RESPONSE OF IMMATURE INFLORESCENCE FOR *IN VITRO* REGENERATION IN COCONUT (*Cocos nucifera* L.)

By
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THESIS

Submitted in partial fulfilment of the requirement for the degree of

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(Plant Biotechnology)

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2006

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DECLARATION

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

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Certified that this thesis entitled **"Response of immature inflorescence for *in vitro* regeneration in coconut (*Cocos nucifera* L.)"** is a record of research work done independently by **Mrs. Siny C.V.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

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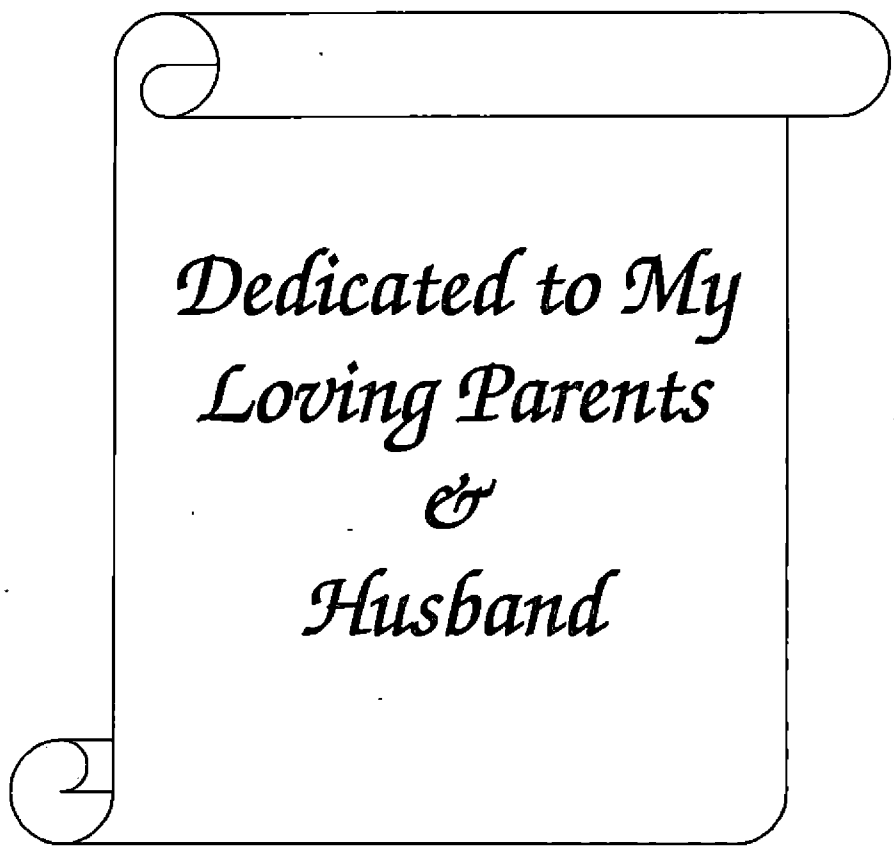

Dedicated to My
Loving Parents
&
Husband

ABBREVIATIONS

BA	Benzyl Adenine
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
GA	Gibberellic acid
TDZ	Thidiazuron
2iP	2 isopentinyl adenine
Kin	Kinetin
2,4-D	2,4 Dichlorophenoxy acetic acid
NAA	Naphthalein Acetic Acid
AC	Activated Charcoal
h	hour
min	minutes
MS	Murashige and Skoog's medium
ppm	Parts per million
%	Per cent
^{0}c	Degree celcius
$g\ l^{-1}$	Grams per litre
mgl^{-1}	Milligram per litre
µM	Micro molar
ml	Milli litre
HCl	Hydrochloric acid
psi	Pounds per square inch
M	Molar
mm	Milli metre
NaOH	Sodium hydroxide
cm	Centi metre
p^{H}	Hydrogen ion concentration

TABLE OF CONTENTS

Chapter	Title	Page. No.
1	INTRODUCTION	1-2
2	REVIEW OF LITERATURE	3-20
3	MATERIALS AND METHODS	21-36
4	RESULTS	37-80
5	DISCUSSION	81-96
6	SUMMARY	97-99
	REFERENCES	
	APPENDICES	
	ABSTRACT	

LIST OF TABLES

Table No.	Title	Page No.
1.	Composition of various basal media tried for *in vitro* culture of coconut	23
2.	Surface sterilization treatments tried for the inflorescence explants of coconut (*Cocos nucifera* L.)	29
3.	Pre-treatments tried to overcome polyphenol interference	30
4.	Growth regulator combinations tried for direct organogenesis in coconut explants	32
5.	Growth regulator combinations tried for callus induction in coconut	33
6.	Media additives tried for callus induction and proliferation	34
7.	Growth regulator combinations tried for callus regeneration	34
8.	Effect of surface sterilization on culture establishment of different explants in coconut (3 weeks after inoculation)	38
9.	Response of explants in different basal media in culture establishment of coconut	43
10.	Effect of different basal media in browning of explants	44
11.	Effect of pretreatments on reducing polyphenol interference	47
12.	Effect of different concentrations of activated charcoal in reducing browning of explant	47
13.	Effect of different media supplements in reducing browning of inflorescence explants	48
14.	Response of different combinations of auxins and cytokinins for direct organogenesis	51

Sl.No.	Title	Page No.
15a	Effect of 2,4-D on callus induction and browning of rachillae and immature anther explants	53
15b.	Effect of 2,4-D on callus induction and browning of male and female flowers of coconut	54
16.	Effect of 2,4-D along with other auxins (Picloram, NAA, IAA)	60
17.	Effect of 2,4-D along with TDZ and 2iP	62
18.	Effect of different concentrations of picloram and TDZ in callus induction of coconut inflorescence explant	65
19.	Combinations and concentrations of growth regulators giving response to coconut inflorescence explants	68
20.	Effect of different levels of sucrose on frequency of callus induction of anthers	69
21	Effect of different media additives on callus induction of immature anther	70
22	Effect of chilling treatment on callus induction and survival percentage of immature anther	73
23	Influence of varying concentrations of gelling agents on anther swelling	74
24	Subculture response of somatic embryo of *Coconut nucifera* in various media combinations	77

LIST OF PLATES

Plate No	Title	Page No.
1	Inflorescence of different size from unopened leaf axil used as explant source for *in vitro* culturing.	26
2	Inflorescence of coconut containing anthers at pre meiotic stage.	39
3	Rachillae of coconut carrying minute male flowers containing anthers at pre meiotic stage.	39
4	Pollen mother cells of coconut	40
5	Dicolouration of activated charcoal incorporated media due to polyphenol exudation.	45
6	Male flower buds at the time of inoculation & enlargement and opening of male flower buds	50
7	Response of inflorescence explants in Y_3 media containing BA.	50
8	Callus initiation from immature anther in Y_3 medium containing 2,4-D18 mgl^{-1} and Picloram 0.5 mgl^{-1}	55
9	Callus induction and embryiod formation from immature anther in Y_3 medium containing 2,4-D15 mgl^{-1}, Picloram 0.5 mgl^{-1} and NAA I mgl^{-1}.	56
10	Callus initiation from rachillae in Y_3 medium containing 2,4-D15 mgl^{-1}, Picloram 1 mgl^{-1} and IAA I mgl^{-1}.	57
11	Callusing of female flower in MS medium supplemented with 2,4-D, Picloram and NAA	58
12	Formation of anther like structure in Y_3 medium containing 2,4-D15 mgl^{-1}, IAA I mgl^{-1} and NAA 1 mgl^{-1}.	59
13	Development of inflorescence primordia in Y_3 medium containing 2,4-D 20 mgl^{-1} and TDZ 0.075 mgl^{-1} .	63

14	Callus induction from immature anther in Y_3 medium containing 2,4-D15 mgl^{-1}, Picloram 0.5 mgl^{-1} and TDZ0.1mgl^{-1}.	64
15	Callus induction from rachillae in Y_3 medium containing 2,4-D15 mgl^{-1}, Picloram 1 mgl^{-1} and TDZ 0.1 mgl^{-1}.	64
16	Pollen grain detected from the anther like structures	72
17	Formation of globular embryo from immature anther derived callus	76
18.	Section of immature rachillae explant showing vascular tissue	79
19.	Section of anther derived calli showing actively dividing parenchymatous cells	79
20.	Vascular bundle formation 6 weeks after callus induction.	80
21.	Vascular bundle formation 8 weeks after callus induction.	80
22.	Vascular connections towards the periphery of callus tissue.	80
23.	Section of callus showing high meristematic activity and embryonic unit.	81

LIST OF FIGURES

Figure No	Figure	Page No
1.	Percentage callus induction and browning of explants using three callus induction media.	67
2.	Effect of 2,4-D concentration on callus induction and browning of inflorescence explants	88
3.	Effect of sucrose levels on anther callusing	92
4.	Effect of chilling treatment on callus induction and survival percentage of immature anther	92

LIST OF APPENDICES

Sl. No.	Title
1.	Solubility of different growth regulators
2.	Growth regulator combinations tried for direct organogenesis in coconut explants
3.	Growth regulator combinations tried for callus induction
4.	Growth regulator combinations tried for callus regeneration

INTRODUCTION

1. INTRODUCTION

Coconut, *Cocos nucifera* L. is a majestic perennial palm grown extensively in numerous islands and also in the humid coastal tracts of tropical countries. In India coconut is grown in an area of 19.32 lakh hectares with an annual production of 12147.6 million nuts (2003-2004). The national productivity is about 6285 nuts/ha (Samsudeen *et al.*, 2006).

The coconut palm, rightly known as the 'kalpa vriksha' or 'the tree of heaven' provides many necessities of life, including food and shelter. Of all the tropical palms providing numerous useful products, it is perhaps the outstanding one and is mainly cultivated for the nuts used for culinary purposes. Also, two important commercial products copra and fiber from nuts are obtained. The trunk of the mature palm is used as timber for constructing houses and plaited leaves are used for thatching houses, fencing etc. Water from tender coconut is a refreshing and delicious drink. In Kerala, the extraction of coir from husk of nuts and the manufacture of coir products provide employment for thousands of people, particularly in the backwater tracts where facilities for retting husks are available. The coconut shell is largely used as a fuel and for the production of charcoal and making a variety of curios. The shell flour is used as filler in plastics. Thus every part of the palm is useful in one way or another.

Improvement of perennial crops in general and coconut in particular is a long drawn, slow and difficult process, because of its long juvenile phase, prolonged interval between generation, heterozygous nature of the crop, lack of asexual method of rapid multiplication and vast land area required for the experimentation because of low planting density. In spite of these limitations, India has been in the forefront of coconut breeding and coconut improvement research. The extensive natural variability due to allogamy and the wide range of genetic potential of palms in the available germplasm offer immense scope for genetic improvement in this crop.

Due to high heterozygosity and sexual mode of propagation, segregation occur in every generation which is the main constraint in fixation of superior traits of elite palms. Standardisation of viable protocol for *in vitro* clonal propagation would open up tremendous possibilities for meeting the requirement for quality planting material and in breaking down productivity barriers. A reliable regeneration system is also an important pre requisite for successful genetic transformation.

Research on coconut tissue culture is a challenging task with only some of the problems having been solved. The major objectives include solving the problem of phenolic exudation using antioxidants other than activated charcoal, production of embryogenic calli and regeneration of plants. Considering the wide variation observed in coconut germplasm for productivity, propagation of elite mother palms through somatic tissue culture will go a long way in solving the problem of poor genetic stock. Besides, this technique would also help in the rapid propagation of elite hybrids. The success obtained in zygotic embryo culture and its use in germplasm collection has been one of the major achievements in this direction. Any breakthrough in coconut biotechnology would be of great importance to all coconut growing countries.

Attempts made so far in coconut have not yielded positive results for *in vitro* multiplication. The exploitation of totipotency for *in vitro* regeneration would be much easier in tissues, which are at transition stage that express high levels of nucleic acids and gene expression. Coconut being a sturdy plant with single apical meristem, immature inflorescence would be a better option to regenerate whole plants. Immature anther with pollen mother cells at early stage could be the best material at transition stage to attempt in vitro regeneration. If proper media and conditions can be identified for successful regeneration of diploid plants from immature inflorescence, it would definitely be a break through in improvement of the majestic coconut palm.

Keeping all these in view, the present study was planned with the objective to find out the response of immature inflorescence for somatic embryogenesis and regeneration of diploid plants.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The coconut palm with its tall, slender, uniformly thick and massive crown with larger number of leaves, bearing bunches of nuts in their axils, is one of the most beautiful and useful tree in the world. It is grown in more than 80 tropical countries and it is the most important of all the cultivated palms.

A survey conducted by CPCRI scientists has brought to light several elite disease free coconut palms showing unusually high yields (over 470 nuts/year), although many of these are growing in root wilt affected 'hot spots' of Kerala (Iyer *et al.*, 1979). If such rare single super palms could be propagated clonally through *in vitro* approaches, this would certainly open up tremendous possibilities for breaking the yield barrier in coconut.

2.1 VEGETATIVE PROPAGATION

At present coconut palm is only sexually propagated because of some morphological and physiological peculiarities. It has only one stem and does not produce any adventitious shoots, sucker or tiller. Axillary buds develop into inflorescence only and the single, terminal vegetative bud is the apex, which builds the whole tree. Therefore the traditional methods of vegetative propagation (cutting, grafting) are of no use. Ramification phenomenon as well as transformation of inflorescence, spikes or even flowers into vegetative buds (Davis, 1969) has been observed very rarely. Though attempts at rooting these vegetative shoots have been successful (Sudasrip *et al.*, 1978), the problem of reversion towards frutification is still unanswered and no routine method of propagation has been developed so far. Trials of aerial layering were conducted on the crown, but although this technique envisages the rejuvenation of specific genotypes, it cannot lead to their multiplication. There is a report on development of several offshoots from young seedlings by splitting the apical meristem, but further studies showed that the neomeristem formation was of

adventive origin (Balaga, 1975). However, the results of these experiments cannot be directly exploited for propagating elite palms.

Thus the only possibility of vegetative propagation is through *in vitro* regeneration. Studies concurring its exploitation have already been developed in the United Kingdom, India, Indonesia, Sri Lanka, Philippines, United States and France. Considering the morphological peculiarities of the coconut, tissue culture seems to be the most suitable technique.

2.2 *IN VITRO* PROPAGATION

Though tissue culture techniques have made it feasible to vegetatively propagate several plant species (Murashige, 1974), the success in culturing woody species in general (King, 1974) and monocotyledonous plants in particular, has been limited to only a few cases (Staritsky, 1970; Bhojwani *et al.*, 1977). Nevertheless, the feasibility of producing large numbers of plants from small tissue has prompted the use of such techniques in exploring ways to multiply elite trees.

2.2.1 General aspects of plant tissue culture

Schleiden (1838) and Schwann (1839) postulated the cell theory, which revealed the totipotent nature of cells. The concept of regeneration of plants from *in vitro* cultured cells can be traced to the German Botanist, Haberlandt (1902) who predicted about totipotency in plants. Skoog and Miller (1957) with their discovery of auxins and cytokinins made a landmark in the history of plant tissue culture. They put forth the concept of hormonal control of organ formation and showed that root and shoot differentiation was a function of auxin-cytokinin ratio and that it could be regulated by altering the relative concentration of these growth regulator in the medium. Many pioneer investigators like White (1934), Gautheret (1939), Nobecourt (1939), Miller *et al.* (1956), Reinert (1958), Steward *et al.* (1958), Bergmann (1960) and Vasil and Hildebrandt (1965) have contributed concepts for the

successful development of plant tissue culture. A completely defined nutrient medium for plant tissue culture was developed by Murashige and Skoog (1962).

Several aspects of plant tissue culture are being applied in agriculture, which include the production of haploid plants, secondary metabolite production, embryo rescue techniques etc. However, the best commercial application of tissue culture is the production of true to type plants at a very rapid rate compared to the conventional methods (Levy, 1981). Vasil and Vasil (1980) reported that the tissue culture derived plantlets grow faster and mature earlier than seed propagated plants.

At present the coconut is propagated entirely by seed. Since this palm is generally cross pollinated and heterozygous, the resulting variation between seedlings (Davis, 1969) is a serious problem for a plantation crop having a life of 50 years or more . Blake (1990) has reviewed the use of tissue culture for propagation of other commercially important palms, such as oil palm (*Elaeis guineensis* Jacq) (Jones, 1974; Rabecault and Martin, 1976) and date palm (*Phoenix dactylifera* L.) (Reynolds and Murashige, 1979; Tisserat, 1982). Several attempts have been made in the past to develop a viable protocol for *in vitro* culturing in coconut also.

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 of donor plant, type of explant, age of explant, surface sterilization, culture medium, plant growth regulators and other additives, pH, light, temperature and relative humidity etc. (Brown and Thorpe, 1986).

2.3.1 Genotype of the donor plant

One of the constraints in tissue culture with coconut seedlings or mature palms is the genotypic difference in culture response (Iyer, 1993). The pertinacious

difference in callogenesis responses of 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 reported by Verdeil *et al.* (1994). Hybrids PB 121 and PB 111 gave the best results. Reproducible obtention of callogenesis and whole somatic embryos from immature leaf or inflorescence fragments in 20 to 25 years old PB 121 hybrid palms was reported by Verdeil *et al.* (1992). Monfort (1985) obtained embryoids from anthers of PB 121 and PB 111.

2.3.2 Type of explant

Various explants such as zygotic embryos (Abraham and Thomas, 1962; Gupta *et al.*, 1984; Bhalla-Sarin *et al.*, 1986), stem (Apavatjrat and Blake, 1977), leaf (Pannetier and Buffard-Morel, 1982; Raju *et al.*, 1984), immature inflorescence (Zaid and Tisserat, 1983) and Plumule (Rajesh *et al.*, 2004) have been used for coconut tissue culture for the last three decades. Inflorescence tissues can be harvested with limited damage to the upper parts of the donor plant and these are enclosed within tightly clasped spathe, which prevent infestation by fungi and bacteria and avoid chemical damage from sterilization solutions. Previous studies have shown that immature inflorescence tissue is an excellent starting material (explant) for coconut embryogenesis. Orense *et al.* (1993) reported that immature inflorescence was the most responsive to callus induction due to the presence of numerous meristematic points.

2.3.3 Age of explant

In addition to palm maturity, the physiological maturity of the explant is also considered as a critical factor in obtaining embryogenic cultures. Previous findings showed that leaf explants from young coconut palms responded better to *in vitro* techniques than those from older plants (Verdeil *et al.*, 1989). Moreover immature embryos have been shown to respond better than mature zygotic embryos (Karunaratne and Periyapperuma, 1989). Immature inflorescence corresponding to the

fourth from the youngest open frond has been found most responsive to callus induction (Orense *et al.*, 1993) 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. Inflorescence with external spathe length 10 and 25 cm gave the best callusing (Verdeil *et al.*, 1994).

2.3.4 Sterilization of explant

The maintenance of aseptic or sterile conditions is essential for successful tissue culture procedures. Plant material can be surface sterilized by a variety of chemicals. It is the eradication of micro organisms with the aid of chemicals. 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 were surface sterilized with 0.1 per cent $HgCl_2$ for 20 minutes and leaf and rachillae, with 0.05 per cent $HgCl_2$ for 10 minutes (Gupta *et al.*, 1984). Verdeil *et al.* (1994) sterilized the inner spathe of inflorescence for 10 minutes in a hypochlorite solution and then removed it under aseptic condition.

2.3.5 Culture medium

Successful *in vitro* culture as a means of plant propagation depends largely on the choice of nutrient medium including its chemical composition and physical form (Murashige, 1974). The widely used medium contains the Murashige and Skoog (1962) MS formulation with certain modification. Eeuwens (1976) standardized a medium for successful callus induction from various coconut explant sources like stem, leaf and inflorescence. Thanh-Tuyen and De Guzman (1983) reported that Blaydes medium was suitable for coconut anther culture. The major constituents of tissue culture medium are mineral salts, carbon as energy source, vitamins and plant growth regulators.

2.3.5.1 Carbon

A carbon energy source is inevitable in any tissue culture media. Sucrose is the most widely accepted carbon source. Besides serving as carbohydrate source, it regulates osmolarity of the culture media and also plays a role during morphogenesis (Sopory, 1979). 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) of coconut.

