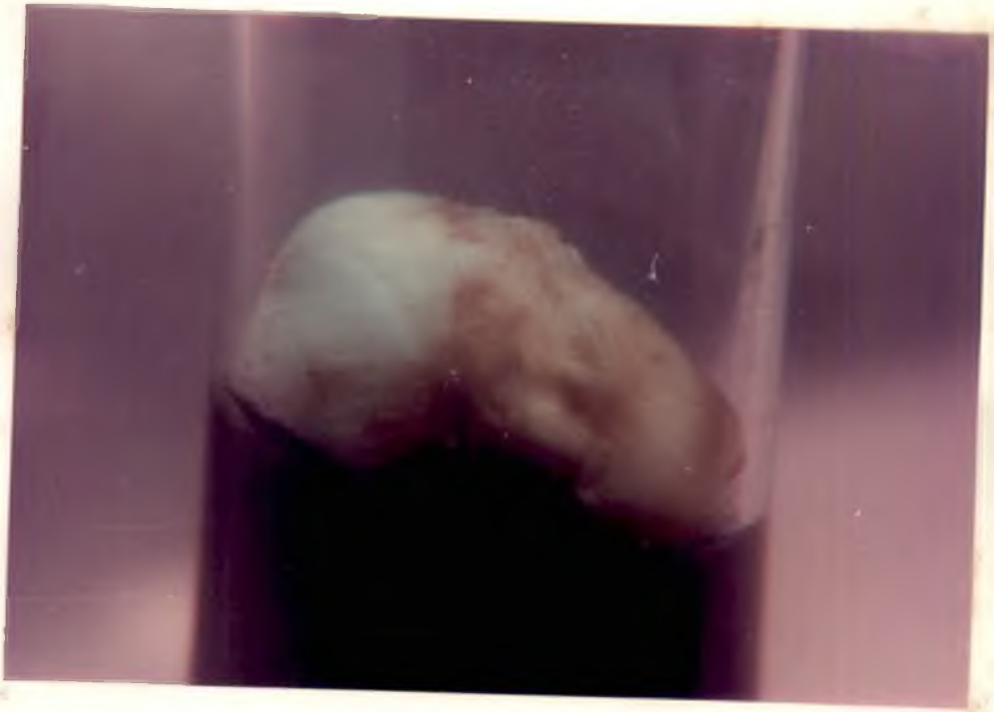
Table 12. Effect of media supplements on callus induction and proliferation in immature zygotic embryos of coconut

Medium	Concentration of 2,4-D mg l^{-1}	Additives	Callusing percentage		Callus index 120 DAI	Nature of callus
			90 DAI	120 DAI		
Y_3	33		--	5	0.75	White, compact
	44		--	35	3.5	White, compact
	55		40	60	6.0	Creamish yellow, compact
	66		--	30	6.0	Cream, friable, nodular
	77		25	50	15.0	White, friable, nodular
	80		--	50	17.5	White, friable, nodular with embryogenic potential
	100		--	50	10.0	White, compact
	33	Casein hydrolysate 200 mg l^{-1}	--	66.6	13.32	Creamish yellow, friable, nodular, embryogenic
	33	Casein hydrolysate 50 mg l^{-1}	--	20.0	4.0	Creamish yellow, nodular
	33	$AgNo_3$ 0.1%	--	--	--	--
		0.2%	--	--	--	--

DAI - Days After inoculation
- no callusing

Plate 4. Plantlet developed from zygotic embryo in Y_3 liquid medium

Plate 5. Expansion of cotyledon into a hard mass in Y_3 medium + 2,4-D 30 mg l^{-1}



Friable, nodular callus with high embryogenic potential was obtained at high concentration of 2,4-D in Y_3 medium after 120 days of incubation. Compact callus was obtained at low (33, 44, 55 mg l^{-1}) and high (100 mg l^{-1}) 2,4-D whereas, friable, nodular callus with high embryogenic potential was obtained at medium levels (66, 77, 80 mg l^{-1}) of 2,4-D. Callus index was maximum at 80 mg l^{-1} (17.5) followed by 77 mg l^{-1} (15) and 100 mg l^{-1} (10). The lowest callus index (0.75) was recorded at 33 mg l^{-1}.

Effect of additives

When 200 mg l^{-1} casein hydrolysate was incorporated into the Y_3 medium supplemented with 33 mg l^{-1} 2,4-D, creamish yellow, friable, nodular embryogenic callus was obtained (Plate 7) and when the level of casein hydrolysate was reduced to 50 mg l^{-1}, callus with the same morphology was obtained at lower rate. Addition of silver nitrate (0.1 and 0.2 per cent) did not alter the response.

From the results, it was found that friable, nodular, embryogenic callus can be produced from immature embryos using 2,4-D at higher concentration or with lower level of 2,4-D supplemented with casein hydrolysate.

4.4.1.2 Callus induction in immature rachillae

The effect of altering the concentrations of NAA at 0.1, 2.0 and 4.0 mg l^{-1} was examined in MS, B_5, Blaydes, Y_3 and MS + Y_3 media. No response was observed in MS, Blaydes, B_5 and MS + Y_3 media supplemented with 0.1 and 2.0 mg l^{-1} NAA. By contrast, in Y_3 medium, the tissues enlarged with thickened tepals. At 4.0 mg l^{-1} NAA, in all the basal media, creamish brown enlarged male flowers were obtained with slight callusing.

Plate 6. Spongy, nodular callus induced from immature zygotic embryo in B_5 medium + 30 mg l^{-1} 2,4-D

Plate 7. Friable, nodular callus induced from immature zygotic embryos in Y_3 medium supplemented with 2,4-D and casein hydrolysate

When IAA or 2iP was used in Y_3 basal medium, callusing was not observed but the rachis enlarged in size with thick, semi-opened, white male flowers.

Following a series of preliminary tests, the effect of concentrations of 2,4-D was examined in MS, B_5, Blaydes, Y_3 and MS + Y_3 media and the results are shown in Table 13. Maximum callus index of 88 was recorded in Y_3 and MS + Y_3 medium supplemented with 30.0 mg l^{-1} 2,4-D. Callusing was not obtained at 5.0 mg l^{-1} 2,4-D in all the media tried. The callus produced at various concentrations of 2,4-D was white and friable.

Since Y_3 medium was found to be superior, higher concentrations of 2,4-D were incorporated into Y_3 basal medium and the response was noticed (Table 14). The callus index was maximum when 33 mg l^{-1} 2,4-D was used (Plate 8). When 2,4-D was increased to 40 mg l^{-1}, the tissues turned brown, and 13 per cent of the cultures produced brown compact tissues without any regeneration potential (Plate 9). The results indicated that 2,4-D at 5.0 mg l^{-1} did not produce callus but the flowers were enlarged and thickened and in the higher concentrations of 2,4-D, the inflorescence tissues succumbed to deliterious effect.

After expansion of the cultured tissues, certain abnormal projections were induced from flower meristems present on the immature rachillae (Plate 10a and 10b). But further growth and development was not achieved.