2.3.5.2 Growth regulators

Plant growth regulators are essential for manipulation of growth and development of explants *in vitro*. Their concentration and ratio in the medium often determines the pattern of development in culture (Krikorian, 1982). 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 was reported to play an important role in the growth of coconut explants (Gupta *et al.*, 1984) and 2,4-D was found to be essential for callus induction (Tahardi, 1987).

2.3.5.3 Other media additives

Activated charcoal is an essential media additive for coconut tissue culture (De Guzman *et al.*, 1971; Karunaratna *et al.*, 1985). Casein hydrolysate or glutamic acid 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. But role of these complex organic compounds were reported to be unpredictable and with poor repeatability and therefore it has been recommended to avoid their use as far as possible (Gamborg and Shyluk, 1981).

2.3.6 **pH of the medium**

In general, plant cells in culture require an acidic medium with an initial range of 5.5 to 5.8 (Gamborg and Shyluck, 1981). In coconut, pH of the culture media was adjusted to 5.7 using potassium hydroxide or sodium hydroxide before autoclaving (Branton and Blake, 1983).

2.3.7 **Culture conditions**

Light, temperature and humidity conditions provided inside the tissue culture room plays a significant role in success of any tissue culture technique.

2.3.7.1 Light

The light intensity, quality and duration are the three major factors affecting the growth of *in vitro* culture (Murashige, 1974). Coconut cultures should be kept in darkness inside an incubator controlled at 28 ± 2°C until well developed calluses were obtained (Sugimura and Salvana, 1989).

2.3.7.2 Temperature

Yeoman (1986) reported that the usual environmental temperature at the original habitat of a particular species should be taken into consideration while regulating the temperature of *in vitro* systems. The optimum temperature for coconut is reported to be 30°C (Branton and Blake, 1983).

2.3.7.3 Humidity

Humidity is rarely a problem except in arid climates, where rapid drying of medium occurs. This can be reduced by the use of tightly closed containers, covering

closures such as foam or cotton wool plugs with aluminium foil. In climates with high humidity, de-humidifier in the culture room may be advantageous (Yeoman, 1986).

2.4 EXUDATION FROM THE EXPLANT

During the course of growth and development *in vitro*, plant tissues not only deplete the nutrients that are incorporated in the medium but also release toxic substances. These substances such as phenols may have profound physiological effects on the cultured tissues. Browning of the tissues and the adjacent medium was assumed to be due to the oxidation of polyphenols and formation of quinones which were highly reactive and toxic to the tissues (Maier and Metzlier, 1965).

The prominent problem of browning has been noticed in coconut tissue culture by Apavatjrat 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 Mc cown, 1980; Mathew *et al.*, 1987).

2.5 METHODS TO OVERCOME POLYPHENOL INTERFERENCE

2.5.1 Pretreatments given to explants

To minimize browning, Murashige (1974) has suggested the presoaking of explants in ascorbic acid and citric acid solutions and adding them to the culture medium to curtail the oxidation of the phenolics. Zaid and Tisserat (1983) soaked date palm explants in an antioxidant solution (150 mg l^{-1} citric acid and 100 mg l^{-1} ascorbic acid) prior to the surface sterilization treatments. Areza *et al.* (1993) soaked the inflorescence tissue in antioxidant, viz. citric acid (50 mg l^{-1}) and ascorbic acid (100 mg l^{-1}) prior to slicing and culturing in Y_3 medium supplemented with activated charcoal, which resulted in less browning.

2.5.2 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 minimized (Sugimura and Salvana, 1989). One mm thick slices gave 32 per cent browning while 0.5 mm thick slices gave 11 per cent only.

2.5.3 Use of media additives

The use of charcoal is preferred over L-cystein hydrochloride and other reducing agents, such as ascorbic acid, citric acid, dithiothreitol, glutathione and mercapto ethanol (George and Sherrington, 1984) because the latter are often toxic to the plant tissues at higher concentrations (Sharma *et al.*, 1980). Inclusion of activated charcoal reduced the availability of hormonal substances. It is therefore, necessary to apply an abnormally high concentration of auxins (Tisserat, 1979; Zaid and Tisserat, 1983) up to a certain level not toxic to the tissues.

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 media prevents the phenolic exudation in coffee (Sreenath *et al.*, 1995) and in rubber (Seneviratne and Wijesekara, 1996).

2.5.4 Selection of growth regulators and carbon sources

The lowest browning incidence was observed when the level of 2,4-D ranged from 20-30 ppm and with 0.25 per cent activated charcoal incorporated in the callus induction medium (Sugimura and Salvana, 1989). Excessive levels of 2,4-D were 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 were 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).

2.5.5 Selection of basal medium

Eeuwens and Blake (1977) suggested that browning could be eliminated by a nutritionally balanced medium.

2.5.6 Age of media

Age of media also affects browning (Ebert and Taylor, 1990). While most explants on one day old medium had died or showed only minimal growth, a slightly better response was observed with 5 day old medium. The best results, however, were obtained with 9 day old medium. These results indicated that fresh medium, might contain toxic levels of some of the chemical compounds such as 2,4-D which was subsequently adsorbed by activated charcoal thus preventing damage to the explants (Ebert, 1993).

2.5.7 Frequency of subculturing

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) reported that reculturing the date palm tissues to fresh medium after a short period of incubation decreased the browning. Excision of brown explant parts during culture was also advocated to prevent this problem (Smith and Thomas, 1973).

2.6 CALLUS INDUCTION

Coconut, like other palms is a difficult crop to manipulate *in vitro*. Eeuwens (1976) standardised a media for successful callus induction from various explant sources like stem, leaf and inflorescence. This led a few laboratories around the world to initiate research on coconut. Most commonly used basal medium at present is Y_3 formulation (Eeuwens, 1976). 2,4-D is the common auxin used for induction of callus. The level of 2,4-D used has been varying. Branton and Blake (1983) reported that 22 mg l^{-1} 2,4-D was required for nodular calloid formation from external layers of floral meristems, while from immature zygotic embryos calli were obtained with 26 to 55 mg l^{-1}. Using the same medium subsequently Arachi and Weerakoon (1997) obtained compact and highly embryogenic calli from floral explants.

2,4-D at 50 mg l^{-1} along with 2 mg l^{-1} kinetin in presence of activated charcoal gave calli from endosperm (Kumar *et al.*, 1985). Where as in another investigation addition of 1 mg l^{-1} 2iP and BAP along with 22 mg l^{-1} 2,4-D in presence of activated charcoal (2.5 g l^{-1}) was reported to produce calli from immature inflorescence (Branton and Blake, 1983). Other auxins and polyamines as well as supplementation with cytokinins have also been reported to be useful. 2,4,5-T on inflorescence explants (Buffard-Morel *et al.*, 1992) NAA along with IAA on leaf explants (Raju *et al.*, 1984) and IAA along with glutamic acid on zygotic embryos (Karunaratne *et al.*, 1985) induced callus formation.

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 explant tissues (Chandralekha, 1997).

Verdeil *et al.* (1989) found that the callogenesis percentage varies according to the physiological age of explant and significantly according to the auxin

concentration of the medium. Callus induction from anthers and rachillae did not give repeatable response (Bhalla-sarin *et al*, 1986).

If seedling cores or pieces of inflorescence rachillae were placed in high levels of auxin then organized growth was partially or totally inhibited and callus often initiated. Callus was formed over a range of cytokinin and sucrose levels, with the determining factors being the presence or absence of charcoal and the level of auxin. Of the auxins used, 2,4-D was the most effective in producing callus, IAA and IBA were generally ineffective, with NAA sometimes giving callus and sometime giving roots (Blake and Eeuwens, 1982).

2.7 SOMATIC EMBRYOGENESIS

In monocots, immature inflorescence, immature embryos or mature seeds were the choice of explants for initiating embryogenic cultures (Bhaskaran and Smith, 1990).

The first report of somatic embryogenesis was given by Reinert (1959) in carrot cultures. Though the positive results were limited to a few species, this was recognised as a more rapid mode of plant regeneration (Evans *et al.*, 1981). There were two routes for somatic embryogenesis as described by Sharp *et al.* (1980). The first in which embryos were formed directly from the explant without the callus formation. The second in which, it required a callus formation, and on callus, embryos were formed. Level of growth regulators in the culture medium, particularly the lower levels of auxin increased the chance for the embryo formation or somatic embryogenesis (Skoog and Miller, 1957; Hussey, 1986). It required an auxin medium for the induction of embryos and a medium devoid of growth regulators for embryogenesis (Amirato, 1983).

Verdeil *et al.* (1994) reported successful embryo maturation through somatic embryogenesis from coconut inflorescence explants, which further regenerated into plantlets. They cultured immature inflorescence on an agar medium

supplemented with activated charcoal (0.2 per cent) and a range of 2,4-D concentration (0.15-0.35 mM). Globular white callus emerged from immature floral meristems, depending on inflorescence age and 2,4-D levels.

When immature rachille tissues were used as explant source, they developed into leaf or root like growth via embryogenesis (Branton and Blake, 1983; Gupta *et al.*, 1984). Blake (1990) reported that high 2,4-D levels and activated charcoal were necessary for callus induction whereas gradual lowering of 2,4-D level was associated with embryo production in immature inflorescence. Cueto *et al.* (1997) cultured inflorescence explants onto callus initiation medium with 1 x 10^{-4} M 2,4-D and 1 x 10^{-6} M 2iP and 2.5 g l^{-1} activated charcoal. Increasing level of 2,4-D up to 2 x 10^{-4} M induced calloid multiplication. The gradual reduction in the 2,4-D levels and simultaneous increase in 2iP levels (to 5 x 10^{-5} M) induced the formation of somatic embryo. Plantlets with single and multiple shoots were regenerated after 17 months in medium when 2iP was used as cytokinin source for calloids and somatic embryos induction and BAP for embryoid maturation and germination.

Adventitious plantlets were obtained from rachilla tissue of *Phoenix dactylifera* cultured on a modified Murashige and Skoog medium containing 3 mg l^{-1} 2- isopentyl adenine, 0.1-100 mg l^{-1} α-naphthalene acetic acid or 2,4-D, and 3 g l^{-1} activated charcoal. Additions of auxins were necessary to induce explants to produce callus, adventitious plantlets and roots. Plantlets were obtained from explants cultured 3-4 months *in vitro* (Tisserat and De Mason, 1980).

When immature inflorescence of oil palm (*Elaeis guineensis*) var. Pisifera was inoculated into MS medium containing 0.3 per cent (w/v) activated charcoal and 475 μM 2,4-D, a hard yellow callus proliferated after 2 to 3 months at the base of the shoot like structures. After 81 weeks of culture, an embryogenic tissue characterized by compact consistency and pearly white colour was observed in tissues derived from very young inflorescence. This compact embryogenic tissue differentiated into normal

somatic embryos when transferred into regeneration medium containing NAA (15 μM) and ABA (2 μM). Normal plantlets were removed from these somatic embryos after 8 weeks on regeneration medium (Teixeira *et al.*, 1994).

To induce formation of somatic embryos, Bamboo inflorescence were inoculated on MS medium supplemented with 2 mg l^{-1} kinetin and 3 mg l^{-1} 2,4-D. The somatic embryos germinated and went through a juvenile phase and developed into complete plantlet (Lin *et al.*, 2003).

High frequency somatic embryogenesis and plant regeneration were achieved from immature anthers of *Hevea brasiliensis* on MS medium supplemented with 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} Kn. Maximum number of somatic embryos were produced on medium supplemented with 0.7 mg l^{-1} kin and 0.2 mg l^{-1} NAA. Further development of the embryos into plantlets was achieved on a hormone free medium (Das *et al.*, 2003).

Karun *et al.* (2004) reported culturing of arecanut inflorescence (*Areca catechu* L.) in MS basal medium. Picloram was reported to be the most suitable callogenic agent. Also they found that serial transfer of explants from high to low auxin concentration was essential for sustained growth of callus and somatic embryo induction. Somatic embryogenesis was achieved in hormone free MS medium whereas somatic embryos were germinated in MS medium supplemented with cytokinin, 20 μM BA was found to be the best.

2.8 ANTHER CULTURE

Studies on the anther culture of coconut are much less than those on embryo culture. So the review in this chapter has been confined mostly to tree crops and solanaceous crops. *In vitro* anther culture technique has enabled the production of large number of haploid plants. This technique has been applied to over 200 species of

higher plants (Maheswari *et al.*, 1982; Keller *et al.*, 1987). Anther culture technique has also enabled the production of diploid plants (Jayasree *et al.*, 1999).

Tulecke (1953) was the foremost scientist who observed that mature pollen grains of a gymnosperm *Ginko biloba* could be cultured to proliferate and form haploid callus. In line with this Guha and Maheswari (1964) were the first to report on the direct development of embryos from microspores of *Datura innoxia* through anther culture. Harn and Kim (1972) obtained callus from the anthers of *Prunus armeniaca* which represented the first favourable response on anther culture of a tree species.

The prominent tropical tree species in which anthers were amenable to *in vitro* culture for the first time were *Coffea arabica* (Sharp *et al.*, 1973); *Pyrus* spp. (Jordan, 1975); *Annona squamosa* (Nair *et al.*, 1983); *Cassia* spp. (Bajaj, 1983); *Hevea brasiliensis* (Jayasree *et al.*, 1999) etc.

Thanh-Tuyen and De Guzman (1983) reported anther culture technique in coconut. They reported pollen embryos from anther cultures and designated them as globular, heart shaped and torpedo.

2.8.1 Factors influencing embryogenesis

The ability to induce totipotency in anther cultures is greatly influenced by several factors. Many workers have regenerated callus and plantlets from anthers of different plant species and have elucidated information on various factors which might influence the success of androgenesis. According to Sopory and Maheswari (1976) the factors, which significantly influence the induction of androgenic plants, are the genotype of the explant, stage of development of microspores at the time of inoculation and the culture medium with conditions of culture incubation.

2.8.1.1 Genotype

The genotypes of the donor plants have been reported to have a profound effect on embryogenic response. The physiological basis of such genetic control of embryogenesis is not clearly understood. In wheat, embryogenesis seems to be controlled by at least three dominant genes (Agache *et al.*, 1989). In coconut, Monfort (1985) reported that embryoid formation is more from anthers of Malayan Yellow Dwarf x West African Tall (PB 121) and West African Tall x Rennell Tall.

2.8.1.2 Physiology of donor plant

The age and physiological status of the donor plant, determined by prevailing growth conditions, markedly affect embryogenic response of cultured anthers. Although no generalization can be made on the factors conducive for embryogenesis, growth conditions such as temperature, light intensity and duration, and nutritional status of plant affect the frequency of embryogenesis (Palmer and Keller, 1997). Generally plants grown under low temperatures respond better (Lo and Pauls, 1992) than those grown under high temperature.

2.8.1.3 Composition of medium

Thanh – Tuyen and De Guzman (1983) reported that Blaydes medium was suitable for coconut anther culture. The carbohydrate component in the medium seems to play an important role (Palmer and Keller, 1997). In several species the frequency of embryo induction increases with increasing concentration of carbohydrate source (Huang and Keller, 1989). Sucrose has so far been the most effective carbohydrate, although maltose has been reported to be a better carbohydrate source for some species, in particular cereals (Hoekstra *et al.*, 1992). Other factors such as pH, nitrogen source and gelling agent also affect the efficacy of induction.

Use of activated charcoal in the medium, which adsorbs toxic metabolites of the medium, has proven beneficial in many species (Buter *et al.*, 1993). Other cultural conditions, which affect embryogenic response are density of anthers, temperature, culture aeration, light (quality and intensity) and photoperiod. In several species, the initial culture temperature was reported to be critical for pollen embryogenesis; initial culture at 30 to 35°C for 12 to 72 hrs followed by maintenance of cultures at 20 to 25°C, generally give optimal response. Initial high temperature seems to facilitate induction of embryos, while subsequent lower temperature promotes further embryo growth (Palmer and Keller, 1997).

2.8.1.4 Stage of anther development

For most species, the stage of anther development at the time of culturing is critical for induction of embryogenesis. It has been established that there is an optimum development stage that corresponds to the late uninucleate to early binucleate state of development, during which large number of microspores could undergo embryogenesis (Kott, 1997; Pechan and Keller, 1988). Further to this it has been proposed that non-embryogenic microspores produce inhibitory substances that suppress embryo development in the embryogenic microspores (Bors, 1984; Kott *et al.*, 1988). In rubber somatic embryogenesis and plantlet regeneration was achieved from anthers at premeiotic stage (Jayasree *et al.*, 1999)

2.8.1.5 Pretreatment of anthers/ flower buds

Pretreatment of anthers / flower buds at low temperature (3 to 5°C) is effective in inducing pollen embryos in several species. Low temperature shock markedly improved embryogenic response in *Nicotiana tabacum*, *Datura metel*, *Brassica napus* and *Hyoscyamus niger* (Maheswari *et al.*, 1982). In Petunia hybrida pretreatment of anthers at 6°C for 48 hrs doubled the percentage of anthers producing embryos (Malhotra and Maheshwari, 1977). Similarly cold treatment of

anthers/spikelet/spike of many cereals for varying periods has been beneficial for embryogenesis (Huang and Sunderland, 1982).

2.9 IN VITRO ROOTING

Although a number of plants root easily in culture, shoots of most species lack a root system. Rooting can be achieved either by sub culturing to a medium lacking cytokinins with or without rooting hormone, or by treating the shoots with rooting hormone as in conventional cuttings (Yeoman, 1986). The concentration of rooting hormone required is often critical to provide sufficient stimulus to initiate roots while preventing the excessive formation of callus (Yeoman, 1986). Generally auxin favours rhizogenesis. Among the auxins, NAA has been the most effective one for induction of rooting (Ancora *et al* ., 1981). Shoots obtained from coconut zygotic embryos were transferred to Y3 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.

2.10 HARDENING AND PLANTING OUT

Tisserat (1982) reported that there was direct relationship between the size of the initial explant and the survival in soil. Direct transfer to green house conditions with out an intermediate humidity stage was not feasible. Physical, chemical, and biological properties of the potting mixture were also important in the plantlet establishment (Kyte and Briggs, 1979). Thorough washing of the plantlets to remove the traces of nutrients and sterilizing the potting mixture eliminate serious problems of fungal infection (Anderson, 1980; Gupta *et al.*, 1984) transferred the seedlings obtained from zygotic embryos of coconut to poly bags containing mixture of soil, sand and compost (3:3:1) and incubated at 25°C with 16hrs photoperiod for four weeks and later transferred to glass house.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Response of immature inflorescence for *in vitro* regeneration in coconut (*Cocos nucifera*)" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara from January 2005 to September 2006. Details regarding the experimental materials used and the methodologies adopted for various experiments are described below.

3.1 Culture medium

3.1.1 Chemicals

The major elements, minor elements, vitamins and growth regulators required for the preparation of media were of analytical grade and were procured from M/s Sisco Research Laboratories, M/s Merck India Ltd., M/s Sigma – Aldrich chemicals Pvt.Ltd. and Himedia laboratories Pvt. Ltd.

3.1.2 Glasswares

Borosilicate glass wares of Corning/ Borosil were used for the requirement. The glasswares were cleaned initially by soaking in potassium dichromate solution for 12 hrs followed by thorough washing with tap water in order to remove completely all traces of potassium dichromate solution. They were further cleaned with 0.1% teepol (detergent) solution and were washed thoroughly with water and then rinsed with double distilled water. These were dried in hot air oven at 80°C for 24 hrs and were stored away from dust and contaminants.

3.1.3 Composition of Media

The most widely accepted MS medium (Murashige and Skoog, 1962), Y_3 medium (Eeuwens, 1976) and Blaydes medium (Blaydes, 1966) were used in the

present study. The nutrient medium included inorganic salts, vitamins, growth regulators, organic supplements and a carbon source (The composition of different basal media is given in Table 1).

3.1.4 Preparation of tissue culture media

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of plant tissue culture media. Separate stocks were prepared by dissolving required quantities of macronutrient, micro nutrient and vitamins in distilled water and were stored under refrigerated conditions (4°C)

A clean steel vessel, rinsed with distilled water was used to prepare the medium. Aliquots from all stock solutions were pipetted into the vessel. To this, the required quantity of sucrose, inositol and growth regulators were added. The desired volume was made up by adding distilled water. The pH of the medium was adjusted between 5.5 to 5.8 using 0.1 N NaOH or HCl.

For solid medium, agar was added at 0.75 per cent (w/v) concentration, after adjusting the pH. The medium was stirred and heated to melt the agar and was poured when hot into culture vessels and plugged with non adsorbent cotton. Test tubes containing media were sterilized in an autoclave at 121°C by applying 15 psi pressure for 20 min. The medium was allowed to cool to room temperature and stored in culture room until used.

3.1.5 Preparation of growth regulators

To prepare a 1 mg ml^{-1} of stock solution 100 mg of the plant growth regulator was added to a 100 ml volumetric flask along with 2 to 3 ml of solvent (NaOH/ethanol) to dissolve the growth regulator. Once completely dissolved the volume was made up with double distilled water. Stirring the solution while adding water may be required to keep the material in solution. One ml of the stock solution

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

Ingredients mg l^{-1}	MS (A)	Blaydes (B)	Y_3 (C)
Major elements			
NH_4NO_3	1650	1000	-
KNO_3	1900	1000	2020
KCl	-	65	1492
KH_2PO_4	170	300	-
$MgSO_4.7H_2O$	370	35	247
$Ca(NO_3)_2.4H_2O$	-	347	-
NH_4Cl	-	-	535
$NaH_2PO_4.2H_2O$	-	-	312
$CaCl_2.2H_2O$	440	-	294
$FeSO_4.7H_2O$	27.85	42	13.90
Na_2 EDTA. $2H_2O$	37.25	32	37.25
Micro elements			
KI	0.83	0.8	8.3
H_3BO_3	6.2	1.6	3.1
$MnSO_4.4H_2O$	22.3	4.4	11.2
$ZnSO_4.7H_2O$	8.6	1.5	7.2
$CuSO_4.5H_2O$	0.025	-	0.16
$COCl_2.6H_2O$	0.025	-	0.24
$NiCl_2.6H_2O$	-	-	0.024
Vitamins			
Nicotinic acid	0.5	0.5	1.0
Pyridoxine HCl	0.5	0.5	1.0
Glycine	2.0	2.0	1.0
Thiamine HCl	0.1	0.1	1.0
Calcium d Pantothenate	-	-	1.0
Biotin	-	-	1.0

A – Murashige and Skoog, (1962); B – Blaydes, (1966); C – Eeuwens, (1976).

was added to 1 litre of medium to obtain a final concentration of 1.0 mg l^{-1} of the plant growth regulator in the culture media. Solubility of different growth regulator is given in Appendix 1.

3.2 Transfer area and aseptic manipulation

All the aseptic manipulations such as surface sterilization of explants, preparation and inoculation of explants and subsequent subculturing were carried out under the hood of a clean 'Thermadyne' laminar air flow cabinet. The working table of the laminar airflow cabinet was first sterilized by wiping with absolute alcohol and then by putting on the UV light for 20 min. The Petri dishes, forceps and scalpel used for the inoculation were first steam sterilized in an autoclave at 15 psi at 121°C for 20 min and then flame sterilized before each inoculation. Hands were also scrubbed with ethyl alcohol before inoculation.

3.3 Culture conditions

The cultures were incubated at 26 ± 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 Source of explant

Immature inflorescences of coconut were collected from the varieties West Coast Tall (17 years old) and Chavakad Green Dwarf (15 years old) grown in Coconut Development Farm, Vellanikkara.

3.5 Explants

Different explants were tried for organogenesis/embryogenesis. Inflorescence at different stages was used for taking explant. The size of the inflorescence used was approximately of 1, 3, 5, 10, 15, 20, 30, 35, 40, 45 and 50 cm (Plate 1). Immature inflorescence having anthers at premeiotic stage was identified and such inflorescence was used to scoop out different explants like rachillae, female flower, male flower and anthers at premeiotic stage. The floral primordia was scooped out from very young inflorescence of 5 to 15 cm size to study its response in selected media.

3.5.1 Rachillae

Rachillae from the top, middle and bottom of the inflorescence were used as explant. The size of rachillae used as explant ranged between 0.5 to 5 mm in thickeness.

3.5.2 Female flower

Female flower from the bottom, middle and top of the rachis were used as explant. Diameter of the female flower ranged between 0.2 to 1 cm.

3.5.3 Male flower

Spikelet at the top, middle and bottom of the rachillae were used as explant, since the maturity of the flower vary at different parts of the rachillae.

3.5.4 Anthers at pre meiotic stage

Cross-section of the male flowers at different development stages were taken to observe the anthers at the early stage, pollen mother cell stage (pre-meiotic stage) and microspore stage. Those which were suspected to be at pollen mother cell stage were

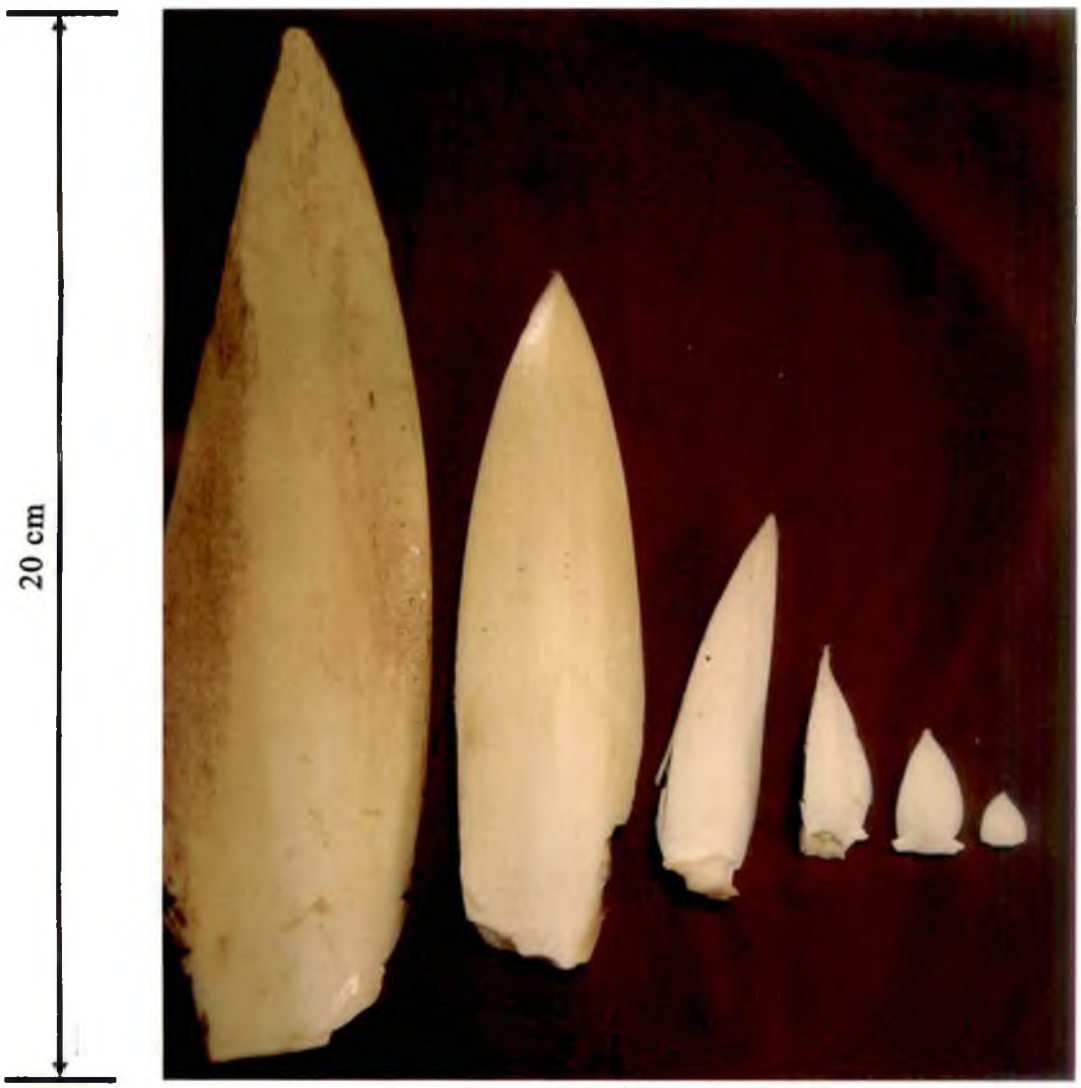


Plate 1. Inflorescence of different size from unopened leaf axil used as explant source for *in vitro* culturing

further subjected to cytological analysis to confirm the stage of pmc to be ready to undergo meiotic division.

Those flower buds which are suspected to be in the pmc stage were separated and fixed in carnoys solution (ethyl alcohol, chloroform and acetic acid in the ratio 6:3:1). After 24 hours of fixation the flower buds were washed thoroughly and then the stage was identified through a series of treatment (Vanessa, 2005) given below.

1. Dissected previously fixed flower buds and collected the anthers.
2. Immature anthers were gently crushed and put in acetocarmine stain in an eppendorf tube (1.5ml).
3. Vortexed for a few minutes (2-3) to shake the microspore mother cells/ tetrads/ immature pollen grains out of the anther walls.
4. The tube was placed on a heat block set at approximately 50°C for 1 hr.
5. The tube was vortexed again for a few seconds (2-3) and removed all large debris from the stain.
6. Centrifuged for 1 minute at 10,000 rpm to get a light pellet of pollen mother cell/ tetrads etc. Poured off the excess stain and debris.
7. Using a micropipette 15 µl of the pelleted cells were transferred to a clean slide with a drop of stain/glycerin and covered with a cover slip.
8. The slide was placed between layers of blotting paper and thumb pressed and viewed for pollen mother cells.

After correlating the anthers at pre meiotic stage to the size of flower bud, similar flower buds were used to dissect out anthers for culture initiation.

3.6 Culture establishment

3.6.1 Surface sterilization

Surface sterilization was carried out under perfect aseptic conditions in the laminar airflow chamber. The washed explants were put into the sterilant and kept

immersed for the required period. They were continuously agitated manually to ensure thorough contact of the explants with chemical. The different surface sterilization treatments tried for the explants are listed in Table 2. The explants after surface sterilization were rinsed five times thoroughly with sterilized distilled water to remove traces of the sterilant from the surface of the explant.

3.6.2 Standardization of pretreatments 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, various treatments were tried to overcome the browning of the immature anthers. Effects of different concentrations of activated charcoal (0.05, 0.1, 0.15, 0.2, 0.25, 0.3%) and different antioxidants were tried by soaking and using as media additives. Details are given in the Table 3.

3.7 Direct organogenesis

The male flower bearing rachillae from the surface sterilized immature inflorescence were sliced transversely (0.5 to 5 mm thickness) and were used for culturing in different media combinations. Anthers were taken carefully from the surface sterilized male flowers and were inoculated. The surface sterilized female flowers were cut into small pieces and were inoculated and incubated under conditions mentioned earlier.

3.7.1 Basal media

MS, Blaydes and Y_3 media were tried for organogenesis. The solid media were supplemented with 5 percent sucrose, 0.75 percent agar and 0.2 per cent activated charcoal.

Table 2. Surface sterilization treatments tried for the inflorescence explants of coconut (*Cocos nucifera* L.)

Treatment No.	Explant	Sterilant	Concentration (%)	Duration in minutes
1	Male flowers	Nil	Nil	Nil
2	Rachillae	Nil	Nil	Nil
3	Alcohol wiping over the spathe	Ethyl Alcohol	70	1
4	Soaking the male flowers and female flowers	$HgCl_2$	0.1	1 2 5 7 10 12 15
5	Soaking the male flowers and female flowers	Ethyl Alcohol	70	1 3 7 12 15
6	Soaking the rachillae	$HgCl_2$	0.1	1 3 5 7 10 12 15

Table 3. Pre-treatments tried to overcome polyphenol interference

Sl. No.	Antioxidant	Concentration (%)
A	**Soaking of the explants**	
1	Ascorbic acid	0.01
2	PVP	0.1
3	Citric acid	0.01
4	Sterile distilled water	-
B	**Media additives**	
1	Ascorbic acid	0.01
2	PVP	0.1
3	Activated charcoal	0.2
4	Citric acid	0.01
5	Ascorbic acid + Activated charcoal	0.01 + 0.2
6	PVP + Activated charcoal	0.1 + 0.2
7	Citric acid + Activated charcoal	0.01 + 0.2

3.7.2 Growth regulators

Growth regulators like auxin and cytokinins were incorporated into the basal media. The details of various combinations are given in Table 4. Explants include male flower, rachillae, anther at premeiotic stage and female flower.

3.8 Indirect organogenesis

3.8.1 Callus induction

Immature rachillae, male flower, female flower and anthers at premeiotic stage were used for callus induction in different basal media viz. MS, Y_3 and Blaydes.

3.8.2 Growth regulators

Different basal media were supplemented with different levels of growth regulators such as auxins and cytokinins. Details of various growth regulator combinations tried for indirect organogenesis are given in Table 5.

3.8.3 Media additives

The effect of media additives on regeneration was studied. The media additives included casein hydrolysate, glutamic acid, Mannitol, phloroglucinol and tender coconut water for callus induction and proliferation. The details of different combination are given in Table 6.

3.8.4 Carbohydrate

Sucrose was incorporated in the media as carbohydrate source. Different levels of sucrose (2 to 10 per cent) were tried to find the favourable concentration of sucrose optimum for callus induction and multiplication.

Table 4. Growth regulator combinations tried for direct organogenesis in coconut explants

Basal medium	Growth regulator combinations
MS, Y_3 and Blaydes	BAP (1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 15 mg l^{-1}) Kinetin (0.25, 0.5, 1, 2, 4, 8, 10,12,14,15 mg l^{-1}) 2iP (0.25, 0.5, 1, 2, 3, 4, 5 mg l^{-1}) NAA (0.5, 1, 1.5, 2 mg l^{-1}) + BA (1, 2, 3, 4, 5, 8 mg l^{-1}) NAA (0.5, 1, 1.5, 2 mg l^{-1}) + 2iP (0.25, 0.5, 1, 2, 4, 5 mg l^{-1}) IAA (1, 2, 3, 4 mg l^{-1}) + kinetin (0.025, 0.5, 1, 2, 3, 4, 5 mg l^{-1})

Table 5. Growth regulator combinations tried for callus induction in coconut

Basal medium	Growth regulator combinations
MS, Y_3 and Blaydes	2,4-D (0.25, 0.5, 1, 1.5, 2, 2.5, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 mg l^{-1}) NAA (0.5, 1, 1.5, 2, 3, 4 mg l^{-1}) IAA (0.5, 1, 2, 3, 4 mg l^{-1}) 2,4-D (0.25, 0.5, 1, 1.5, 3.5 mg l^{-1}) + Kinetin (0.25, 0.5, 1, 1.5, 2, 2.5 mg l^{-1}) IAA (1, 2, 3, 4 mg l^{-1}) + NAA (0.5, 1, 1.5, 2 mg l^{-1}) + BA (1, 2, 3, 4 mg l^{-1}) IAA (1, 2, 3, 4 mg l^{-1}) + Kinetin (0.25, 0.5, 1, 2, 4 mg l^{-1}) NAA (0.5, 1, 1.5, 2 mg l^{-1}) + IAA (1, 2, 3, 4 mg l^{-1}) + 2ip (0.25, 0.5, 1, 2 mg l^{-1}) 2,4-D (15, 18, 20, 22, 25 mg l^{-1}) + Picloram (0.25, 0.5, 1, 1.5, 2 mg l^{-1}) + IAA (0.5, 1, 2.5, mg l^{-1}) + NAA (1, 2 mg l^{-1}) + TDZ (0.025, 0.05, 0.075, 0.1, 0.5, 1.5, 2 mg l^{-1}) 2.4-D 1 mg l^{-1} + NAA (10, 15, 20 mg l^{-1}) + IAA 1 mg l^{-1} + TDZ 0.1 mg l^{-1} 2,4-D 1 mg l^{-1} + IAA (5, 10, 15 mg l^{-1}) + NAA 1 mg l^{-1} + TDZ 0.1 mg l^{-1} 2,4-D 15 mg l^{-1} + Picloram 0.5 mg l^{-1} + NAA 1 mg l^{-1} + TDZ 0.1 mg l^{-1} + Dicamba (0.25, 0.5, 1, 1.5 mg l^{-1})

Table 6. Media additives tried for callus induction and proliferation

Basal medium	Media additives
Y_3 + 2,4-D 15 mg l^{-1} + Picloram 0.5 mg l^{-1} + NAA 1 mg l^{-1} +TDZ 0.1 mg l^{-1}	Casein hydrolysate (0.5, 1, 2%) Mannitol (0.5, 1, 2%) Malt extract (0.5, 1, 2%)
Y_3 + 2,4-D 25 mg l^{-1} + IAA 1 mg l^{-1} + NAA 1 mg l^{-1} + TDZ 0.1 mg l^{-1}	Glutamine (100, 500 mg l^{-1}) Coconut water (15,30 %) Phloroglucinol 200 mg l^{-1}

Table 7. Growth regulator combinations tried for callus regeneration

Sl.No.	Growth regulator combinations
1	2,4-D 5 mg l^{-1} + Picloram 0.2 mg l^{-1} + IAA 0.25 mg l^{-1} + TDZ 0.2 mg l^{-1} + 2ip (0.5, 1, 2, 3, 4 mg l^{-1})
2	2,4-D 5 mg l^{-1} + Picloram 0.2 mg l^{-1} + IAA 0.25 mg l^{-1} + TDZ 0.1 mg l^{-1} + Zeatin (0.25, 0.5, 1, 2 mg l^{-1})
3	2,4-D 15 mg l^{-1} Picloram 0.5 mg l^{-1} + NAA 1 mg l^{-1} + TDZ 0.1 mg l^{-1} + Spermine (1, 2 µM)
4	2,4-D 15 mg l^{-1} + Picloram 0.5 mg l^{-1} + NAA 1 mg l^{-1} + TDZ 0.1 mg l^{-1} + Putrescine (5, 10 mM)
5	2,4-D 15 mg l^{-1} + Picloram 0.5 mg l^{-1} + NAA 1 mg l^{-1} + TDZ 0.1 mg l^{-1} + 2ip (0.5, 1, 2, 3, 4,5 mg l^{-1})
6	2,4-D 15 mg l^{-1} + Picloram 0.5 mg l^{-1} + NAA 1 mg l^{-1} + TDZ 0.1 mg l^{-1} + Zeatin (0.25, 0.5, 1, 2 mg l^{-1})

3.8.5 Influence of solidifying agents on callus induction of anthers

Influence of different concentrations of agar (6.5, 7.0, 7.5, 8 g l^{-1}) and phytagel (1.4, 1.6, 1.8, 2.0 g l^{-1}) on callus induction and performance of anther cultures were studied.

3.8.6 Effect of chilling treatment

Inflorescence was incubated at 4°C for varying time periods to study the effect of chilling treatment on *in vitro* response.

3.9 Embryogenesis/ Organogenesis

The calli were subcultured at four weeks interval to the same media and to media having different combinations of growth regulators (Table 7). The response of the calli was observed and recorded at fortnightly intervals.

The embryos formed were separated and kept for germination. Media combinations attempted for germination are presented below. The morphological changes to the embryo were observed at weekly intervals.

Media combinations tried for germination of embryo

Basal Y_3

Basal Y_3 +2,4-D 5 mg l^{-1} +Picloram 0.2 mg l^{-1} + NAA 0.25 mg l^{-1} +TDZ 0.1 mg l^{-1}

Basal Y_3 + BAP 1 mg l^{-1}

Basal Y_3 + BAP 3.0 mg l^{-1}

Basal Y_3 + Zeatin 0.5 mg l^{-1} + ABA 1 mg l^{-1}

Basal Y_3 + coconut water

Coconut water

3.10 Histological studies of callus

Rachillae explants of 45 cm length and calli at different stages of growth (6 and 8 weeks after callus induction) were selected for histological studies. The hand sections were taken, stained with saffranine and mounted in a drop of glycerine for microscopic observations. Photographs were taken under bright field using a Nikon50 research microscope.

RESULTS

4. RESULTS

The results of the study on "Response of immature inflorescence for *in vitro* regeneration in coconut (*Cocos nucifera* L.) are presented in this chapter.

4.1 Explant preparation

Different types of explants such as male flowers, female flowers, rachillae and anthers at premeiotic stage obtained from healthy immature inflorescence were used for inoculation. The unopened inflorescence collected from the field was immediately taken to the laboratory, where they were first washed thoroughly in tap water to remove all the dirt and soil particles. Explants were taken from inflorescence with and without surface sterilization. When the explants were extracted directly from the washed, unopened spathe; only 70 per cent of the cultures survived and 30 per cent were contaminated with bacteria and fungi (Table 8).