When different combinations of 2,4-D and kinetin were tried in different basal media, callusing was not observed from immature rachillae explants.

Table 13. Effect of various levels of 2,4-D supplemented onto different basal media on callus induction and proliferation in immature inflorescence rachillae

Concentration of 2,4-D mg l^{-1}	Basal medium									
	Callus percentage					Callus index at 60 DAI				
	MS	B_5	Blaydes	Y_3	MS+Y_3	MS	B_5	Blaydes	Y_3	MS+Y_3
5	-	-	-	-	-	-	-	-	-	-
10	20.0	13.3	13.3	20.0	20.0	8.0	5.32	5.32	8.0	8.0
20	40.0	13.3	40.0	40.0	20.0	32.0	10.64	24.0	40.0	20.0
30	66.6	66.6	73.3	80.0	80.0	73.15	66.6	73.3	88.0	88.0

- no callusing

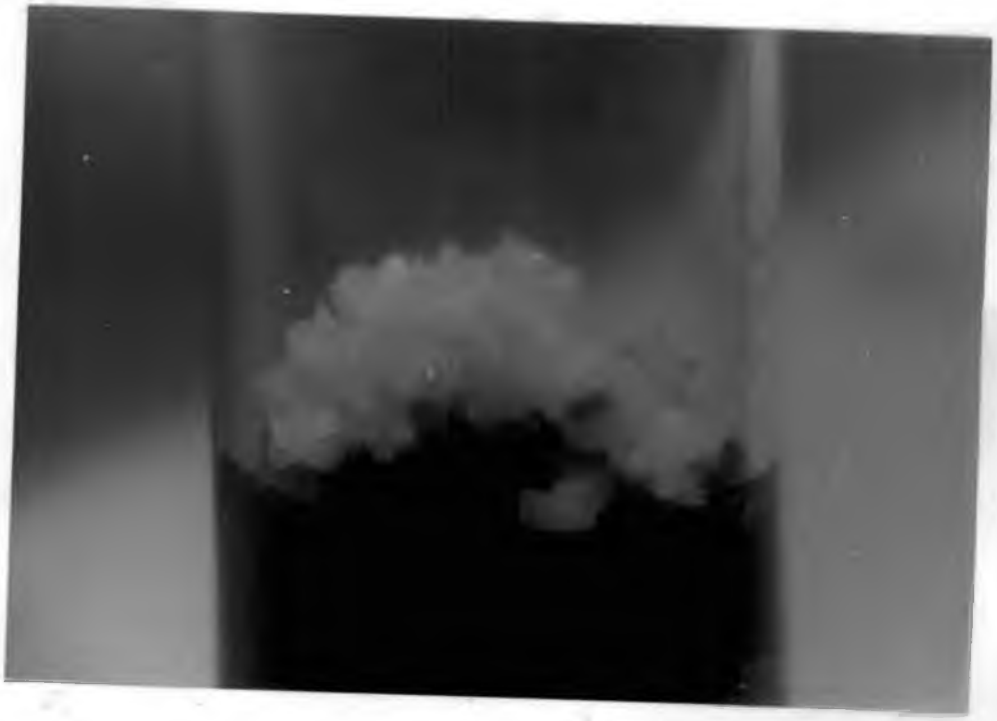
Table 14. Effect of different concentrations of 2,4-D on callus induction and proliferation in immature rachillae explants of coconut

Basal medium	Concentration mg l^{-1}	Days taken for callus initiation	Callusing (percentage)		Callus index 60 DAI	Nature of callus
			30 DAI	60 DAI		
Y_3	5	--	--	--	-	-
	11	35	--	20	8.0	White, friable
	22	35	--	40	40.0	,,
	30	26	40	80	88.0	,,
	33	26	40	80	104.0	,,
	40	30	--	13	3.9	Brown compact
	44	--	--	--	--	+
	55	--	--	--	--	+
	66	--	--	--	--	+
	77	--	--	--	--	+

- no callusing
+ turned brown and dried

Plate 8. Callus induced from immature inflorescence rachillae in medium supplemented with 2,4-D 33 mg l^{-1}

Plate 9. Brown, compact callus induced from immature inflorescence rachillae in Y_3 medium + 2,4-D 40.0 mg l^{-1}



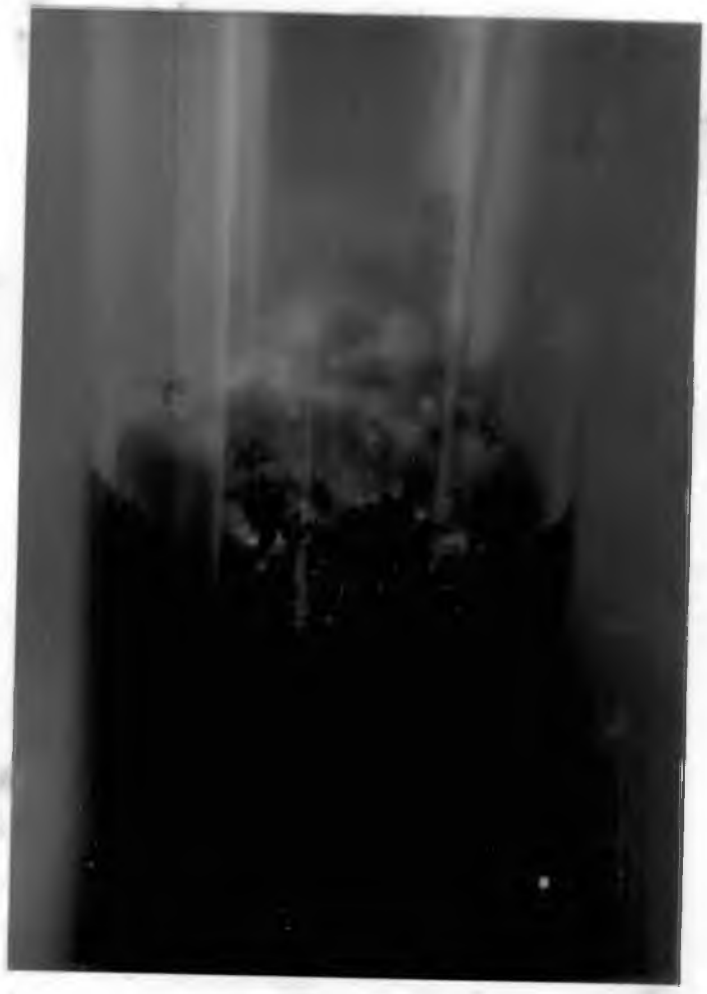
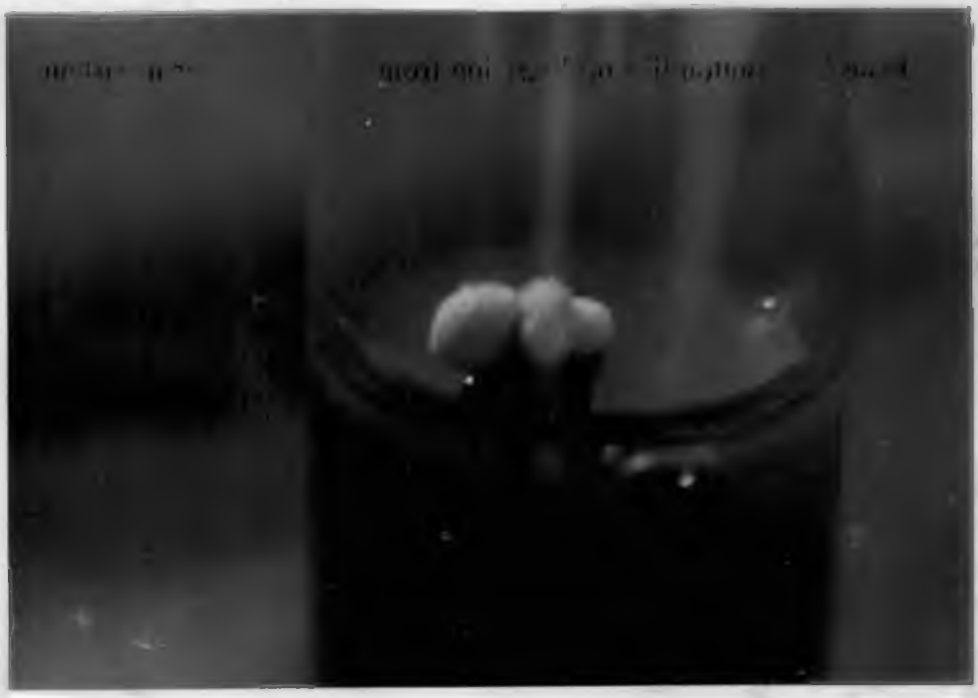