4.2 Fixing the premeiotic stage of anthers for inoculation

For using anther as explant, inflorescence selection was done by correlating the premeiotic stage with the length of inflorescence. Microscopic examination of anthers revealed that in inflorescence with a length of 40-50 cm, (Plate 2 and 3) anther lobes contain pollen mother cells (Plate 4) in just before the meiotic division, whereas inflorescence of length more than 55 cm showed the tetrad stage. Hence anthers dissected from inflorescence of 40 to 50 cm length were used to initiate culture.

4.3 CULTURE ESTABLISHMENT

4.3.1 Surface sterilization

The results of surface sterilization of explants using various sterilants are presented in Table 8. Since the flowers are enclosed inside the tightly clasped

Table 8. Effect of surface sterilization on culture establishment of different explants in coconut (3 weeks after inoculation)

Explant	Sterilant	Concentration (%)	Duration (minutes)	No. of explants inoculated	Fungal/ bacterial contamination (%)	Survival (%)
Male flowers	-	-	-	100	30	70
Female flowers	-	-	-	25	30	70
Rachillae	-	-	-	100	30	70
Entire spathe	Alcohol wiping	70	1	100	-	100
Male flowers	Soaking in $HgCl_2$	0.1	1	100	-	100
			2	100	-	100
			5	100	-	100
			7	100	-	100
			10	100	-	100
			12	100	-	90
			15	100	-	75
Female flowers	Soaking in $HgCl_2$	0.1	1	25	-	100
			2	25	-	100
			5	25	-	100
			7	25	-	100
			12	25	-	80
			15	25	-	72
Male flowers	Soaking in alcohol	70	1	100	-	100
			3	100	-	100
			7	100	-	90
			12	100	-	80
			15	100	-	100
female flowers	Soaking in alcohol	70	1	25	-	100
			3	25	-	100
			7	25	-	100
			12	25	-	90
			15	25	-	80
Rachillae	Soaking in $HgCl_2$	0.1	1	100	-	100
			3	100	-	100
			5	100	-	100
			7	100	-	100
			10	100	-	100
			12	100	-	95
			15	100	-	80

Plate 2. Inflorescence of coconut containing anthers at premeiotic stage

Plate 3. Rachillae of coconut carrying minute male flowers containing anthers at premeiotic stage

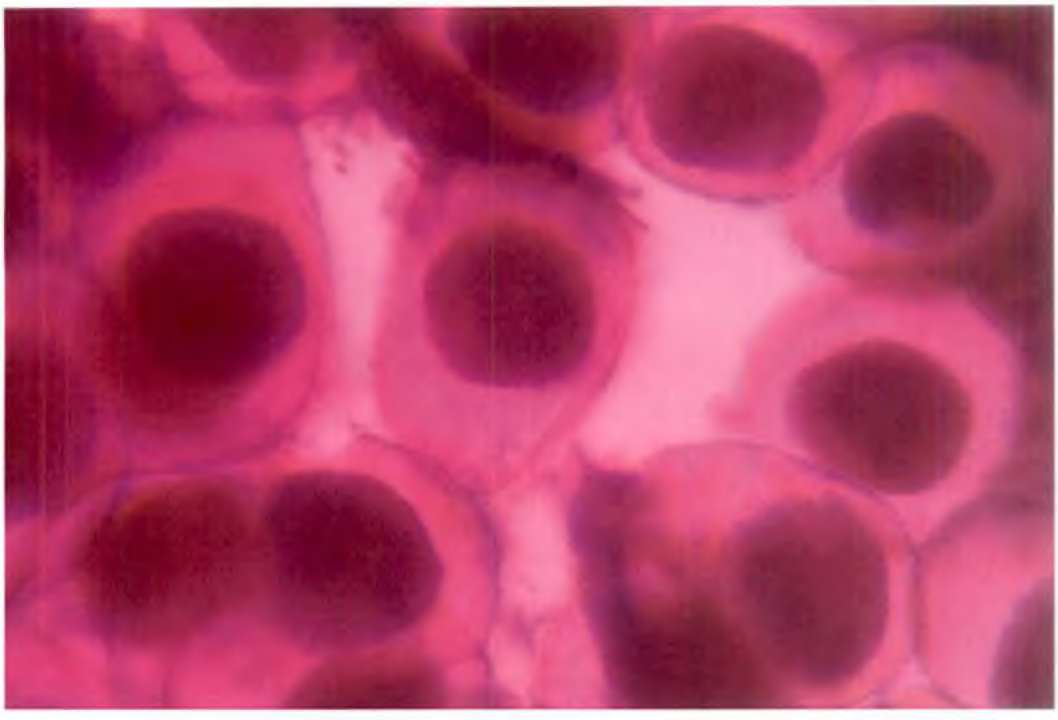


Plate 4. Pollen mother cells of coconut (100x)

spathe only mild sterilization procedure was required. All the different treatment like soaking the explants in 0.1 per cent $HgCl_2$ or 70 per cent alcohol or wiping the entire spathe with 70 per cent alcohol gave 100 per cent culture survival. The time of treatment varied from 1 to 12 minutes but even the minimum time of 1 minute soaking was found sufficient to prevent any contamination and there was 100 per cent culture survival.

Increasing the time of soaking or the concentration of the sterilant adversely affected the survival of the explants. The explants did not survive in higher concentration of the sterilant or at longer periods of sterilization. Soaking the explant in any chemical for more than 12 minutes was not desirable. It resulted in the browning and death of the explants. Even though higher concentration of the chemical sterilants fully controlled the contamination, it affected the survival of the explant as well.

4.3.2 Effect of different basal media and genotype

The effect of three different basal media on the culture establishment of the different explants of coconut is presented in Table 9. The explants responded better in Y_3 medium. In MS medium, positive response was shown only by female flowers in which callus induction was observed in 12 per cent of cultures. In Blaydes media, none of the explants tried responded. Y_3 media was identified as the best medium for *in vitro* culture of coconut. There was not much difference between Chavakad Green Dwarf and West Coast Tall in culture response. Callus induction percentage was almost same in both cultivars except in the callus induction of female flower, where WCT only responded in MS media.

4.3.3 Effect of different inflorescence explants on culture establishment

Among the four different explants tried, namely male flowers, anthers at pre meiotic stage, rachillae and female flowers, better response was observed for male

flowers and female flowers of immature inflorescence. However such explants only enlarged in size in Y_3 media without dedifferentiation. Immature female flowers of the variety WCT alone recorded callus induction. The rachillae and anthers at premeiotic stage also recorded callus initiation when cultured in Y_3 medium.

4.4 STANDARDISATION OF TREATMENTS TO OVERCOME POLYPHENOL INTERFERENCE

The exudation of polyphenols from the explants adversely affected their survival (Plate 5).

4.4.1 Effect of basal media in reducing polyphenol interference

Three different basal media such as MS, Y_3 and Blaydes media were assessed. Browning was least (35 per cent) in Y_3 media compared to MS (42 per cent) and Blaydes (45 per cent) media 3 weeks after inoculation (Table 10). So Y3 medium was selected and various treatments were tried to overcome the browning of immature anthers. The intensity of polyphenol exudation decreased in all the cases under dark condition.

4.4.2 Effect of pretreatments in reducing polyphenol interference in immature anther explants

Results of the pre-treatments tried are given in Table 11. Among the treatments, soaking the explant with 0.01 per cent ascorbic acid for 4 hrs was found to be the most effective for reducing the browning of the explants with a survival of 54.27 percentage followed by pretreatment with PVP (52.25%). The other two treatments viz., soaking with citric acid and sterile water also improved the survival of explants compared to control but the effect was less than that of ascorbic acid and PVP.

Table 9. Response of explants in different basal media in culture establishment of coconut

Medium*	Explant	No. of explants inoculated	Cultures responding (%)		Response
			Chavakad Green Dwarf	West Coast Tall	
MS	Male flower	100	Nil	Nil	-
	Female flower	25	Nil	12	Callus initiation
	Rachillae	100	Nil	Nil	-
	Anthers at premeiotic stage	100	Nil	Nil	-
Y_3	Male flower	100	51	48	Enlargement of male flower
	Female flower	25	48	52	Bulging of female flower
	Rachillae	100	17	20	Callus initiation
	Anthers at premeiotic stage	100	31	29	Callus initiation
Blaydes	Male flower	100	Nil	Nil	-
	Female flower	25	Nil	Nil	-
	Rachillae	100	Nil	Nil	-
	Anthers at premeiotic stage	100	Nil	Nil	-

* Sucrose, growth regulators and media additives added were the same in the cultures with a particular explant in different basal media

Table 10. Effect of different basal media in browning of explants

Basal media	Explant	Browning of explant (%)	Average (% of browning)
Y_3	Male flower	35	35
	Female flower	32	
	Rachillae	38	
	Immature anther	35	
MS	Male flower	41	42
	Female flower	38	
	Rachillae	46	
	Immature anther	43	
Blaydes	Male flower	44	45
	Female flower	42	
	Rachillae	49	
	Immature anther	45	

Fifty anthers (5 per tube) and 10 other explants (1 per tube) were tried per treatment

Plate 5. Discolouration of activated charcoal incorporated media due to polyphenol exudation

4.4.3 Effect of different media supplements in reducing browning of immature anther explants

The effect of activated charcoal alone and in combination with other antioxidants were investigated. Among the different concentrations of activated charcoal tried for the purpose, adding 0.2 per cent activated charcoal to the media was found to be ideal in reducing browning of explant (Table 12). Hence media containing 0.2 per cent activated charcoal combined with other antioxidants were tried. Among the different media supplements tried, activated charcoal (0.2 per cent) combined with PVP (0.1 per cent) was found to be the most effective in nullifying the polyphenol interference with 64 per cent explant survival (Table 13) followed by 0.01 per cent ascorbic acid and 0.2 per cent activated charcoal. Hence in the later investigations, Y_3 medium supplemented with 0.2 per cent activated charcoal and 0.1 per cent PVP was selected.

4.5 DIRECT ORGANOGENESIS

Immature female flowers, male flowers, rachillae and anthers at premeiotic stage were cultured to analyse their potential for organogenesis in various basal media.

4.5.1 Effect of cytokinins

Different levels of kinetin (0.25, 0.5, 1, 2, 4, 8, 10, 15 mg l^{-1}) were tried for induction of direct organogenesis in various explants. But none of the treatments showed positive response in different explants irrespective of the media tried (MS, Y_3, Blaydes).

Media containing BA showed high percentage of browning. At lower levels of BA (up to 2 mg l^{-1}), the male flower buds present on the immature rachillae pieces enlarged, thickened and opened with cream colour (Plate 6). At higher concentration, BA exhibited severe browning and death of the different explants in all

Table 11. Effect of pretreatments on reducing polyphenol interference

Basal medium	Treatment	Concentration (%)	Survival percentage of cultures 4 weeks after incubation
Y_3	Ascorbic acid	0.01	54.27
	Citric acid	0.1	48.70
	PVP	0.1	52.25
	Sterile distilled water	-	45.00
	Control	-	35.60

Fifty immature anthers (5 per tube) were tried per treatment

Table 12. Effect of different concentrations of activated charcoal in reducing browning of explant

Basal medium	Concentration of activated charcoal (%)	Survival percentage of cultures 4 weeks after incubation
Y_3	0.05	38.60
	0.1	42.90
	0.15	49.50
	0.2	56.15
	0.25	54.05
	0.3	52.00

Fifty immature anthers (5 per tube) were tried per treatment

Table 13. Effect of different media supplements in reducing browning of immature anther explants

Basal medium	Media supplements	Concentration	Survival percentage of cultures 4 weeks after incubation
Y_3	PVP	0.1	51.00
	Activated charcoal	0.2	56.05
	Citric acid	0.01	48.70
	Citric acid + Activated charcoal	0.01 + 0.2	58.50
	PVP + Activated charcoal	0.1 + 0.2	64.00
	Ascorbic acid + Activated charcoal	0.01 + 0.2	61.00

Fifty immature anthers (5 per tube) were tried per treatment

the basal media tried (Plate 7). Above 5 mg l^{-1}, anthers turned brown and later dried one month after incubation in all the media tried.

Different levels of 2iP (0.25, 0.5, 1, 2, 3, 4, 5 mg l^{-1}) were tried for direct organogenesis in various explants. All the explants showed enlargement in Y_3 media. But the other two media showed no response in all the explants.

4.5.2 Effect of combinations of auxins and cytokinins

Different concentrations and combinations of auxins (IAA and NAA) and cytokinins (BA, kinetin and 2iP) were attempted for direct organogenesis in different basal media. Male and female flowers enlarged in size in certain combinations of auxins and cytokinins tried. Anthers remained fresh without any change for a period of 30 days after incubation and later they turned brown. Inflorescence rachillae turned brown within 2 weeks of incubation without any response (Table 14). Even though different combinations and concentrations of growth regulators were tried (Appendix 2) organogenesis was not achieved from any part of the inflorescence.

4.6 INDIRECT ORGANOGENESIS

4.6.1 Callus induction

Callus inducing ability of five different auxins namely 2,4-D, NAA, IBA, IAA and picloram and cytokinins 2iP and TDZ were evaluated at various concentrations in different basal media.

4.6.2 Role of 2,4-D in callus induction

The presence of 2,4-D was found to be essential for the callus induction of all the explants tried. Among the various concentrations of 2,4-D tested, better induction and growth of callus in immature anthers and rachillae could be obtained at 15 to 30 mg l^{-1}. At higher concentrations (above 35 mg l^{-1}), severe browning of tissue

Plate 6.

Male flower buds at the time of inoculation

Enlargement and opening of male flower buds

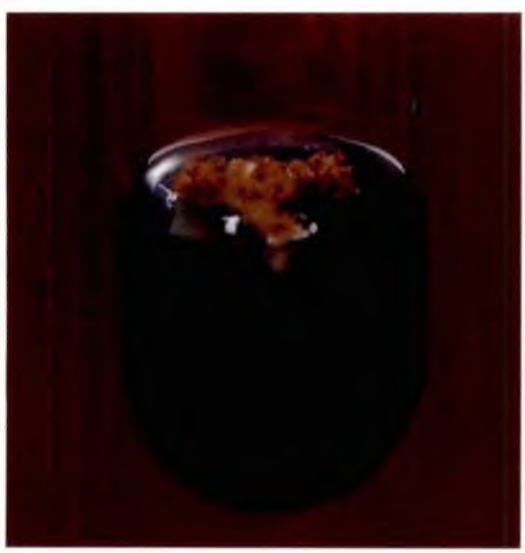


Plate 7. Response of inflorescence explants in Y_3 medium containing BA

Table 14. Response of different combinations of auxin and cytokinin for direct organogenesis

Media	Explant	No. of explant inoculated	Response at 2 weeks interval		
			2	4	6
NAA 2 mg l^{-1} + 2iP 2 mg l^{-1}	Male flower	9	Enlargement of flower	Enlargement of flower	Enlargement of flower
	Female flower	5	Fresh	Enlargement of flower	Enlargement of flower
	Rachillae	9	Turned brown	Turned brown	Turned brown
	Immature anther	45	Fresh	Swelling of anther	Slight browning
NAA 2 mg l^{-1} + BA 5 mg l^{-1}	Male flower	9	Enlargement of flower	Browning	Complete browning
	Female flower	5	Fresh	Enlargement of flower	Enlargement of flower
	Rachillae	9	Slight brownin g	Complete browning	Complete browning
	Immature anther	45	Fresh	Slight browning	Complete browning
IAA 3 mg l^{-1} + Kinetin 2 mg l^{-1}	Male flower	9	Enlargement of flower	Enlargement of flower	Enlargement of flower
	Female flower	5	Fresh	Enlargement of flower	Enlargement of flower
	Rachillae	9	Slight browning	Complete browning	Complete browning
	Immature anther	45	Fresh	Swelling of anther	Slight browning
NAA 1 mg l^{-1} + 2iP 2 mg l^{-1}	Male flower	9	Enlargement of flower	Enlargement of flower	Enlargement of flower
	Female flower	5	Enlargement of flower	Enlargement of flower	Enlargement of flower
	Rachillae	9	Slight browning	Complete browning	Complete browning
	Immature anther	45	Fresh	Swelling of anther	Slight browning

Basal media - Y_3 + 5 per cent sucrose

was observed. When explants were cultured with 2,4-D without activated charcoal, browning was observed in all explants. The inclusion of activated carbon played a key role in minimizing browning. Browning was minimum at range of 25-30 mg l^{-1} 2,4-D. Enlargement of male and female flowers could be obtained at 10-30 mg l^{-1} 2,4-D. Effects of 2,4-D concentration on callus induction and browning of explant are given in Table15 a & b.

4.6.3 Effect of 2,4-D along with other auxins

Anthers cultured in 0.5 mg l^{-1} Picloram added media containing 2,4-D gave callus initiation (Plate 8) whereas for rachillae explants required 1 mg l^{-1} of Picloram for callus induction. Above 1 mg l^{-1} callus induction decreased. Picloram was found to be essential for callus induction of coconut inflorescence explants.

Medium containing 2,4-D 15 mg l^{-1}, Picloram 0.5 mg $l^{-1}$and NAA 1 mg l^{-1} gave maximum callus induction (20%) for immature anther (Plate 9). Whereas 2,4-D along with Picloram gave only 15 per cent callus induction. Hence it was found that NAA 1 mg l^{-1} increased the callus induction of immature anther.

Callus induction of rachillae explants was obtained in Y_3 medium containing 2,4-D 15 mg l^{-1}, Picloram 1 mg l^{-1} and IAA 1 mg l^{-1}(Plate10). IAA 1mg l^{-1} was found to be needed for the callus induction of rachillae instead of NAA 1 mg l^{-1} for immature anther. Male flower responded by enlarging in a medium containing 2,4-D 15 mg l^{-1}, Picloram 0.5 mg $l^{-1}$and NAA 1 mg l^{-1}. Female flower responded by inducing callus in MS medium containing 2,4-D, Picloram and NAA (Plate 11).

Anther like structures were formed from the immature anther at higher concentration of 2,4-D (22 to 25 mg l^{-1}) along with IAA and NAA (Plate12). Effects of 2,4-D along with other auxins are given in the Table 16.