Plate 10a. Flowering type growth from a single flower meristem

Plate 10b. Button like proliferation from a single flower meristem



GA was supplemented at 1.0, 2.0 and 5.0 mg l^{-1} to Y_3 basal medium and it caused elongation of rachillae pieces and the flowers were semi opened with thickened tepals. When 200 mg l^{-1} phloroglucinol and 15 per cent v/v coconut water was added to Y_3 medium in addition to GA (1.0, 2.0 and 5.0 mg l^{-1}), the male flower buds were opened and were enlarged with thickened, cream coloured parts. Inside the thickened tepals white spongy tissue structure was produced (Plate 11).

Effect of additives

When silver nitrate (0.1%) was added to Y_3 medium supplemented with 22.0, 33.0 and 44.0 mg l^{-1} 2,4-D, brown coloured friable callus was obtained with high polyphenol exudation.

When 200 mg l^{-1} casein hydrolysate or 100 mg l^{-1} L-glutamine or 200 mg l^{-1} phloroglucinol was added in addition to 33 mg l^{-1} 2,4-D, white friable callus was obtained.

Aminoacid mixture consisted of L-glutamine, L-asparagine and L-aspartate each at the rate of 100 mg l^{-1} supplemented to the medium caused decrease in the callusing and the rachillae explants turned to yellowish green colour. When the aminoacid mixture was added with 55 mg l^{-1} 2,4-D, severe browning and death of the tissues were observed. Addition of coconut water to the medium did not alter the response.

4.4.1.3 Callus initiation in anther

Various treatments tried for callus initiation in coconut anther are shown in Table 7. When NAA 2.0 mg l^{-1} was supplemented to Blaydes medium, the

anthers swelled a little and remained fresh for 35 days but callusing was not observed. Eventhough several treatments were tried in Blaydes medium, anthers turned dark brown within 45 days of incubation. When 500 mg l^{-1} L-glutamine, 9 per cent sucrose, 15 per cent coconut water and 2.0 mg l^{-1} 2,4-D were supplemented into Blaydes medium without agar and activated charcoal, cream, friable ring like structures were produced from the pollengrains released out of the brown anthers (Plate 12).

4.4.1.4 Tender leaves

Callusing was not obtained from tender leaves eventhough different concentrations of 2,4-D and NAA were tried. At lower levels of 2,4-D and NAA, the explants remained green without any callusing. The response of leaf explants in different treatments are given in Table 16. There was no difference between the cultures kept under dark and light except that green colour developed under light.

4.4.2 Indirect organogenesis/embryogenesis from the callus

The callus obtained from zygotic embryos were subcultured to media supplemented with 2,4-D (40.0, 25.0, 10.0 mg l^{-1}) or without 2,4-D to induce somatic embryoids. Small globular structures were produced when the 2,4-D level was gradually reduced and when finally cultured to basal medium without growth regulators (Plate 13). But further development was not achieved.

Callus obtained from inflorescence and anther were subcultured to the same medium, basal medium or to media with 2 mg l^{-1} BAP. But none of the treatment combinations induced organogenesis or embryogenesis from the callus.

Table 15. Effect of auxins on callus induction and proliferation in coconut anther

Basal medium	Growth regulator	Concentration (mg l^{-1})	Days taken for callus initiation	Callusing (Percentage)	Morphology of callus
Blaydes +	NAA	1	-	-	-
Sucrose 9% +		2	-	-	-
L-glutamine 500 mg l^{-1}		3	-	-	-
+ Coconut water 15%		5	-	-	-
	2,4-D	1	-	-	-
		2	62.0	18.75	Cream, friable with ring like structures
		3	-	-	-
		5	-	-	-

- no response

Table 16. Effect of auxins on callus induction and proliferation in leaf explants of coconut

Medium	Growth regulator	Concentration mg l^{-1}	Response of cultures 3 MAI	Survival of cultures 4 MAI (%)
Y_3	2,4-D	1	Remain fresh without callusing	87.5
		5	,,	87.5
		10	,,	62.5
		20	Margins turned brown	37.5
	NAA	1	Remain fresh without callusing	100.0
		2	,,	75.0
		3	,,	75.0

MAI - Months After Inoculation

Plate 11. Proliferation of callus in Y_3 medium supplemented with phloroglucinol 200 mg l^{-1}, coconut water 15% and GA 2.0 mg l^{-1}

Plate 12. Cream, friable ring like structures released out of the cultured anthers

Plate 13. Globular proliferation on callus induced from immature zygotic embryos

Plate 14. *In vitro* seedling from zygotic embryo transferred to mud pot

4.5 Hardening and planting out

The seedlings germinated from mature zygotic embryos were planted in mud pots filled with sterile sand and were hardened (Plate 14). They survived for a period of three weeks and later all of them dried up.

4.6 Biochemical attributes

4.6.1 Total phenol content and polyphenol oxidase activity (PPO) of various explants and *in vitro* cultures (Table 17)

4.6.1.1 Explants

Inflorescence exhibited the lowest polyphenol oxidase activity per minute (0.002) and leaves the maximum (0.309). Variation in PPO activity of inflorescence, anther and embryo was negligible.

Embryo expressed the lowest (0.00213) specific activity and leaves the highest (1.0065). Variation in specific activity between inflorescence and anther was negligible.

Phenol content was maximum in anther (11.698%) and minimum in leaves (0.1698%). At the same time phenol content of anther and inflorescence (8.094%) was very high.

4.6.1.2 *In vitro* cultures

Among the *in vitro* cultures, anthers expressed the lowest PPO activity (0.001) and embryo callus the highest (0.01). PPO activity of anther, inflorescence callus and seedling was almost similar.

Table 17. Polyphenol oxidase (PPO) activity and total phenol content in various explants and *in vitro* cultures of coconut

Sample	PPO activity per minute	Specific activity	Total phenol (%)
a) Explants			
Zygotic embryo	0.006	0.00213	0.4231
Inflorescence rachillae	0.002	0.0259	8.094
Anther	0.004	0.0615	11.698
Leaves	0.309	1.0065	0.1698
b) *In vitro* cultures			
Seedling	0.004	0.0367	0.1945
Embryo callus	0.010	0.0986	0.1405
Inflorescence callus	0.002	0.0243	0.1283
Anther	0.001	0.0065	0.1248

Specific activity of polyphenol oxidase enzyme was the lowest in anther and the highest in callus of embryo.The specific activity of various cultures varied between 0.0065 to 0.0986.

Total phenol content of *in vitro* anther and inflorescence callus was in the same range and variation in total phenol in *in vitro* cultures was negligible.

Eventhough the variation of PPO activity of embryo and *in vitro* seedling was negligible, the embryo callus expressed higher PPO activity.

Specific activity of PPO enzyme of embryo was significantly different from that of *in vitro* seedlings and embryo callus. *Ex vitro* and *in vitro* inflorescence showed the same specific activity while that of anther differed significantly in *ex vitro* and *in vitro*.

In general, the phenol content of *in vitro* cultures was low and the lowest percentage was seen in inflorescence and anther.

4.6.2 Estimation of Protein

Protein content in various explants and *in vitro* cultures are presented in Table 18.

4.6.2.1 Explants

Protein content of *ex vitro* embryo, inflorescence and leaf varied significantly. The lowest protein content (0.065%) was seen in anther which was almost similar to that of inflorescence (0.077%). *Ex vitro* embryo had the highest amount of protein (2.817%) followed by leaf (0.311%).

Table 18. Protein content in various explants and *in vitro* cultures

Sample	Protein content (%)
a) Explants	
Zygotic embryo	2.817
Inflorescence rachillae	0.077
Anther	0.065
Leaves	0.311
b) *In vitro* cultures	
Seedling	0.109
Embryo callus	0.101
Inflorescence callus	0.082
Anther	0.152

4.6.2.2 *In vitro* cultures

Protein content of *in vitro* seedling, callus of inflorescence and embryo was in the same range. *In vitro* anther (0.152%) showed higher protein content when compared with *in vitro* seedling, callus of inflorescence and embryo.

Protein content in embryo decreased significantly upon germination and callusing under *in vitro* condition. Protein content of inflorescence and callus of inflorescence was almost similar but that of anther increased slightly under *in vitro* condition.

4.6.3 Protein pattern by electrophoresis

Ex vitro embryo showed two bands in electrophoresis (7.5% gel) (Plate 15). No other samples of *ex vitro* and *in vitro* expressed protein banding in 7.5 per cent polyacrylamide gel.

15 Polyacrylamide gel electrophoresis of protein

1. Zygotic embryo
2. Inflorescence rachillae
3. Anther
4. Leaves
5. *In vitro* seeling
6. Inflorescence callus
7. *In vitro* anther

DISCUSSION

Despite the development of new cultivars or hybrids and considerable area expansion in coconut, the productivity of the crop in the country remains static for the last one decade due to urbanization, biotic stress like root (wilt) disease etc. Even in the severe disease prone areas, there are palms which give very good yield and do not manifest any disease symptoms. Large scale production of propagules from such disease free elite palms for replanting in hot spot areas will be very good strategy for increasing the productivity in such areas. However, the success of such attempts are crippled due to want of a viable clonal propagation technique. In view of the problems lurking with the aseptic method of vegetative propagation in coconut, it will be worth investigating the biochemical aspects of *in vitro* recalcitrancy in this crop.

The results obtained from the present investigations on *in vitro* response and causes of recalcitrancy in coconut (*Cocos nucifera* L.) are discussed in this chapter.

5.1 Culture establishment

5.1.1 Surface sterilization

To remove the microbes present on the explants, different surface sterilization treatments were given to various explants. Explants from different parts of the plant may vary in their sensitivity to bleach solutions (George and Sherrington, 1984). Since all the explants except leaves are very delicate, sterilization was given to them with the surrounding tissues or the protective cover intact. As with most plant species, the higher percentage of explant survival reported here

depended on the duration of treatment, kind of explants and sterilants. They are furnished below.

Treatment with maximum survival	Explant	Survival percentage
External spathe nonaseptically with 0.1% mercuric chloride for 20 minutes + rachillae aseptically with chlorine water 3.5 per cent for 1 minute	Immature inflorescence	100
Mercuric chloride 0.1 per cent for 30 minutes	Endosperm cylinders	100
Mercuric chloride 0.1 per cent for 2 minutes	Male flowers	100
Alcohol wiping (70%) + Mercuric chloride 0.1 per cent for 2 minutes	Tender leaves	100

Use of mercuric chloride as surface sterilant for coconut endosperm plugs, rachillae and leaf was reported by Gupta *et al.* (1984).

5.1.2 Effect of treatments to control polyphenol exudation

Coconut explants especially the immature rachillae frequently turn brown shortly after inoculation. Such browning of tissues and medium occurs due to polyphenols which are released when tissues are disrupted. The released polyphenols become the substrate of oxidising enzymes like polyphenol oxidase and peroxidase. Polyphenols can be oxidised either by peroxidases (Mayer and Harel, 1979; Vaughn and Duke, 1984) or by polyphenol oxidases (Mayer and Harel, 1979; Hu and Wang, 1983) to quinones which are highly toxic to the explants. Therefore, coconut tissue culture requires different procedures to avoid problems that are associated with polyphenol oxidation. Same problem has been reported in coconut by several

workers like Apavatjrut and Blake (1977), Fisher and Tsai (1978), Sugimura and Salvana (1989), Areza *et al.* (1993).

Among the various pretreatments tried for reducing the polyphenol interference, soaking and shaking the rachillae pieces in activated charcoal was found to be one of the best procedures, eventhough cent per cent culture establishment was not obtained. The increased survival percentage of the explants is due to the ability of activated charcoal to adsorb inhibiting metabolites released by the plant tissues. Similar result has been reported by Fridborg and Eriksson (1975) and Pierik (1987). Pretreatment with ascorbic acid or PVP considerably increased the survival of explants. Use of ascorbic acid or PVP for soaking the explants to reduce browning has been already reported by Gupta *et al.* (1980) and D'Silva and D'Souza (1993), respectively. It is thus made clear that the polyphenol browning of explants could be refrained through the addition of additives or antioxidants. Use of coconut water or sucrose solution slightly improved the survival of explant tissues.