Table 15a. Effect of 2,4-D on callus induction and browning of rachillae and immature anther explants

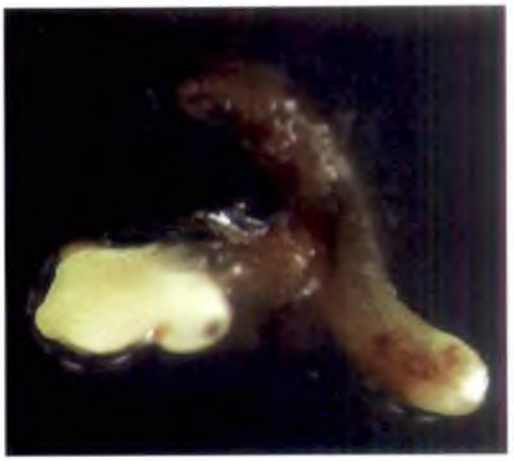
2,4-D concentration (ppm)	No of explants inoculated	Callus induction(%) of rachillae and immature anther	Browning after 2 months (%)	
			rachillae	immature anther
1-5	9	Nil	80	70
5-10	9	Nil	76	68
10-15	9	Nil	72	65
15-20	9	33.33	67	60
20-25	9	22.22	64	57
25-30	9	11.11	60	55
30-35	9	Nil	67	58
35-40	9	Nil	70	64
40-45	9	Nil	74	67
45-50	9	Nil	77	72

Immature anther (5 per tube), Rachillae (1 per tube)

Table 15b. Effect of 2,4-D on callus induction and browning of male and female flower of coconut

Concentration of 2,4-D (ppm)	No. of explants inoculated	Response after 2 months (Enlargement of explant)		Percentage of browning after 2 months	
		Male flower	Female flower	Male flower	Female flower
1 - 5	10	Nil	Nil	70	66
5 - 10	10	Nil	Nil	66	62
10 - 15	10	20	20	64	60
15 - 20	10	30	40	62	59
20 - 25	10	20	10	59	57
25 - 30	10	10	10	56	54
30 - 35	10	Nil	Nil	61	58
35 - 40	10	Nil	Nil	65	62
40 - 45	10	Nil	Nil	68	66
45 - 50	10	Nil	Nil	70	69

Anther at pollen mother cell stage (10x)



Callus initiation from anther lobe (10x)

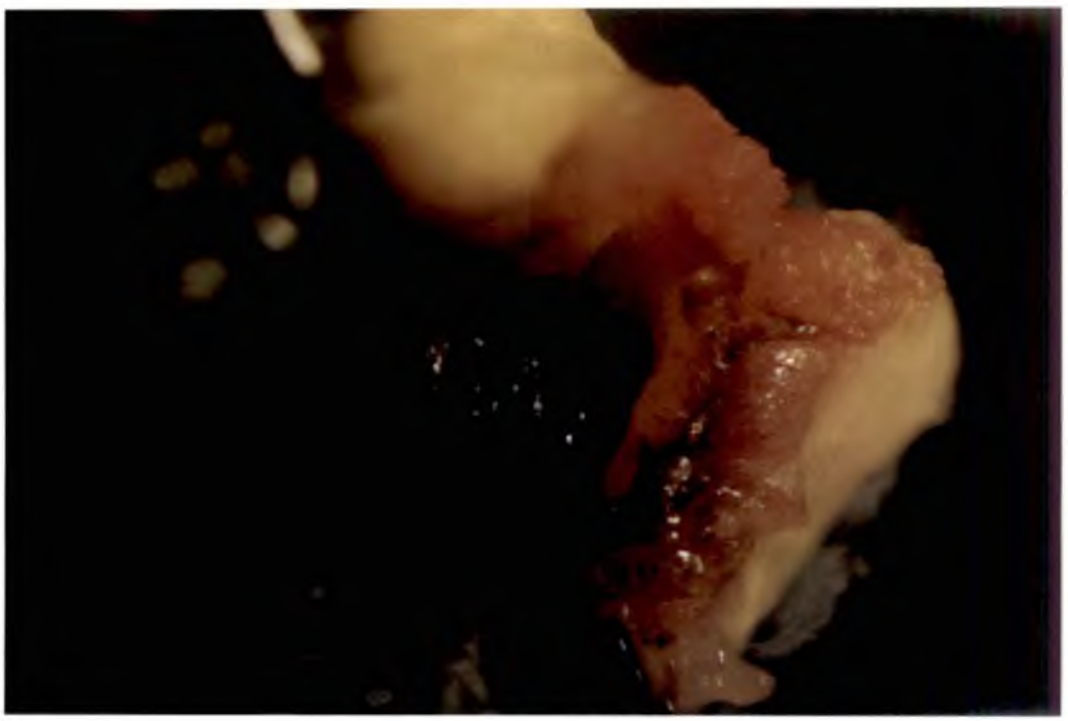


Plate 8. Callus initiation from immature anther in Y_3 medium containing 2,4-D 18mg l^{-1} and picloram 0.5mg l^{-1} (10x)

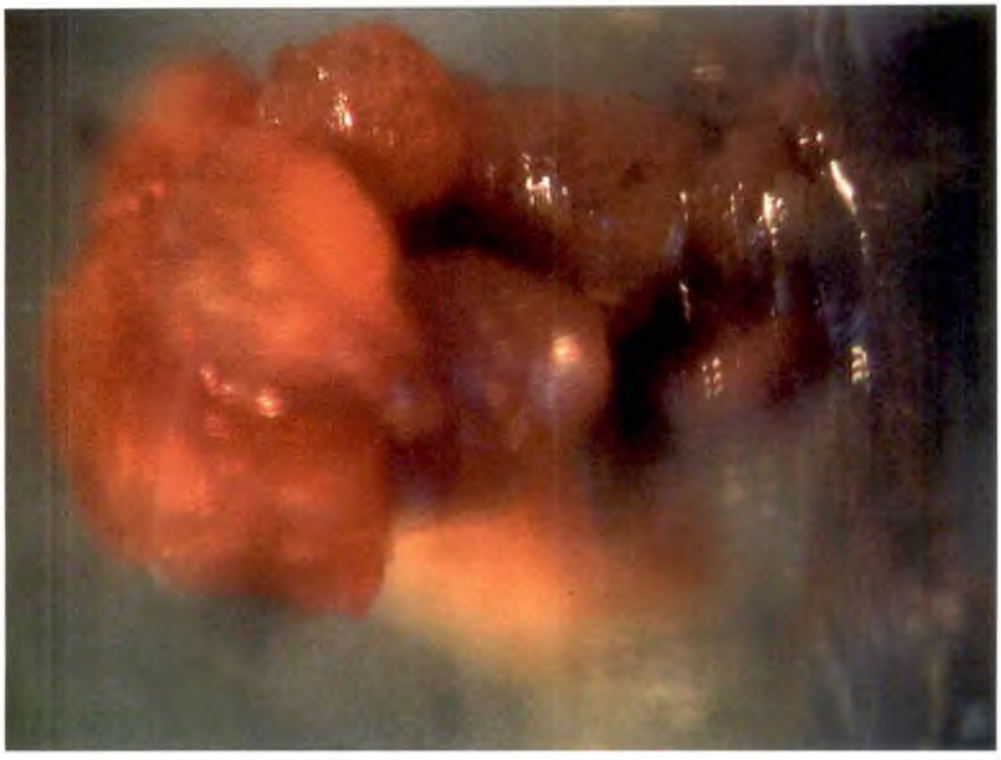
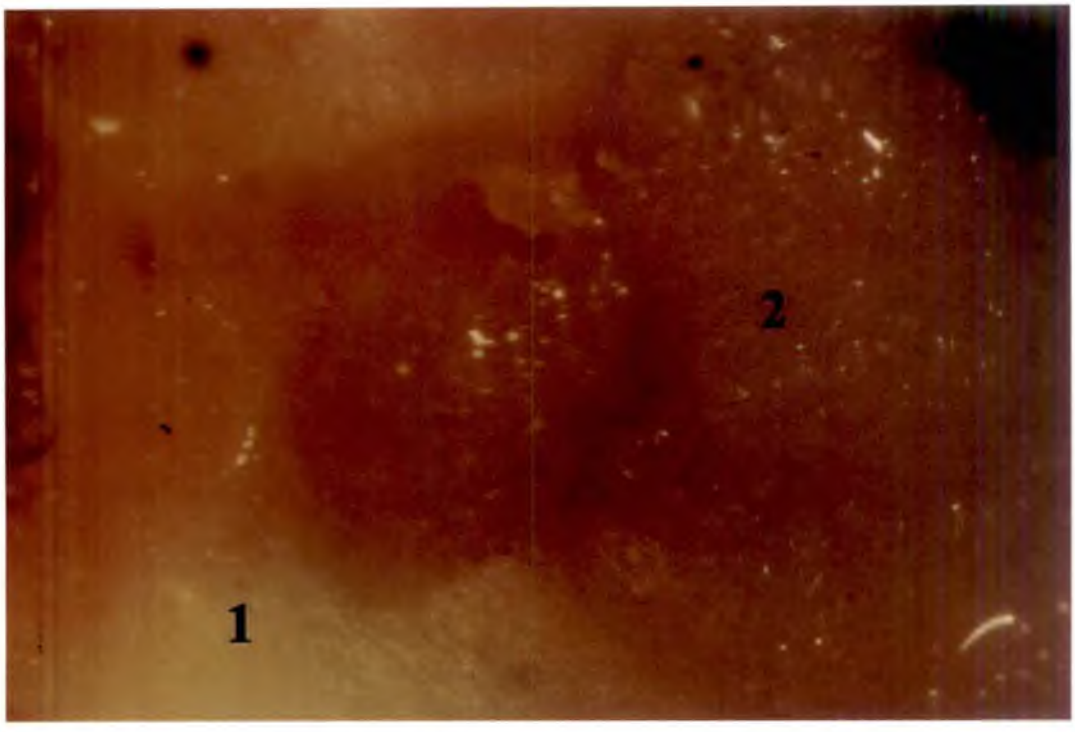


Plate 9. Callus induction and embryoid formation from immature anther in Y_3 medium containing 2,4-D 15mgl^{-1}, picloram 0.5mgl^{-1} and NAA 1mgl^{-1} (10x)



1 Rachillae (explant)

2 Callus

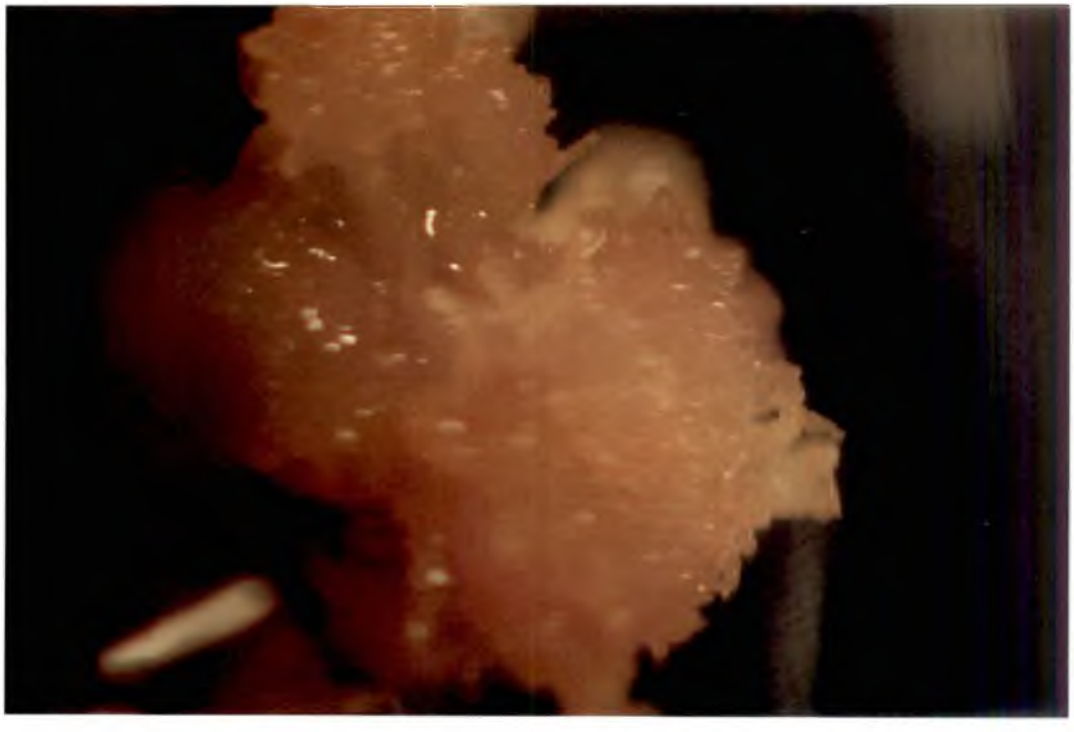


Plate 10. Callus initiation from rachillae in Y_3 medium containing 2,4-D 15mgl^{-1}, picloram 1mgl^{-1}and IAA 1mgl^{-1} (10x)

Female flower at the time of inoculation

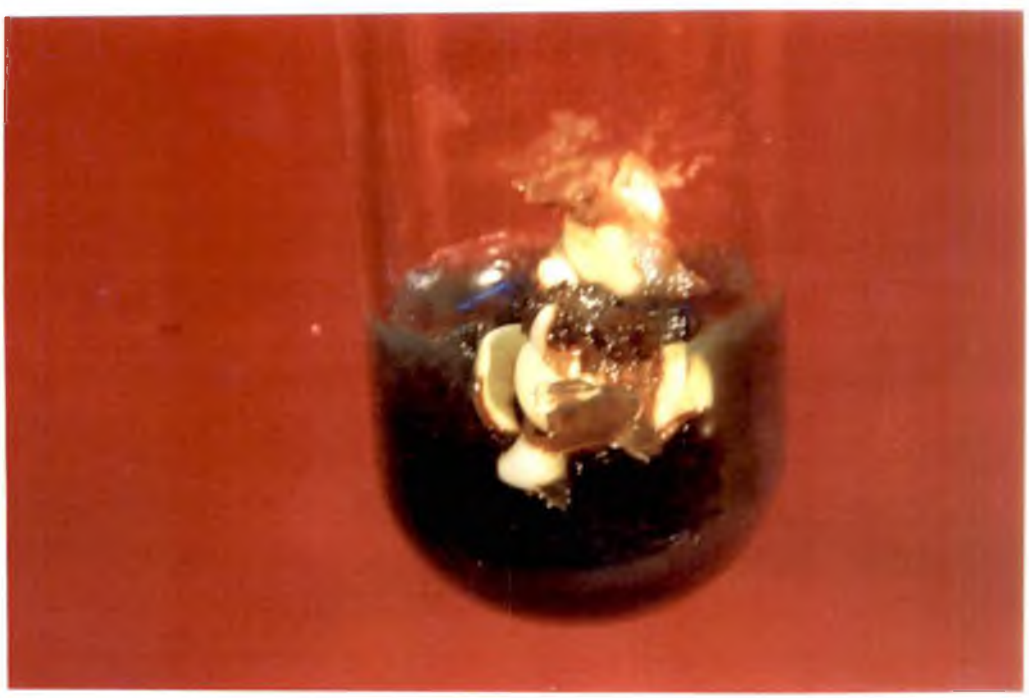
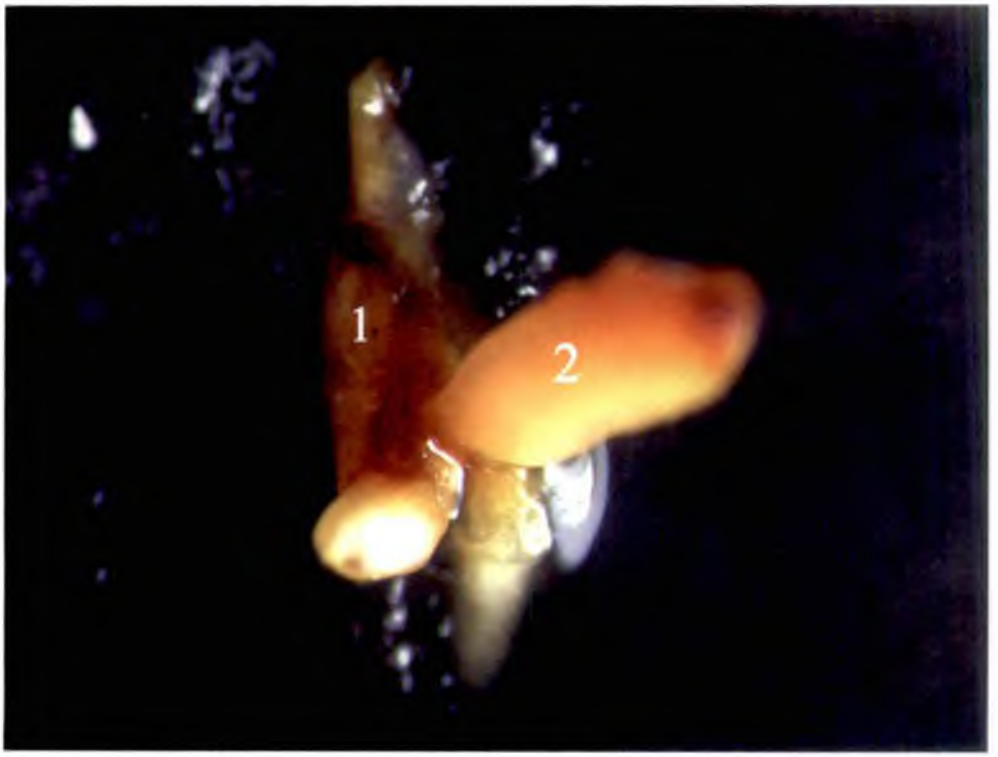


Plate 11. Callusing of female flower in MS medium supplemented with 2,4-D, picloram and NAA



1. Anther (explant)

2.Anther like structure

Plate 12. Formation of anther like structure from immature anther in Y_3 medium containing 2,4-D 25mgl^{-1} + IAA 1mgl^{-1} + NAA 1mgl^{-1}

Table 16. Effect of 2,4-D along with other auxins (Picloram, NAA, IAA)

Sl. No.	Explant	Growth regulator mg l^{-1}	Response	% of response
1	Immature anther	2,4-D 18+ Picloram 0.5	Callus induction	15
2	Female flower	MS + 2,4-D 15 + Picloram 0.2 + NAA 1	Callus induction	10
3	Immature anther	2,4-D 25 + IAA 1 + NAA 1	Formation of anther like structure	10
4	Immature anther	2,4-D 22+IAA 2	Formation of anther like structure	10
5	Rachillae	2,4D 15 + Picloram 1 + IAA1	Callus induction	10
6	Male flower	2,4-D 15 + Picloram0.5 + NAA1	Enlargement of male flower	25
7	Immature anther	2,4-D 15 + Picloram 0.5 + NAA 1	Callus induction	20

Basal media- Y_3 + 5 per cent sucrose

50 anthers (5 per tube) and 10 other explants (1 per tube) were tried per treatment.

4.6.4 Effect of 2,4-D along with TDZ and 2iP

Lower concentration of cytokinin appears to be necessary in the production of calloid. Effects of 2,4-D along with TDZ and 2iP are given in the Table 17. The frequency of callus induction was increased and the browning of explants reduced when TDZ was added along with auxin (2,4-D) in the callus induction medium. Callus induction was significantly influenced by the concentration of TDZ. Concentration of 0.1 mg l^{-1} was the most effective for promoting callus induction.

The segment of inflorescence primordia when inoculated in Y_3 media supplemented with 2,4-D 20 mgl^{-1} and TDZ 0.075mgl^{-1} responded with elongation and enlargement. The inflorescence primordia of size 0.25cm elongated and developed in to 2.3cm with in 2 months. Rudimentary protuberance were observed on the central axis representing spikelet (Plate 13) .

2,4-D along with 2iP was found to have no response for coconut inflorescence explants.

4.6.5 Effect of other growth regulator combinations in callus induction

Effect of different concentrations of Picloram and TDZ is shown in Table 18. At higher concentration of TDZ (2 mg l^{-1} and above) rapid browning and eventual death of explant was observed. Maximum callus induction was achieved in a medium containing picloram 0.5 mg l^{-1} and TDZ 0.1 mg l^{-1} (33.3%) for immature anther (Plate 14), where as rachillae explants required higher concentration of picloram (1mg l^{-1}) and TDZ 0.1 mg l^{-1}(Plate 15).

Basal Y_3 was used to study the effect of 2,4-D, TDZ and picloram on callus induction and browning of explants. 2,4-D 15 mg l^{-1} alone resulted in 11.1 per cent callus induction where as 15 mg l^{-1} 2,4-D along with 0.1 mg l^{-1} TDZ resulted in 22.1 per cent callus induction. 2,4-D alone resulted in 60 per cent browning where as

Table 17. Effect of 2,4-D along with TDZ and 2iP

Sl. No.	Explant	Growth regulators mg l^{-1}	Response	Percentage of response
1	Immature anther	2,4-D 15 + TDZ 0.1	Callus induction	30
2	Immature anther	2,4-D 15 + TDZ 0.075	Callus induction	20
3	Immature anther	2,4-D 25 + TDZ 0.075	Formation of anther like structure	40
4	Immature anther	2,4-D 20 + TDZ 0.1	Formation of anther like structure	30
5	Immature anther	2,4-D 22 + TDZ 0.5	Formation of anther like structure	20
6	Floral primordia	2,4-D 20 + TDZ 0.075	Enlargement of floral primordia	10
7	Female flower	2,4-D 15 + TDZ 0.1	Nil	Nil
8	Male flower	2,4-D 15 + TDZ 0.1	Nil	Nil
9	Rachillae	2,4-D 15 + TDZ 0.05	Callus induction	10
10	Rachillae	2,4-D 15 + TDZ 0.1	Callus induction	20
11	Immature anther	2,4-D 15 + 2iP 1	Nil	Nil
12	Immature anther	2,4-D 15 + 2iP 2	Nil	Nil
13	Rachillae	2,4-D 15 + 2iP 1	Nil	Nil

Basal media- Y_3 + 5 per cent sucrose

50 anthers (5 per tube) and 10 other explants (1 per tube) were tried per treatment.