The intensity of polyphenol browning was highest in B_5 medium and the lowest in Y_3 medium. The lowest intensity of polyphenol browning and the highest tissue enlargement in Y_3 medium together contribute to its suitability to coconut tissue culture. According to Eeuwens (1976), presence of reduced nitrogen in addition to nitrate appears to be essential for satisfactory growth of coconut tissues under *in vitro* condition and high content of iodine and macro and micro elements may also enhance the growth of tissues. Successful reports on coconut tissue culture in Y_3 medium have been reported by Gupta *et al.* (1984), Sugimura and Salvana (1989), Kalamani and Rangasamy (1990), Areza *et al.* (1993), Verdeil *et al.* (1994). The

lowest response in B_5 medium may be due to the absence of reduced nitrogen in B_5 medium as reported by Eeuwens and Blake (1977).

The intensity of polyphenol oxidation was less under dark condition than under light. Creasy (1968), found that the activity of enzymes concerned with both biosynthesis and oxidation of phenols was increased by light.

Since the survival of tissues was low even after the pretreatments tried, various media additives were used to reduce polyphenol interference.

Among the various media additives, intensity of polyphenol browning was decreased and the survival of tissues was increased effectively by adding ascorbic acid or PVP in the media. This confirms the result obtained by Siqueira and Inoue (1991) in coconut. Similar results have been reported in many other crops by Bhaskar (1991), Sreenath *et al.* (1995) and Seneviratne and Wijesekara (1996). Among the treatments, addition of activated charcoal to the media resulted in 98.0 per cent survival of the explants after four weeks of incubation. The key role of activated charcoal in minimizing the browning has been reported in coconut by Sugimura and Salvana (1989) and Areza *et al.* (1993) and in date palm by Tisserat (1979) and Sharma *et al.* (1980).

5.2 Direct organogenesis

Mature and immature zygotic embryos, immature rachillae and anthers were used in various basal media for organogenesis.

5.2.1 Effect of cytokinins

Incorporation of BAP produced single shoot from mature and immature

zygotic embryos. BAP at 1.0 mg l^{-1} was found to be superior to kinetin for shoot production from the embryos. Cytokinin is important to the growth of shoot tissue and BAP is the most promotive cytokinin (Eeuwens and Blake, 1977). The effect of cytokinin may vary according to the compound used, the type of cultures and the plant species from which it was derived (George and Sherrington, 1984).

The minute male flower meristems present on the immature rachillae were enlarged in size and opened with thickened tepals. BAP above 5.0 mg l^{-1} resulted in browning of the rachillae explants. Similar result has been obtained in coconut by Ebert (1993) also. Cytokinins are known to stimulate the synthesis of polyphenols (Bergmann, 1964; Asahira and Nitsch, 1969).

Anthers turned brown without any response. Similarly Ebert (1993) reported that after two weeks from initial culture, most anthers turned brown.

5.2.2 Effect of auxins and cytokinins

Mature zygotic embryos produced plantlets with good shoot and root systems in media supplemented with low NAA and high BAP whereas immature embryos did not produce roots.

When NAA and kinetin were added to the medium, higher percentage (60%) of plantlets were produced with good shoot and root systems from mature zygotic embryos. Germination followed the usual *in vivo* pattern, the widening of cotyledonary sheath and the emergence of plumule being followed by the emergence of radicle. Sixty percentage of the mature embryos germinated when 2.0 mg l^{-1} NAA and 1.0 mg l^{-1} kinetin were added to the Y_3 medium. When the concentration of NAA was altered to 1.5 mg l^{-1}, the percentage of germination was decreased to

50.0 and when the concentration of kinetin was increased to 2.0 mg l^{-1}, none of the embryos germinated. The result showed that proper balance between auxin and cytokinin is necessary for successful plantlet production. This is in support of the view of George and Sherrington (1984) that balance between auxin and cytokinin is most often required for the formation of shoot and root meristems and the requisite concentrations of each type of regulant differ greatly according to the kind of plant being cultured, cultural conditions and the compound used. Successful zygotic embryo culture in coconut has been reported using Y_3 medium supplemented with NAA 1.5 mg l^{-1}, kinetin 1.0 mg l^{-1} and activated charcoal 0.1 per cent (Kalamani and Rangasamy, 1990).

The minute, male flower buds on the rachillae were opened and enlarged but anthers turned brown after one month of inoculation.

5.2.3 Effect of liquid media

It was observed that germination of zygotic embryos was very poor and the germinated ones remained stunted in growth when liquid medium was used. Assy-Bah (1986) obtained plantlets from embryos only in the presence of agar and activated charcoal. In contrast, successful plantlet production from zygotic embryos in liquid phase has been reported by Tahardi and Warga-Dalem (1982), Karunaratne *et al.* (1985) and Ashburner *et al.* (1991).

The immature rachillae showed no sign of growth and turned brown. In contrast, Blake and Eeuwens (1982) reported that unshaken liquid medium was better than shaken or agar medium for inducing coconut flower meristems to grow into 'shootlets'. Anthers in liquid medium remained fresh for longer time compared to that in solid medium. Similar reports are there by D'Souza and Mallya (1993).

Direct organogenesis has not been achieved eventhough various treatments were tried for different explants. Reports are not available on the differentiation of shoot buds directly in coconut tissue culture. Rao and Ganapathi have also commented the same in 1993.

5.3 Callus induction and proliferation

5.3.1 Immature zygotic embryos

Lower levels of NAA and 2,4-D were inefficient for inducing callus from mature zygotic embryos. Higher concentration of 2,4-D was required to initiate nodular embryogenic callus because the availability of growth regulators is decreased by the addition of activated charcoal in the medium. Paranjothy and Othman (1982) had to use 10 times the level of auxin that would otherwise have been effective to initiate callus of oil palm in the presence of activated charcoal.

At high level of 2,4-D, friable, nodular growth was obtained whereas at medium level, compact callus was obtained. Karunaratne and Periyapperuma (1989) reported that only very low levels (10-20 μM) of 2,4-D was required for callus initiation.

Addition of casein hydrolysate 200 mg l^{-1} considerably reduced the concentration of 2,4-D required to initiate and proliferate friable, nodular callus. This may be due to some unknown stimulatory factor supplied by casein hydrolysate as reported by Inoue and Maeda (1982). Addition of silver nitrate 0.1 and 0.2 per cent did not give favourable response. Joseph (1997) observed rhizogenesis from callus of *Kaempferia galanga* when silver nitrate was supplemented at higher concentration.

5.3.2 Immature rachillae

Addition of NAA and IAA at various concentrations did not initiate callusing in rachillae but the tissues were enlarged and thickened. Further development was not at all observed.

Higher concentrations of 2,4-D was required for inducing callus from rachillae explants. Among the different basal media, callus index was maximum when 30 mg l^{-1} 2,4-D was supplemented to Y_3 and MS + Y_3 medium. Eeuwens (1976) obtained growth of coconut tissues on MS + Y_3 medium equal to that on Y_3 medium.

It was found that callus index was maximum when 33.0 mg l^{-1} of 2,4-D was supplemented in Y_3 medium. Blake and Eeuwens (1982) reported that 2,4-D was the most effective in producing callus, IAA and IBA were generally ineffective and NAA sometimes induced callus or roots.

After expansion of the cultured tissues, instead of callus induction, certain abnormal proliferations were induced. This asynchronous development was early noticed in coconut by Eeuwens and Blake (1977), Branton and Blake (1983) and Sugimura and Salvana (1989) and the different response may depend on the developmental stages of the undifferentiated male flowers at different parts of the rachillae.

Increasing the concentrations of 2,4-D above 33.0 mg l^{-1} caused severe browning and due to high polyphenol oxidase activity drying of the tissues indicating their sensitivity to 2,4-D. According to Verdiel *et al.* (1989), coconut tissue is

highly sensitive to chlorinated auxins which are essential for callus formation. This sensitivity is revealed by intense browning leading to explant necrosis.

2iP or combination of 2,4-D and kinetin were found to be ineffective to initiate or proliferate callusing.

The male flower meristems present on the rachillae were enlarged and elongated when GA was supplemented. Addition of coconut water and phloroglucinol enhanced the growth of flower meristems.

Addition of silver nitrate with various levels of 2,4-D produced brown, friable callus with increased polyphenol oxidation. Addition of organic nitrogen supplements like casein hydrolysate or L-glutamine enhanced the callus proliferation. Smith and Thomas (1973) and Eeuwens (1978) suggested that organic nitrogen supplements stimulated growth of cultured palm tissues. Phloroglucinol, an auxin synergist, also enhanced the growth of tissues. However, addition of the amino acid mixture (L-glutamine, L-aspartate and L-asparagine) was found to be unsuitable for callus proliferation. The result is contradictory to that reported by Eeuwens (1978).

5.3.3 Anther

Blaydes medium was found to be the best for coconut anther culture. Similar result has been obtained by Thanh-Tuyen and De Guzman (1983). Callusing from microspores was noticed only in the presence of additives like L-glutamine, coconut water, 2,4-D and increased concentration of sucrose. Addition of amino acids such as glutamine, proline and serine considerably increased the percentage of embryonic anthers (Sangwan, 1983). Repeatability of microspore callusing was not observed.

5.3.4 Tender leaves

Callusing was not observed eventhough various treatments were tried. Callogenesis from leaf explants has been reported by Verdeil *et al.* (1989). Possible variations in the physiological maturity of different regions of the spindle leaf tissues may cause variation in response of tissues *in vitro* (Iyer, 1993).

5.4 Indirect organogenesis/embryogenesis

Development of organoids did not occur from the callus produced from various explants eventhough some successful reports on coconut organogenesis are there (Gupta *et al.*, 1984; Bhalla-Sarin *et al.*, 1986).

Friable callus with embryogenic potential obtained from immature zygotic embryos were subcultured to media supplemented with lower levels of 2,4-D or without 2,4-D. Globular structures were produced but further development was not achieved. Callus from rachillae turned brown after 2-3 subcultures and regeneration became impossible. Callus from microspores continued to proliferate.

5.5 Biochemical attributes

A knowledge on biochemical attributes is indeed warranted for an effective breakthrough in breaking the recalcitrant nature of coconut for *in vitro* mass multiplication response.

Coconut palm contains phenolic compounds abundantly. The content varies with plant parts and age of the plant. There is a significant difference in the phenolic (tanin) content of different plant parts in some apricot varieties. This is in

agreement with the report of Misirly *et al.* (1995). Growth inhibition is mostly severe in species that naturally contain high levels of tannin or other hydroxy phenols. In the natural state, phenols play an important role in plants, the hormone balance compartmentalization, disease resistance and protection of injured tissues from infection (Crompton and Preece, 1986). Coconut varieties which are susceptible to root (wilt) contain high amount of phenols (Joseph and Jayasankar, 1974; CPCRI, 1975). Total phenol and orthohydroxy phenols are more in coconut palms affected by Thanjavore wilt also (Suriachandraselvan *et al.*, 1993).

In the present study, it is observed that 0.25 per cent activated charcoal was insufficient to control the inhibition by secondary products for anther and inflorescence rachillae, at the same time, browning of anther and rachillae explants occurred one month after incubation and after 2-3 subcultures, respectively and regeneration became very difficult. The phenols and oxidizing enzymes of intact tissues are apparently in separate pools or in compartments within cells and upon wounding, they are released and the enzyme process occurs. Similarly, Vaughn *et al.* (1988) reported that polyphenol oxidase is a plastid enzyme which is in inactive form and gets activated only when released into the cytoplasm following cell injury. Irreparable growth inhibition occurs when phenols are oxidised to highly active quinone compounds which then cyclise, polymerise and oxidise proteins to form increasingly melanic compounds (George and Sherrington, 1984).

Germination as well as callusing of zygotic embryos of coconut palm was observed when there was high protein, medium PPO activity with a specific activity of 0.00213 and low phenol content. Eventhough inflorescence rachillae had good callus formation, after 2-3 subcultures, browning effect was observed due to

PPO activity with very low protein content. Tender leaves with high PPO activity and low phenol and medium protein did not exhibit any response of regeneration. They remained green due to the presence of high chlorophyll content and masking of the colour of oxidised products.

High PPO activity, medium phenol and protein content in embryo callus inhibited the regeneration due to the production of highly toxic quinones, eventhough embryogenic callus was produced. Browning effect was not observed because of the high PPO activity. Due to low PPO activity, phenol and protein, regeneration from inflorescence callus did not occur. Very low content of PPO activity, total phenol and high content of protein were observed in anther under *in vitro* conditions. Regeneration was not observed due to phenolic interference.

From the study it was found that a proper balance between PPO activity, phenol and protein content are very essential for regeneration and further development. Very high and low activity of PPO was inhibitory for regeneration. Low PPO activity and high phenol content as observed in inflorescence rachillae and anther and high PPO activity and low phenol as observed in leaf explants; both the conditions may not give a favourable result. In this case, if the protein content is high, regeneration may occur because of the presence of protein in the development of plants. Just the reverse case may also happen when proteins of inhibitory nature occur in the plant cells.

Presence of two bands in 7.5 per cent gel may be an indication of the presence of proteins ranging from 30 to 50 thousand Daltons in *ex vitro* embryo. These proteins may have an important role in the germination and callusing of zygotic embryos of coconut palm. Other explants and *in vitro* cultures did not express protein banding in 7.5 per cent gel and there was no regeneration in the absence of the above proteins.

5.6 Hardening and planting out

Seedlings germinated from zygotic embryos were hardened under controlled conditions. Unfortunately, none of them survived beyond three weeks of planting out. Similar result has been obtained by Assy-Bah (1986). But later Assy-Bah *et al.* (1989) could successfully plant out them.

5.7 Constraints in achieving repeatable results in coconut tissue culture

Eventhough work on coconut tissue had been conducted for more than three decades in various laboratories using different tissue explants, sporadic and hardly any repeatable success has been reported. Palm tissue culture is far more difficult than other tissue culture work and the callus growth from explants of palmae is slow compared to other species, facts previously noted by Staritsky (1970) and Reuveni and Kipinis (1974), respectively.