Inflorescence primordia at the time of inoculation

Inflorescence primordia three weeks after inoculation

Plate 13. Development of inflorescence primordia in Y_3 medium containing 2,4-D 20mgl^{-1} and TDZ 0.075mgl^{-1}

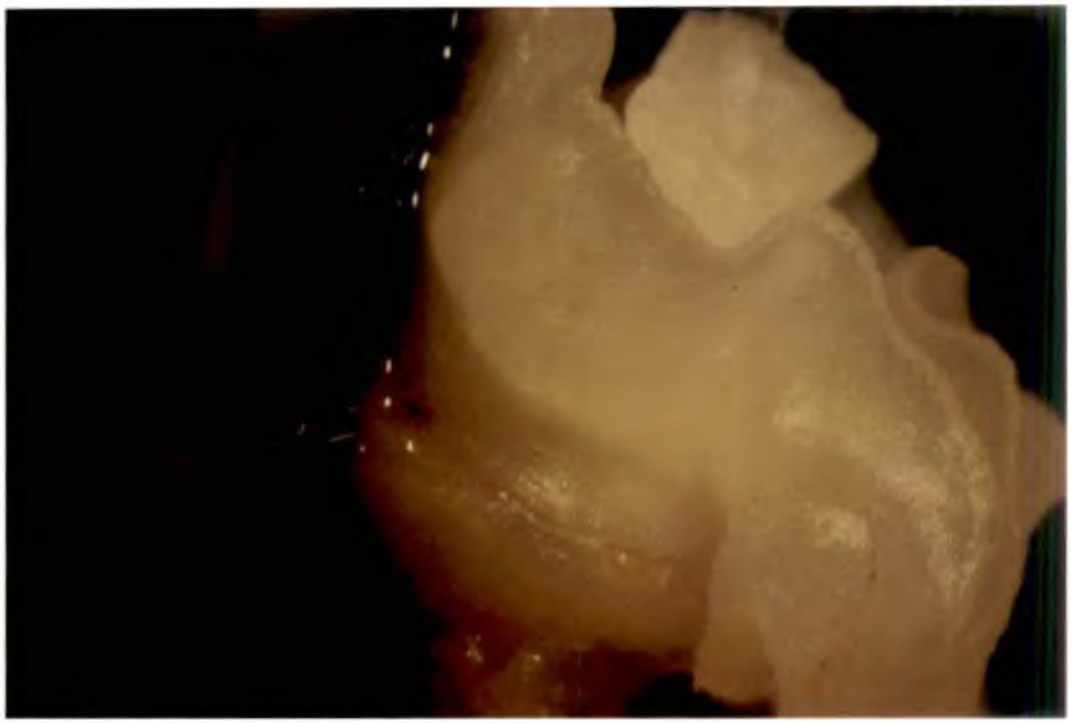


Plate 14. Callus induction from immature anther in Y_3 medium containing 2,4-D 15mgl^{-1}, picloram 0.5mgl^{-1} and TDZ 0.1mgl^{-1} (10x)

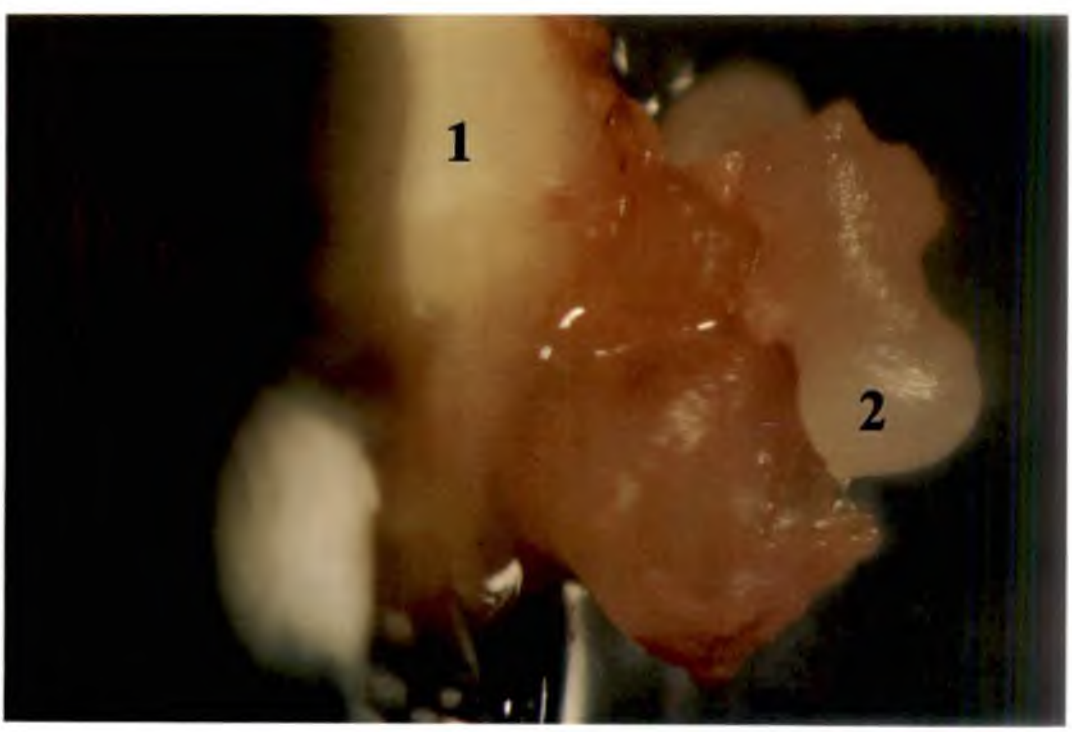


Plate 15. Callus induction from rachillae in Y_3 medium containing 2,4-D 15mgl^{-1}, picloram 1mgl^{-1} and TDZ 0.1mgl^{-1} (10x)

1. Rachillae (explant)
2. Callus

Table 18. Effect of different concentrations of picloram and TDZ on callus induction of coconut inflorescence explant

Sl.No.	Growth regulator combination mg l^{-1}	Response
Anther		
1	Picloram 0.5 + TDZ 0.1	Embryogenic callus induction (33.3%)
2	Picloram 1 + TDZ 0.1	Callus induction (22.2%)
3	Picloram 2 + TDZ 0.5	Enlargement of anthers
4	Picloram 2 + TDZ 1.5	Browning of explant
5	Picloram 2 + TDZ 2	Rapid browning of explant
Rachillae		
6	Picloram 0.5 + TDZ 0.1	Callus induction (22.2%)
7	Picloram 1 + TDZ 0.1	Callus induction (33.3%)
8	Picloram 1.5 + TDZ 0.1	Remaining as fresh
9	Picloram 2 + TDZ 1.5	Rapid browning of explant
10	Picloram 2 + TDZ 2	Rapid browning of explant

Medium - Y_3 + 2,4-D 15 mg l^{-1} + 5 per cent sucrose
Forty five anthers (5 per tube) and nine rachillae (1 per tube) were tried per treatment.

2,4-D along with TDZ resulted in 50 per cent browning after 4 weeks of incubation. Picloram along with 2,4-D and TDZ resulted in higher percentage of callus induction (33.3 %). But browning was not affected by the addition of Picloram. Percentage of callus induction and browning of explants using the three callus induction media is shown in (Figure 1)

Other growth regulators like Dicamba, zeatin, kinetin, IBA and GA were found to have no response on coconut inflorescence explants. Among the different combinations of growth regulators tried, (Appendix 3) responsive media containing auxin and cytokinin are shown in the Table 19.

4.6.6 Effect of sucrose on callus induction

The effects of levels of sucrose on the frequency of callus induction (Table 20.) revealed that 5 per cent sucrose was the optimum concentration for maximum callus induction in immature anther. Callus induction was observed at sucrose levels ranging from four to seven per cent sucrose concentration. There was no callus induction below four and above seven per cent of sucrose.

4.6.7 Effect of media additives

Various growth supplements like coconut water, casein hydrolysate, mannitol and glutamic acid were added along with other growth regulators in different concentration to find out their effect on culture establishment and growth (Table 21).

There was higher percentage (40%) of callus induction when Mannitol (1%) was added to 2,4-D, Picloram and TDZ containing medium. Moreover it also reduced the browning of the explant thus maintaining the cultures fresh for longer periods (3 months).

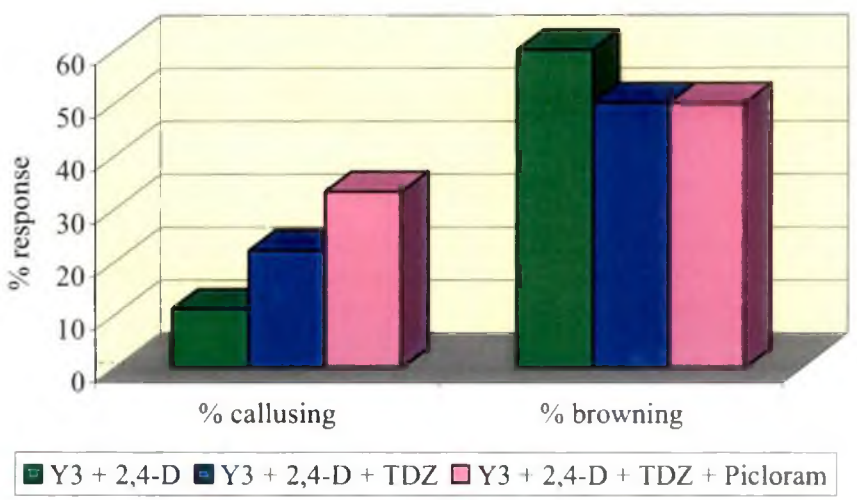


Fig. 1. Percentage callus induction and browning of explants using the three callus induction media

Table 19. Combinations and concentrations of growth regulators giving response to coconut inflorescence explants

Sl. No.	Explant	Growth regulator (mg l^{-1})	Response							
			1 month	2 months	3 months	4 months	5 months	6 months	7 months	8 months
1	Anther	2,4-D 18, picloram 0.5	Swelling of anther	Swelling of anther	Callus induction	Callus proliferation	Slight browning of callus	Complete browning of callus	Complete browning of callus	Complete browning of callus
2	Anther	2,4-D 15, picloram 0.5, NAA I, TDZ0.1	Swelling of anther	Swelling of anther	slight browning of anther	Callus induction	Callus proliferation	Slight browning of callus	Embryo formation	Browning of embryo
3	Rachillae	2,4-D 15, picloram I, IAA I, TDZ 0.1	Nil	Increase in size	Increase in size	Callus induction	Slight browning of callus	Complete browning of callus	Complete browning of callus	Complete browning of callus
4	Anther	2,4-D 15,NAA 1, TDZ 0.1	Swelling of anther	Swelling of anther	Slight browning of anther	Callus induction	Complete browning of callus	Complete browning of callus	Complete browning of callus	Complete browning of callus
5	Female flower	MS + 2,4-D 15, picloram 0.2, NAA I	Bulging of flower	Bulging of flower	Callus induction	Slight browning of callus	Complete browning of callus	Complete browning of callus	Complete browning of callus	Complete browning of callus
6	Floral primordia	2,4-D 20, picloram 0.5, IAA I, TDZ 0.1	Nil	Enlargement of floral primordia	Enlargement of floral primordia	Slight browning	Complete browning	Complete browning	Complete browning	Complete browning
7	Anther	2,4-D 25, IAA I, TDZ 0.075	Swelling of anther	Swelling of anther	Formation of anther like structure	Slight browning	Complete browning	Complete browning	Complete browning	Complete browning
8	Anther	2,4-D 25, IAA 0.5, TDZ 0.075	Swelling of anther	Swelling of anther	Formation of anther like structure	Slight browning	Complete browning	Complete browning	Complete browning	Complete browning
9	Anther	2,4-D 20, IAA I, TDZ 0.1, NAA I	Swelling of anther	Swelling of anther	Formation of anther like structure	Slight browning	Complete browning	Complete browning	Complete browning	Complete browning
10	Anther	2,4-D 22, IAA 2, TDZ0.1	Swelling of anther	Swelling of anther	Formation of anther like structure	Slight browning	Complete browning	Complete browning	Complete browning	Complete browning

Table 20. Effect of different levels of sucrose on frequency of callus induction of anthers

Sl.No.	Sucrose level (%)	Callus induction (%)
1	2	Nil
2	3	Nil
3	4	11.1
4	5	33.3
5	6	22.1
6	7	11.1
7	8	Nil
8	9	Nil

Forty five anthers (5 per tube) were tried per treatment

Table 21. Effect of different media additives on callus induction of immature anther

Media additive	Response	% of response
No additive	Callus induction	30
mannitol 0.5%	Callus induction	35
mannitol 1%	Callus induction	40
mannitol 1.5%	Callus induction	30
Coconut water 15%	Callus induction	30
Coconut water 30%	Formation of anther like structure	40
glutamine 100 mg l^{-1}	Callus induction	30
glutamine 500 mg l^{-1}	Callus induction	30
phloroglucinol 200 mg l^{-1}	Inhibited culture growth	Nil
Casein hydrolysate 0.5%	Callus induction	30
Casein hydrolysate 0.5%	Callus induction	30
Casein hydrolysate 2%	Callus induction	30

Basal media- Y_3 + 5 per cent sucrose, 2,4-D 15 mg l^{-1}, Picloram 0.5 mg l^{-1}, NAA 1, mgl^{-1} and TDZ 0.1 mg l^{-1}

Hundred explants were tried per treatment

When tender coconut water was added at two different concentration (15 and 30 per cent) along with other growth regulators, it was found that the lower level (15 per cent) of coconut water had no favourable effect. But at the higher level, anther like structures were formed from the immature anthers (Plate 12). Cytological studies detected pollen grains from this anther like structure (Plate 16). None of the levels of casein hydrolysate, malt extract and glutamic acid used with different combination of growth regulators have any favourable effect on callus induction and proliferation. Phloroglucinol, a phenolic auxin synergist not only gave any favourable results but it reduced the percentage of culture establishment when incorporated in the medium.

4.6.8 Effect of chilling treatment

Pretreatment of inflorescence at 4°C for 24hrs and 30 hrs doubled the percentage of callus induction. Similarly cold treatment reduced the browning of explant considerably and increased the survival percentage (Table 22). Treatment of flower buds above 30 hrs decreased the callus induction and culture survival.

4.6.9 Influence of solidifying agent on anther development

Influence of agar and phytagel as solidifying agents in the basal medium of Y_3 + 5 per cent sucrose + 2,4-D 15 mg l^{-1}, picloram 0.5 mg l^{-1}, NAA 1 mg l^{-1} and TDZ 0.1 mg l^{-1} on anther development were studied (Table 23). Varying concentrations of phytagel from 0.14 to 0.2 per cent along with growth regulators were tried and it was found that phytagel at 0.18 and 0.16 per cent was the best with respect to anther swelling (++).

Addition of varying concentrations of agar (0.65 to 0.8%) showed that the performance was best at a concentration of 0.75 per cent with good anther swelling (+++). Agar at 0.75 per cent was found to be superior to phytagel as the solidifying agent in the medium for anther development.

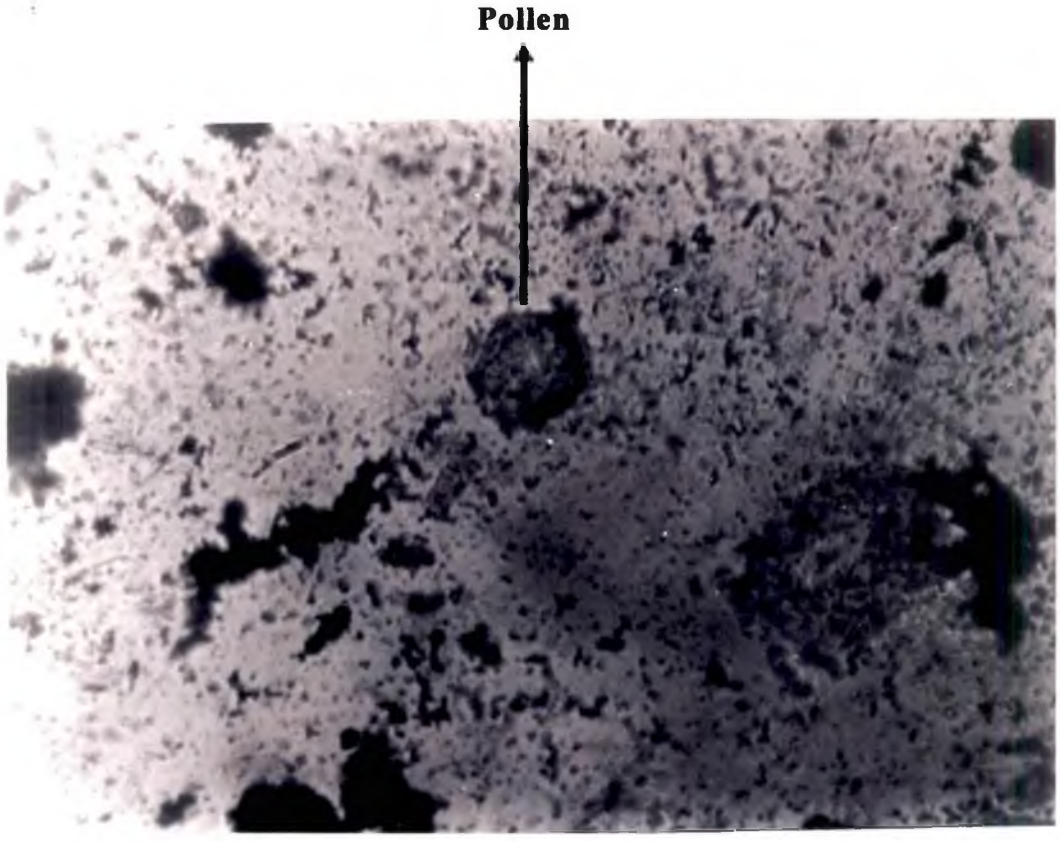


Plate 16. Pollen grain detected from the anther like structure (50x)

Table 22. Effect of chilling treatment on callus induction and survival percentage of immature anther

Sl. No.	Treatment time (hrs)	Callus induction (%)	Survival after 2 months (%)
1	0	20	35
2	3	20	38
3	24	40	51
4	30	40	55
5	48	30	47
6	52	10	43
7	72	10	40

Media- Y_3 + 5 per cent sucrose, 2,4-D 15 mg l^{-1}, Picloram 0.5 mg l^{-1}, NAA 1 mg l^{-1} and TDZ 0.1 mg l^{-1}

Fifty immature anthers (5 per tube) were tried per treatment

Table 23. Influence of varying concentrations of gelling agents on anther swelling

Sl.No.	Gelling agent	Anther swelling
1	0.20% Phytagel	+
2	0.18% Phytagel	++
3	0.16% Phytagel	++
4	0.14% Phytagel	+
5	0.8% agar	++
6	0.75% agar	+++
7	0.7% agar	++
8	0.65% agar	++
9	Control (liquid media)	–

Medium - Y_3 + 5 per cent sucrose, 2,4-D 15 mg l^{-1}, Picloram 0.5 mg l^{-1}, NAA 1 mg l^{-1} and TDZ 0.1 mg l^{-1}

Scoring '++++' very good; '+++' good, '++' moderate; '+' low, '-' noresponse

4.6.10 GROWTH OF CALLUS CULTURE

The callus tissue was separated from the explant, and was subcultured regularly at four-week intervals. The growth of callus was very slow. After 2-3 subcultures callus became dried and dead. Subculturing of callus tissue along with parent tissue also did not favour regeneration. The outer cells turned brown and the callus volume did not change.

4.7 Embryogenesis

The callus induced in different media combinations were subcultured in the same medium as well as to the media with reduced concentration of auxin (Appendix 4). Repeated subculturing of calli in the same medium containing 2,4-D 15 mg l^{-1}, picloram 0.5 mg l^{-1}, NAA 1 mg l^{-1}, TDZ 0.1 mg l^{-1} resulted in formation of globular embryos (Plate 17). Though the frequency was less, embryogenesis was observed in Y_3 medium supplemented with combinations of 2,4-D, picloram, NAA and TDZ. The embryo observed was globular in shape with smooth surface.

4.8 Embryo germination and further growth

The embryo formed was subcultured to various media combinations (Table 24). None of the combinations attempted could support further growth and germination of embryo. They became brown but remained alive. Embryo maturation was observed to be a very slow process.

4.9 Histological study

Sections through immature rachillae explants revealed a ground parenchyma with a ring of several vascular bundles (Plate 18). The bundles were enclosed in thick sclerenchymatous bundle sheaths. Several bud traces were also observed which connect the male flower primordia with the central vascular system.