Palm to palm differences in genotypic responses, geographical location, maturity and nutrition of mother palms, physiological maturity of explants and

polyphenol exudation are the critical factors to be understood for successful coconut tissue culture. Although the addition of activated charcoal to the culture medium proved effective for controlling the polyphenol oxidation, there is no clear understanding of its function or its effect on the availability of other media components. Somatic embryogenesis is the highest when meristematic activity of the explant is at its peak. Unfortunately, in the case of coconut leaf and embryo, this lasts only for a few days and so material available for experimentation is scarce (Karunaratne, 1993). Various factors influencing callus initiation, multiplication, development and regeneration under controlled conditions have to be standardized yet. If successful and reproducible protocols are established, coconut palms with outstanding traits could be propagated and the outlook is bright.

SUMMARY

The present investigations on *in vitro* response and causes of recalcitrancy in coconut (*Cocos nucifera* L.) were carried out during the period 1995 to 1997 in the Tissue Culture Laboratory of the Department of Plantation Crops and Spices and Biochemistry Laboratory, College of Horticulture, Vellanikkara. The salient findings of the study are summarised in this chapter.

1. Among the different surface sterilization treatments tried, the best treatment varied with respect to the type of explant.

2. Soaking the immature inflorescence with the spathe intact in 0.1 per cent mercuric chloride for 20 minutes followed by treating the rachillae without the spathe in 3.5 per cent chlorine water for one minute was found to be the most effective surface sterilization treatment.

3. Endosperm cylinders and male flowers were surface sterilized by soaking them in 0.1 per cent mercuric chloride for 30 minutes and 2 minutes respectively. For tender leaves, wiping them with 70 per cent alcohol followed by 2 minutes treatment with 0.1 per cent mercuric chloride was found to be the best sterilization technique.

4 Culture establishment of coconut explants faced the problem of polyphenol interference.

5. Soaking and shaking the immature rachillae with 0.5 per cent activated charcoal for 24 hours was found to be the best pretreatment for reducing phenolic exudation eventhough cent per cent survival of tissues was not achieved.

6. Among the different media additives tried, activated charcoal 0.25 per cent gave 98 per cent survival of explants after four weeks of incubation.

7. Addition of ascorbic acid or polyvinyl pyrrolidone was found inferior to activated charcoal.

8. Explants cultured in Y_3 medium exhibited the least polyphenol exudation while those in B_5 medium exhibited the highest exudation.

9. Keeping the cultures under dark condition reduced the polyphenol oxidation.

10. Y_3 medium supplemented with 2.0 mg l^{-1} NAA, 1.0 mg l^{-1} kinetin and 0.25 per cent activated charcoal was found to be the best for germination of zygotic embryos.

11. Maximum number of single shoots from zygotic embryos were produced when 1.0 mg l^{-1} BAP was incorporated to Y_3 medium.

12. Higher concentration of BAP caused polyphenol exudation and browning in inflorescence rachillae explants.

13. Liquid medium was found unsuitable for embryo culture and growth of inflorescence rachillae explants but found suitable for anther culture.

14. Direct organogenesis could not be achieved from various explant sources with various treatment combinations tried.

15. 2,4-D was found to be the most effective auxin for callus induction and proliferation in various explants.

16. 2,4-D and NAA at lower levels did not produce callus in zygotic embryos.

17. Higher concentrations of 2,4-D are required to initiate callusing in zygotic embryos.

18. Addition of casein hydrolysate 200 mg l^{-1} reduced the concentration of 2,4-D required to initiate callusing.

19. Addition of silver nitrate 0.1 or 0.2 per cent did not favour callus induction and proliferation.

20. High concentration of 2,4-D was required to produce white, friable callus from inflorescence. However, 2,4-D above 33.0 mg l^{-1} caused drying of the explant tissues.

21. Several abnormal structures were produced from rachillae explants due to the difference in the developmental stages of flower meristems.

22. Gibberellic acid enhanced the growth and enlargement of flower meristems but did not express any effect on callus initiation.

23. Addition of amino acid mixture (L-glutamine, L-aspartate and L-asparagine 100 mg l^{-1} each) did not favour callus initiation and proliferation.

24. Blaydes medium was found suitable for anther culture.

25. Microspores produced cream friable callus with ring like structures in Blaydes medium supplemented with 500 mg l^{-1} L-glutamine, 15 per cent coconut water, 2 mg l^{-1} 2,4-D and 9 per cent sucrose.

26. Leaves failed to induce callus or embryoids in any one of the media combinations tried.

27. Indirect organogenesis or somatic embryogenesis could not be obtained with various treatment combinations tried.

28. Planting out of seedlings germinated from zygotic embryo was not successful.

29. Content of total phenol, polyphenol oxidase activity and protein were different in different explants and *in vitro* cultures.

30. Protein bands obtained by PAGE showed that the zygotic embryo contained proteins with molecular weight ranging from 30 to 50 thousand Daltons. These proteins may have an important role in the regeneration process.

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* Originals not seen

Appendices

ANNEXURE I

Estimation of total phenol

Weighed exactly one gram sample and ground it with a pestle and mortar in 10 ml methanol. Centrifuged the homogenate at 10,000 rpm for 20 minutes and saved the supernatent. Re-extracted the residue with 5 ml methanol and centrifuged for 10 minutes. Pooled the supernatent and again centrifuged for 10 minutes using 5 ml methanol and collected the supernatent and evaporated it to dryness. Dissolved the residue in 5 ml distilled water. Pipetted out one ml sample into 50 ml volumetric flask and made up the volume. From it one ml was pipetted out and two ml distilled water was added. Added 0.5 ml Folin Ciocalteu's phenol reagent and after 3 minutes two ml of 20 per cent sodium carbonate solution was added and mixed thoroughly. Placed the tube in boiling water for exactly one minute, cooled and measured the absorbance at 650 nm against a reagent blank.

A standard curve was prepared using different concentrations of catechol. From the standard curve, the concentration of phenols in the test sample was calculated and expressed as percentage.

ANNEXURE II

Polyphenol oxidase activity

assayed by the method suggested by Malik and Singh (1980)

Protein extraction buffer

Tris buffer	- 21.1995 g
Citric acid	- 2.6267 g
Vitamin C	- 0.5283 g
Cystein HCl	- 0.5268 g
Water to	- 200 ml
pH	- 7.0

Buffer for assay

Monobasic sodium phosphate solution 0.2 M 87.8 ml

Dibasic sodium phosphate solution 0.2 M 12.3 ml

Water to 200 ml; pH 6.0

Substrate

Buffer 100 ml

Catechol 0.01 M (0.11 g/100 ml)

Preparation of the sample

The plant parts collected in ice buckets were washed with distilled water and removed the water using a blotting paper. The *in vitro* cultures were also cleaned well and blotted the water. Extracted one gm of tissue in 5.0 ml of

extraction buffer by grinding in a precooled mortar and pestle. The homogenised material was centrifuged at 18000 rpm for 15 minutes at 5°C. The supernatents were used as enzyme source.