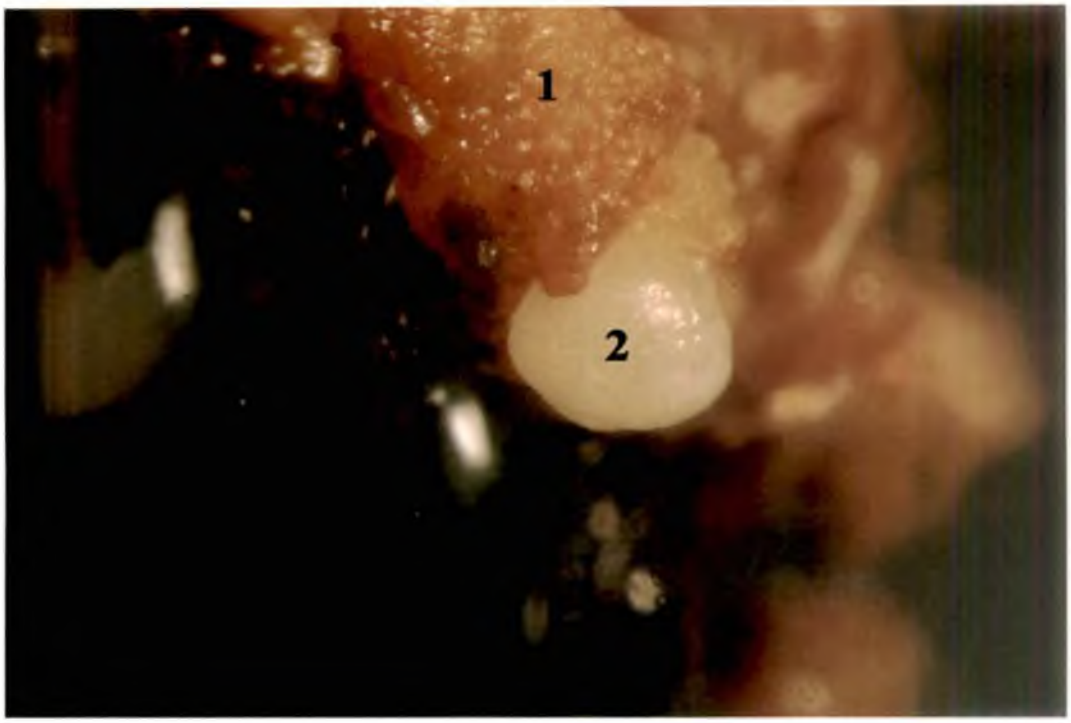


Plate 17. Formation of globular embryo from immature anther derived callus (10x)

1. Callus

2. Embryo

Table 24. Subculture response of somatic embryo of *Coconut nucifera* in various media combinations

Sl.No.	Treatment (subculture media)	Response after	
		1 Month	2 months
1	Basal Y_3	Creamy white, surviving	Brown, surviving
2	Basal Y_3 + 2,4-D 5 mg l^{-1}, Picloram 0.2 mg l^{-1} + NAA 0.25 mg l^{-1} + TDZ 0.1 mg l^{-1}	Creamy white, surviving	Brown, surviving
3	Basal Y_3 + BA 1.0 mg l^{-1}	Creamy white, surviving	Brown, surviving
4	Basal Y_3 + BA 3.0 mg l^{-1}	Creamy white, surviving	Brown, surviving
5	Basal Y_3 + Zeatin 0.5 mg l^{-1} + ABA 1.0 mg l^{-1}	Creamy white, surviving	Brown, surviving
6	Basal Y_3 +coconut water	Creamy white, surviving	Brown, surviving
7	Coconut water	Creamy white, surviving	Brown, surviving

The histological study of early stages of callus showed actively dividing meristematic cells forming a primary callus (Plate19). In some of the calli further development resulted in the initiation of vascular tissues in the form of narrow elongated darkly stained cells, the procambium and then the tracheary elements scattered in the parenchymatous callus tissue (Plate 20). In some of the calli at later stage, the derivatives cells from these parenchyma started differentiating into structures similar to the bud traces found in the explant anatomy (Plate 21). These vascular nodules were found to be concentrated near the peripheral regions of the callus tissue (Plate 22). Later some of these nodules were found to get separated from the ground parenchyma by a layer of epidermal cells forming distinct primordium (Plate 23).

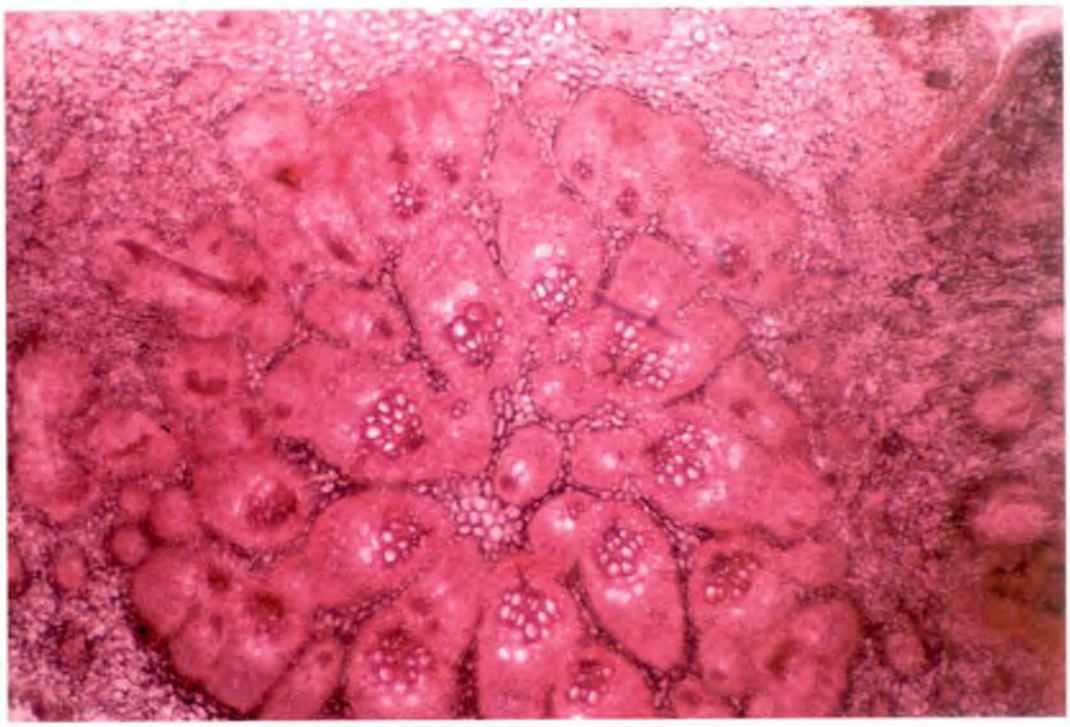


Plate 18. Section of immature rachillae explant showing vascular bundles (50x)

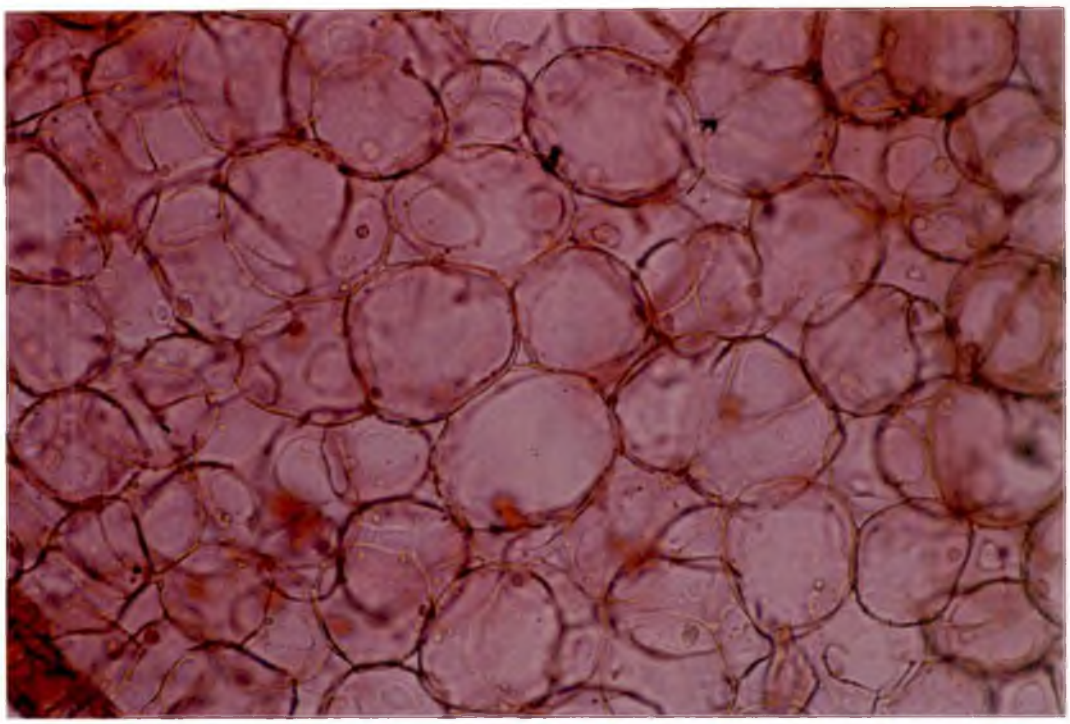


Plate 19. Section of anther derived calli showing actively dividing parenchymatous cells (50x)

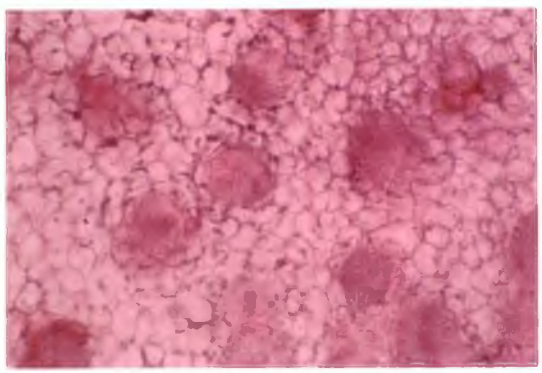


Plate 20.

Vascular bundle formation 6 weeks after callus induction (50x)

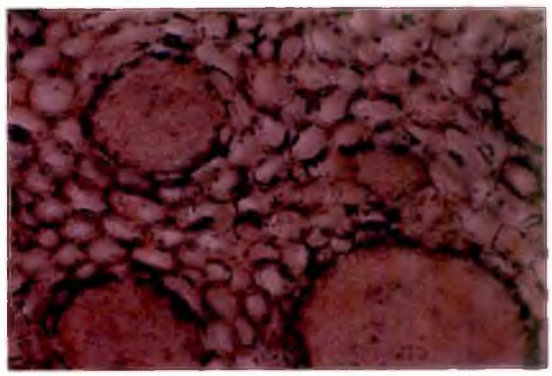


Plate 21.

Vascular bundle formation 8 weeks after callus induction (50x)

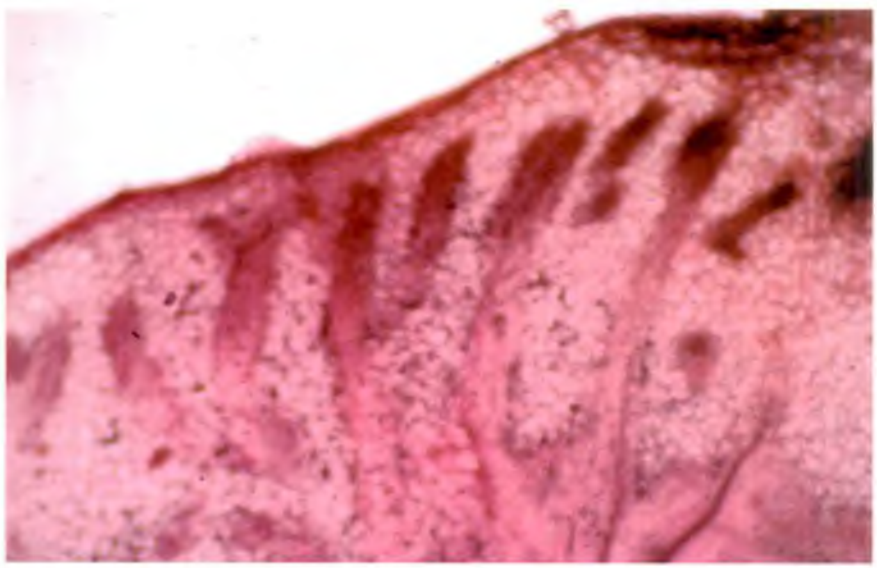
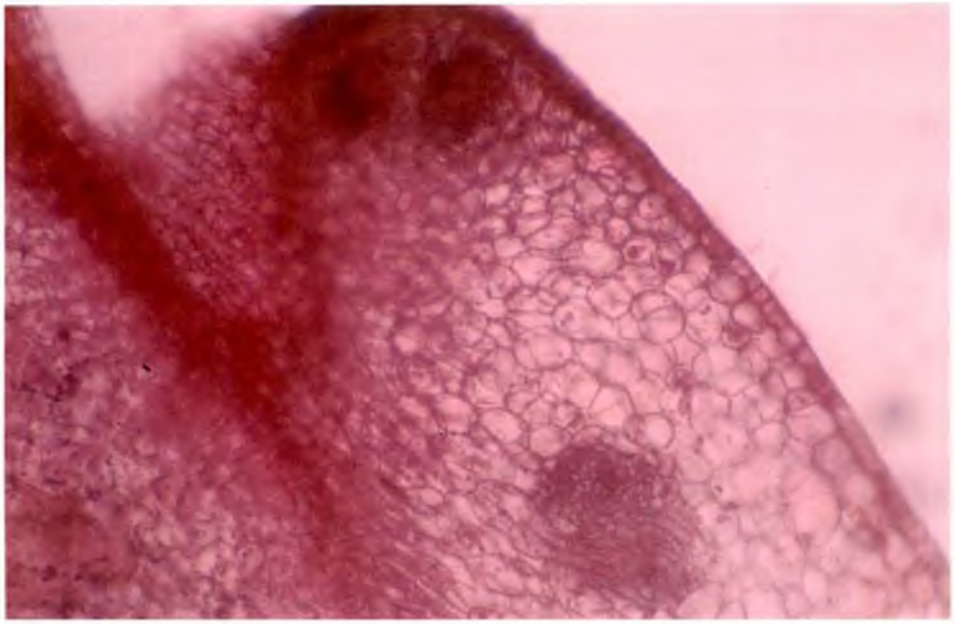


Plate 22. Vascular connections towards the perifery of the callus tissue (50x)



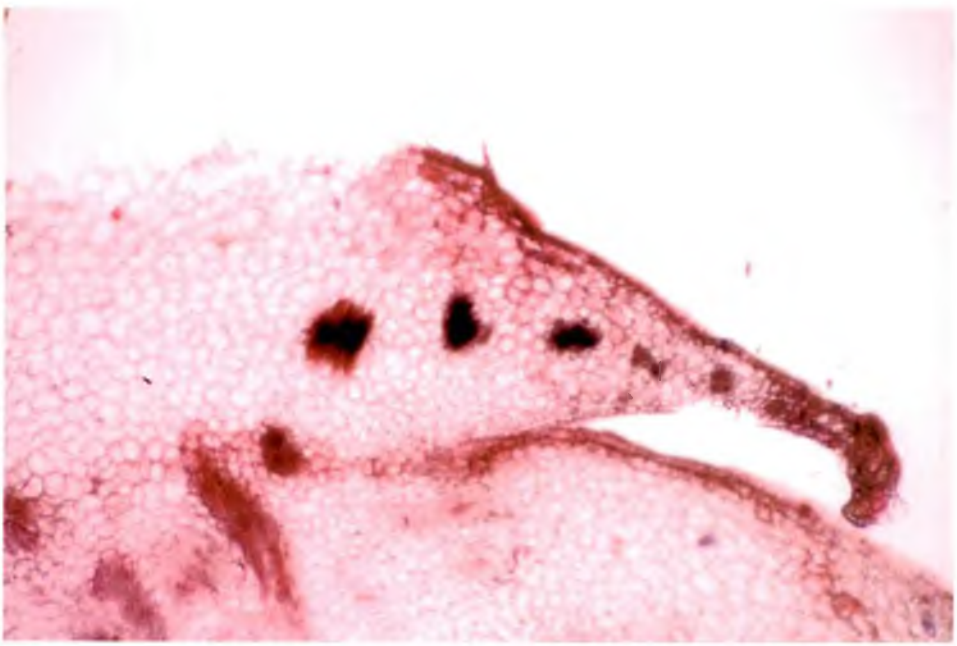


Plate 23. Section of callus showing high meristematic activity and embryonic unit (50x)

DISCUSSION

5. DISCUSSION

Though tissue culture and plantlet initiation in monocotyledons was considered difficult, several investigators have been able to induce plantlets from numerous herbaceous monocotyledons *in vitro* (Constabel *et al.*, 1971). However successful reports of production of plantlets from palms through tissue culture is limited (Eeuwens, 1976; Eeuwens and Blake, 1977; Reuveni, 1972).

The coconut industry is currently burdened with a number of problems such as senility of existing plantations and prevalence of various pests and diseases. Due to its long pre-bearing age, high heterozygosity, long interval between generations and exclusively seed propagated nature, crop improvement in coconut is a difficult and time consuming programme. The predominantly cross-fertilized nature of the palm results in enormous variability in the seedling progenies. Only about 20 per cent of the estimated annual requirement of quality planting material to replace senile and disease ravaged plantations is met. The productivity of a coconut plantation could be increased significantly if high yielding homogeneous materials are available.

In view of these facts, the standardization of *in vitro* propagation technique has great relevance in the production of true to type propagules at a cheaper rate within a reasonable time.

Several attempts have been reported earlier from all over the world on micropropagation of coconut. The crop is reported to be highly recalcitrant with very little response under *in vitro* culture (Eeuwens, 1976; Apavatjrat and Blake, 1977; Sugimura and Salvana, 1989; Areza *et al.*, 1993; Chandralekha, 1997). Based on the leads obtained in the previous reports, the present study was programmed. The results obtained in the efforts taken to standardize the *in vitro* propagation technique in coconut (*Cocos nucifera* L.) at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during 2005-2006 are discussed in this chapter.

5.1 Culture establishment

5.1.1 Selection of explants

Being a monocot, the meristematic growing tips are limited in coconut and hence option goes to other tissues that show high cell division and differentiation. Earlier reports in coconut suggested inflorescence parts as the best explant (Orense *et al.*, 1993). Hence in this study emphasis was given on inflorescence derived explants.

Successful reports in the tree species *Hevea brasiliensis* on somatic embryogenesis and plant regeneration from anther tissue at premeiotic stage inspired us to concentrate upon anthers of such stage in coconut in the present study. The cells at the premeiotic stage could be expected to be highly juvenile since it is getting prepared to enter the reproductive phase and thus high cellular activity with highly functional DNA and RNA.

5.1.2 Surface sterilization

Since the inflorescence is tightly enclosed inside the spathe, only mild sterilization procedure was required. The most effective surface sterilization was achieved by wiping or soaking the explants in 70 per cent alcohol for 1 minute or by soaking them in 0.1 per cent $HgCl_2$ for 1 minute. The immature inflorescence could withstand up to 12 minutes soaking without cell damage. Bonga (1982) has advocated the use of alcohol alone or in combination with other chemicals for disinfection of tree crops.

Exposure of the explants to the sterilant beyond 12 minutes in 0.1 per cent of $HgCl_2$ was found to be deleterious. This is in agreement with findings of Thanh – Tuyen and De Guzman (1983). Use of mercuric chloride as surface sterilant for coconut endosperm plugs, rachillae and leaf has been reported by Gupta *et al.* (1984).

After surface sterilization, rinsing the explants thoroughly with sterile distilled water ensured the removal of the traces of the chemical present on the explants. Hu and Wang (1983) opined that it is necessary to wash the tissues twice or thrice in sterile distilled water to ensure dilution of the chemical. Krishnan and Seeni (1994) also recommended the need for rinsing the explants five to six times in sterile distilled water before inoculation. $HgCl_2$ if retained in explant would definitely interfere with cell division and differentiation under *in vitro* culture.