Procedure

To a clean cuvette of a spectrophotometer 3.0 ml buffered catechol solution was added. The photometer was set to read absorbance at 495 nm. The cuvette was inserted in the cuvette chamber of the spectrophotometer and the absorbance was adjusted to zero. Added one ml enzyme extract to the cuvette, mixed immediately, placed the cuvette in the chamber and recorded the changes in absorbance for every 30 seconds upto 15 minutes.

Calculation

Plotted the increasing absorbance values and from the linear phase of the curve, the increase in optical activity per minute was read. Specific activity of the enzyme was also determined.

$$\text{Specific activity at } 30^{\circ}\text{C for 1 minute} = \frac{\text{Enzyme activity per minute}}{\text{Protein (mg)}}$$

ANNEXURE III

Protein estimation by Lowry's method

Reagent A

Sodium carbonate 2 per cent in 0.1 N sodium hydroxide.

Reagent B

Copper sulphate ($CuSO_4.5\ H_2O$) 0.5 per cent in 1 per cent potassium sodium tartarate.

Reagent C

Reagent A 50 ml and Reagent B 1 ml was mixed prior to use.

Reagent D

Folin Ciocalteu's phenol reagent was diluted to 1:2 reagent : water.

Stock standard

Weighed accurately 50 mg of Bovine serum albumin (Fraction V) and dissolved in 0.1 N NaOH and made upto 50 ml.

Working standard

Diluted 10 ml of the stock solution to 50 ml with 0.1 N NaOH in a standard flask and 1 ml of this solution contains 200 μg protein.

Procedure

Three ml of the supernatent used for the polyphenol oxidase enzyme assay was used for the estimation of protein. To this supernatent 3 ml of 10 per cent Trichloro Acetic acid (TCA) was added. Centrifuged for 20 minutes and poured out the supernatent. To the protein which is precipitated down, 5 ml of 0.1 N NaOH was added to dissolve the protein and this solution was used for estimating the protein.

Pipetted out 0.2, 0.4, 0.6, 0.8 and 1.0 ml of working standard into a series of test tubes. Pipetted out 1 ml of the sample extracts in each tube. Made up the volume to 1 ml in all the test tubes and a tube with 1 ml of distilled water served as blank. Added 5 ml of reagent C to each tube including the blank. Mixed well and were allowed to stand for 10 minutes. Reagent D 0.5 ml was added and mixed well and were incubated at room temperature in the dark for 30 minutes. Blue colour was developed. The reading was taken at 660 nm using a uv-vis spectrophotometer. A standard graph was drawn and calculated the amount of protein in the sample. Expressed the protein content in various samples as percentage.

ANNEXURE IV

Protein pattern by electrophoresis

Poly Acrylamide Gel Electrophoresis (PAGE) using vertical slab gel was carried out for electrophoretic pattern of proteins.

Preparation of the gel

The following stock solutions were prepared

Solution A		Solution B	
Tris buffer	38.3 g	Acrylamide	30.0 g
TEMED	0.46 ml	Bisacrylamide	0.9 g
IN HCl	48 ml	Distilled water to	100 ml
Distilled water to	200 ml		
pH	9.0		

Solution A and B were stored in amber coloured bottles at 0-4°C.

Solution C

Ammonium per sulphate 0.14 g

Distilled water to 100 ml. It is prepared freshly.

Electrode buffer solution

Tris buffer	6 g
Glycine	28.8 g
Distilled water to	1000 ml
pH	8.3

Extraction buffer (same as protein extraction buffer)

Preparation of gel column

Stock A, B and C were pipetted in the ratio 1:1:2, mixed well and was gently injected by a syringe in between glass plates kept in polymerisation stand. Pushed the combs in for making wells and allowed the polymerisation.

After polymerisation, the gels were transferred to electrophoretic apparatus. The upper and lower tanks were filled with precooled electrode buffer. Removed the combs carefully and 20 μl of sample was applied to each well with a transfer pipette. Upper tank was connected to the cathode and the lower one to the anode. The analysis of protein was carried out in anionic system. Bromophenol (0.002%) was added to the upper tank as tracer dye. Electrophoresis was carried at 5°C for 5 hours by using a cooling system. Adjusted the current at 25 mA per slab and kept constant throughout the running.

Preparation of the sample

Freshly collected plant parts and *in vitro* cultures were washed with distilled water and the adhering water was removed using a blotting paper.

Chilled mortar and pestle was used for extraction. One gram sample and 5 ml extraction buffer were used. To chelate the polyphenols, a pinch of insoluble PVP was added. The homogenised material was centrifuged at 15000rpm for 15 minutes in a refrigerated centrifuge at 5°C. After centrifugation, the clear supernatent was collected.

Staining of gels

Immersed the gel in 0.01% Coomassie Brilliant Blue R 250 in 10 per cent TCA for 4 hours. Glacial acetic acid 7 per cent at 50-60°C was used for destaining. Repeated the destaining till clear bands were obtained.

IN VITRO RESPONSE AND CAUSES OF RECALCITRANCY IN COCONUT (*Cocos nucifera* L.)

By

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ABSTRACT OF THE THESIS

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ABSTRACT

A study was undertaken in the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara, during 1995 to 1997 to find out the *in vitro* response and causes of recalcitrancy in coconut (*Cocos nucifera* L.). Young palms of D x T parentage were used as source of explants in the present study. Direct and indirect organogenesis and embryogenesis were tried in various explants like zygotic embryo, immature inflorescence rachillae, anther and tender leaves.

Coconut tissues usually turn brown under *in vitro* condition due to polyphenol interference. Addition of 0.25 per cent activated charcoal reduced the phenolic exudation effectively and was found superior to the addition of ascorbic acid or polyvinyl pyrrolidone. The phenolic exudation was considerably reduced under dark condition.

Y_3 medium was found to be superior for zygotic embryo culture and growth of inflorescence tissues whereas, Blaydes medium was found suitable for anther culture. Y_3 medium supplemented with 2.0 mg l^{-1} NAA and 1.0 mg l^{-1} kinetin was found the best for zygotic embryo culture. Immature zygotic embryos produced friable, nodular callus with higher embryogenic potential. White globular structures were produced when concentration of 2,4-D was gradually reduced but regeneration was not obtained. Immature inflorescence rachillae explants produced friable callus when high levels of 2,4-D was supplemented. Callusing was observed from microspores when L-glutamine 500 mg l^{-1}, 2,4-D 2 mg l^{-1}, coconut water 15 per cent v/v and 9 per cent sucrose were added to the Blaydes medium. But repeat-ability of callus induction was not obtained.

Total phenol content, polyphenol oxidase activity and protein content were different in different plant parts and these factors indirectly affected the response of tissues under *in vitro* condition.

Coconut is admittedly a recalcitrant material as far as tissue culture is concerned. Hence much more concerted efforts are required to develop a viable protocol for the micropropagation of coconut *(Cocos nucifera)* L. Nevertheless, the above mentioned observations on biochemical attributes points, in part, to the possible factors related to the recalcitrant behaviour of coconut *in vitro*.