5.1.3 Basal medium

In the present study, Y_3 medium was observed to be the best basal medium to support inflorescence derived explants of coconut (Table 10). All the explants tried namely rachillae, anthers at premeiotic stage, male flower and female flower responded better in this medium. Compared to other media tried, Y_3 medium contained higher levels of potassium, iodine and nitrate form of nitrogen. The suitability of Y_3 medium for *in vitro* culture of palm has been reported by several other workers Eeuwens (1976) and Branton and Blake (1983) in coconut, Sharma *et al.* (1980) in date palm (*Phoenix dactylifera* L.).

Blaydes media has been reported to give better response for anther culture in coconut (Blaydes, 1966; Chandralekha, 1997). However anthers at premeiotic stage used in the present study did not show any preference to Blaydes media.

5.1.4 Polyphenol interference

Coconut is reported to contain polyphenols that ooze out into the medium during culture. Survival of the explants reduces if polyphenol interference is left unchecked. Explant establishment of coconut thus required special procedures to escape or avoid problems that are associated with polyphenol exudation. Similar problems have been reported in other woody plant species by Lloyd and Mc Cown (1980) and Mathew (1995).

Significant reduction in polyphenol exudation was observed when the cultures were incubated in the dark. Similar observation was reported earlier by Sugimura and Salvana (1989) in coconut. Creasy (1968) has reported that the activity of enzymes concerned with both biosynthesis and oxidation of phenols was increased in presence of light.

Among the various media additives tried in the present study, intensity of polyphenol browning was decreased and the survival of tissues was increased effectively by adding activated charcoal (0.2%) and PVP (0.1%) to the media. This is in agreement with the result obtained by Siqueira and Inoue (1991) in coconut. Similar results have been reported in banana (Bhaskar, 1991), coffee (Sreenath *et al.*, 1995) and rubber (Seneviratne and Wijesekara,1996). 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). It is known to adsorb toxic metabolites released into the medium (Fridborg *et al.*, 1978). The problem of browning in the present study was also reduced by soaking the explant in Ascorbic acid (0.01%). This is in agreement with the report by Areza *et al.* (1993) in coconut.

5.1.5 Response of Explants

Among the four different inflorescence derived explants used in the present study, the immature anther and rachillae responded better under *in vitro* culture. The immature anthers confirmed to be at premeiotic stage responded better than all other explants. The stage of anther development in the present study was confirmed through cytological observations (Plate 4). These anthers are expected to respond better due to their activity (DNA and RNA) before entering reproductive phase. Such an attempt with anthers at premeiotic stage is the first of its kind in coconut. The immature anther under *in vitro* culture responded in three different ways. In the best media identified 40 percentage of cultures remained as such with no size enlargement and were dried within 2 months. Thirty percent of anthers remained

viable with callus induction within 3 months. The cells inside such anthers were observed to be dividing, but not in the sporophytic pathway. There was only undifferentiated cells with no tetrads or pollen grains (Plate 19). Such cells indicated potential for differentiation. Since the cells are at premeiotic stage, the regenerants would be maternal in nature. Few of the anthers (30 per cent) elongated slightly and remained viable for 3 months without any callusing. Cell division was also observed in such anthers with stray cases of pollen development. The number of pollen in such anthers were observed to be very few (2 to 4).

The differential response of the immature anther under *in vitro* culture might be due to the difference in their physiological maturity. Though inflorescence with optimum size was selected for *in vitro* culture, the stage of development of anthers may vary slightly among the male flowers. The significance of correct stage of anther has also been reported in rubber (Jayasree *et al.*, 1999) the only crop in which true to type plants have been regenerated from immature anther at premeiotic stage.

Blake and Eeuwens (1982) have also reported the better response of rachillae explants in coconut. The immature rachillae used as explant consists of several flower primordia, which are active source of growth hormones. Thus higher levels of native growth hormone content might have supported the *in vitro* performance of rachillae. Callusing was observed in 30 percentage of rachillae explants in Y_3 media supplemented with 2,4-D 15 mg l^{-1}, picloram 1 mg l^{-1}, IAA 1 mg l^{-1},TDZ 0.1 mg l^{-1} (Table 19).

The immature male and female flower used as explants did not give any positive response. The flowers recorded slight increase in size through bulging due to development of floral parts (Plate 6).

5.2 Direct organogenesis

The chances for direct organogenesis in coconut is remote since it is a recalcitrant monocot species with no suckering habit and presence of a single growing primordia.

In the present study, the minute male flower primordia present on the immature rachillae enlarged in size under *in vitro* culture and opened with cream colour. Female flowers, anthers at premeiotic stage and rachillae were also found enlarged in various basal media. BAP above 5.0 mg l^{-1} resulted in browning of various explants. Similar result has been reported in coconut by Ebert (1993). Cytokinins are known to stimulate the synthesis of polyphenols (Bergmann, 1964; Asahira and Nitsch, 1969).

Anthers initially enlarged in size and then turned brown without any positive response. Similarly Ebert (1993) reported that after two weeks from initial culture, most of the anthers turned brown in coconut.

Direct organogenesis was not achieved in the present study even though various treatments were tried for different explants. Reports are not available on the differentiation of shoot buds directly in coconut tissue culture.

5.3 Callus induction and proliferation

Indirect organogenesis/ embryogenesis is another option for *in vitro* regeneration in coconut. Attempts were made with four explants for dedifferentiation and redifferentiation. All together 350 media combinations were tried to stimulate cell division in this recalcitrant crop. The response was very slow even at higher levels of growth regulator combinations.

Several reports exist on callus induction and proliferation in coconut. Eeuwens (1976) tried to improve callus formation and growth by optimizing the mineral composition of the culture media. Micropropagation of coconut from any tissue requires a strong auxin stimulus and 2,4-D is the most commonly used auxin for callogenesis and somatic embryogenesis (Blake and Eeuwens, 1982; Hornung, 1995). Verdeil *et al.* (1994) reported that this hormone was essential for the activation and division of the undifferentiated cells in the explant to produce calli.

In the present study, lower levels of 2,4-D was inefficient for inducing callus from inflorescence explants (Table 15). Embryogenic calli was obtained at higher levels of 2,4-D (15 to 30 mg l^{-1}) (Fig 2). The high phenolic interference warranted incorporation of activated charcoal in the culture media of coconut. This might be one reason for adding higher levels of 2,4-D for callus induction. In oil palm the incorporation of activated charcoal in the media is reported to increase the levels of auxin requirement 10 times (Paranjothy and Othman, 1982). Inclusion of activated charcoal reduced the availability of hormonal substance. It is therefore necessary to apply an abnormally high concentration of auxins (Tisserat, 1979; Zaid and Tisserat, 1983) up to a certain level not toxic to tissue.

In the present investigation NAA and IAA at lower concentration i.e. 1 mg l^{-1} induced callus in anther and rachillae respectively. Earlier investigators (Blake and Eeuwens, 1982) have reported IAA and NAA to be less effective for callus induction in coconut. The positive response obtained in the present study might be due to highly immature stage of anther and rachillae used as explant.

Instead of callus induction in the high auxin media certain abnormal proliferations were observed from the anther and inflorescence primordia. It was interesting to note anther like structures developing from immature anther. These structures were confirmed to be anthers due to presence of pollen like structure (Plate 12). This can be referred as recurrent androgenesis – a phenomenon similar to recurrent embryogenesis. The segments of inflorescence primordia were elongated and

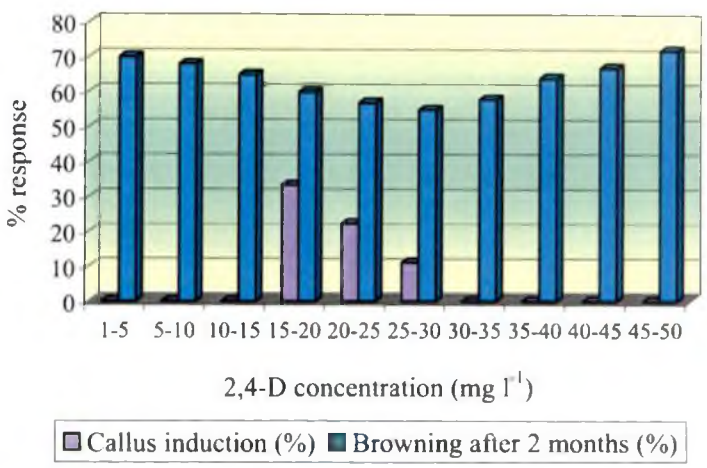


Fig. 2. Effect of 2,4-D concentration on callus induction and browning of inflorescence explants

rudimentary protuberance were observed on the central axis representing spikelet. This differential 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 35 mg l^{-1} resulted in severe browning and high polyphenol oxidase activity that induced drying of the tissues indicating their sensitivity to 2,4-D (Fig 2). According to Verdeil *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. Sugimura and Salvana (1989) have reported low browning incidence in coconut when the level of 2,4-D ranged from 20 to 30 ppm and with 0.25 per cent activated charcoal incorporated in the callus induction medium. Ebert (1993) has also reported excessive levels of 2,4-D to cause browning and growth inhibition in coconut cultures.

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

TDZ is a substituted phenyl urea that has been reported to exert high cytokinin activity and aid regeneration in many woody plant species (Huetteman and Preece, 1993). The use of TDZ in association with 2,4-D was effective for callus induction. Callus induction was significantly influenced by the concentration of TDZ. Concentration of 0.1 mg l^{-1} TDZ along with 2,4-D 15 mg l^{-1} was found to be most effective for promoting callus induction in coconut. There is no report on role of TDZ on *in vitro* culture of coconut. Neuman *et al.* (1998) used 2,4-D and TDZ in the primary media for immature cotyledonary tissue cultures of black walnut. TDZ at higher concentration (above 2 mg l^{-1}) was toxic, causing excessive browning and eventual death of the explant.

Picloram has been used successfully in tissue culture of various plants with no adverse effect on the callus or subsequently regenerated plants (Conger *et al.*, 1983; Fitch and Moore, 1990; Groll *et al.*, 2001). In the present study, 0.5 to 1.0 mg l^{-1}

picloram was found to be needed for callus induction of inflorescence explants. Positive response of picloram was reported for callus formation in Pejibaye palm (Valverde *et al.*, 1987) and *Phoenix canariensis* (Huong *et al.*, 1999). It suggests that there is an effective uptake and mobilization of this growth regulator coupled with rapid metabolization at target site.

5.3.1 Effect of media additives on callus induction

Media additives like coconut water, casein hydrolysate and phloroglucinol were tried so as to study their effect on callus induction.

Enhancement in callus induction by adding casein hydrolysate has been reported by Chandralekha (1997) in coconut. Similar observations have been reported by George and Sherrington (1984) in several plant species both in monocots and dicots. Casein hydrolysate or glutamic acid added to the medium stimulated growth of inflorescence *in vitro* significantly (Blake and Eeuwens, 1982). In the present investigation, addition of casein hydrolysate in the culture medium gave no favourable response in coconut.

Incorporation of tender coconut water in the callus induction media could induce anther like structures. The favourable effects of coconut water in the promotion of growth and differentiation of excised tissues and organ have been attributed to the presence of cytokinins and gibberllin like substances in it (Straus and Rodney, 1960). Blake and Eeuwens (1982) have also reported that addition of coconut water gave an improvement in culture growth.

Phloroglucinol, a phenolic auxin synergist commonly used in tree species, gave no favourable results in coconut. Instead, it reduced the percentage of culture establishment when incorporated in the medium. This result is contradictory to that reported by Chandralekha (1997) in coconut and Mallika *et al.* (1992) in *Theobroma*

cacao. Addition of glutamine was also found to be unsuitable for callus proliferation. The result is contradictory to that reported by Eeuwens (1978) in coconut.

5.3.2 Effect of sucrose on frequency of callus induction

Carbon source in the culture medium was altered at different levels to study their favourable influence. In the present study 5 per cent sucrose was found to be the optimum level for callus induction (Fig 3). Gamborg *et al.* (1974) suggested that the efficiency of nitrate and ammonium ions can depend on sucrose concentration and the effect of cytokinins on cell division may also be dependent on the sugar availability. At higher concentrations, sucrose in addition to being a carbohydrate source may possibly play some other roles like osmoregulation at the time of induction (Binding, 1972).

5.3.4 Influence of solidifying agent in the medium

The type and concentration of solidifying agent affected the swelling of anther considerably (Table 23). Agar at 0.75 per cent was found to support the immature anther. This may be due to the high phenolic interference in phytagel containing media and better absorption of nutrients by the explants from the agar-containing medium.

5.3.5 Effect of chilling treatment

Inflorescence incubated for 24 hrs and 30 hrs at 4°C responded better for callus induction (Fig 4). It has been suggested that cold pretreatments delay the senescence of anthers and destroy the close association of tapetum and pollen pore (Pelletier and Henry, 1974). The sensitivity of anthers to cold stress (Zhang and Croes, 1983) has been proposed to be related to their endogenous proline content. Though there are no favourable reports in tree species, beneficial effects have been reported in other monocot species like rice (Gupta and Borthakur, 1987).

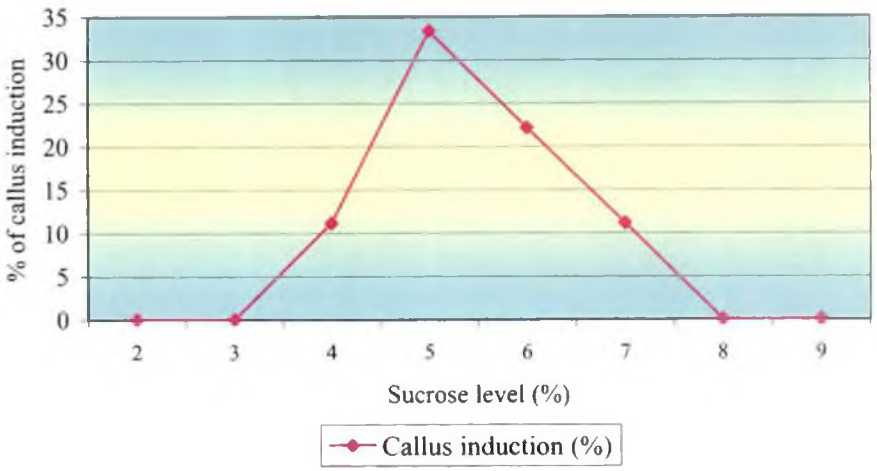


Fig. 3. Effect of sucrose levels on anther callusing

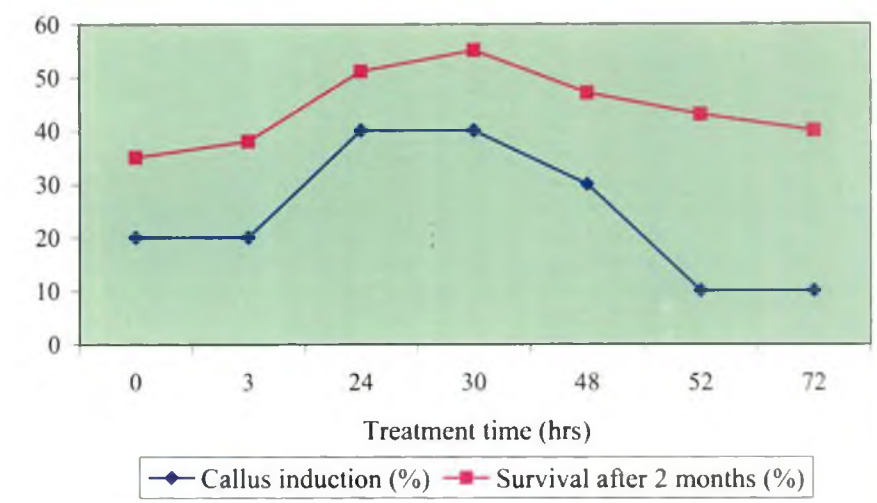


Fig. 4. Effect of chilling treatment on callus induction and survival percentage of immature anther

5.4 Indirect embryogenesis

Somatic embryogenesis was attempted in a number of palms and the positive results include the reports of Verdeil *et al.* (1994) in coconut, Teixeira *et al.* (1994) in oil palm, and Karun *et al.* (2004) in arecanut.

The callus induced on rachillae and anther explants of coconut in the present study were subcultured to the same medium and to different media combinations, so as to induce embryoids. Embryogenesis could be achieved in a medium containing 2,4-D 15 mg l^{-1}, picloram 0.5 mg l^{-1}, NAA 1 mg l^{-1} and TDZ 0.1 mg l^{-1}. Most of the media combinations tried were unfavourable for embryogenesis. The embryoids observed were initially globular in shape with smooth surface.

The embryoids formed in coconut were subcultured to different media for their further growth and maturation. None of the treatment gave favourable results. The embryoids either turned brown or dried up.

Response of coconut for indirect embryogenesis and further manipulations of embryoids so as to obtain normal plantlets was found not encouraging. Somatic embryos formed on the 2,4-D media when transferred to the medium containing BA and coconut water, failed to develop into plantlets. A failure to establish a functional shoot meristem may be the reason of conversion failure in somatic embryos (Nickle and Yeung, 1993; Goeble-Tourand *et al.*, 1993). All the normal procedures for callus induction and embryogenesis were attempted for the crop.

Since the number of embryoids that could be recovered for further manipulations were limited, the treatment combinations tried in the present study were not sufficient to identify the best media that could support further growth of the embryoids. A prolonged growth period has been reported for the development of zygotic embryo (11-12 months). Such a delay can be expected in the development of somatic embryo also. The coconut has a liquid endosperm rich in growth regulators

that support development of zygotic embryo. Further long term studies are required to obtain viable mature embryos from proembryos in coconut.

5.5 Histological study

Histological observation of young rachillae explants consists of parenchyma in the outer cell layer and several vascular bundles in the central zone. Simiar structures were observed in oil palm (Cutler, 1978) and *Musa* spp. (Lee *et al.*, 1997). Histological study of callus showed initiation of vascular tissues in the form of narrow elongated darkly stained cells, the procambium and then tracheary elements scattered in the parenchymatous callus tissue (Plate 20). Sujatha *et al.* (2003) also reported similar developments in the callus tissue of Black pepper. These vascular nodules as designated by Chen and Galston (1967) were found to get separated from the ground parenchyma by a layer of epidermal cells forming distinct primordium. This may be a shoot primordia as reported in several other crops (Azmi *et al.*, 1997; Kallak *et al.*, 1997; and Sujatha *et al.*, 2003) or embryonic unit as reported by Das *et al.* (2003) in rubber and Chand and Singh (2001) in *Hardwickia binata* (Plate 23).

5.6 Conclusion

At present the route of clonal propagation through callus and embryogenesis seems the most hopeful for coconut, especially since oil palm and date palm have been successfully propagated by this method. Coconut is admittedly a recalcitrant crop and *in vitro* regeneration of coconut tissue is being conducted for more than three decades in various laboratories using different tissue explants. However sporadic and hardly any repeatable success has been reported in this crop. Palm to palm difference in geographical locations, 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 exudation there is no clear understanding of its effect on the availability of other media components.

Present observations have clearly shown that the immature inflorescence tissue of coconut is capable of dedifferentiation to form callus. Friable embryogenic calli were obtained from immature anther and rachillae in Y_3 medium containing 5 per cent sucrose and growth regulator combinations of 2,4-D, Picloram, NAA, IAA and TDZ. Incubation for three to four months under darkness was required for the callus induction. Repeated subculturing of calli (3 months) obtained from the immature anther resulted in the formation of somatic embryo. Though the freqeuency was less, embryogenesis was observed in Y_3 medium supplemented with combinations of 2,4-D 15 mg l^{-1}, picloram 0.5 mg l^{-1}, NAA 1 mg l^{-1} and TDZ 0.1 mg l^{-1}. The embryo formed was subcultured to various media combinations for the germination. None of the combinations attempted could support further growth and germination within 3 months of culture period. However, this is the first report of better *in vitro* response of anther at premeiotic stage in coconut. A prolonged growth period has been reported for the development of zygotic embryo (11 to 12 months) in this crop. Such a delay can also be expected in the development of somatic embryo. Hence long term studies are required to obtain viable mature embryos in coconut.

SUMMARY

6. SUMMARY

An investigation on 'Response of immature inflorescence for *in vitro* regeneration in coconut (*Cocos nucifera* L.)' was undertaken in the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2005 to 2006. The salient findings of the study are summarized as follows.

1. Among the different basal media tried, Y_3 medium was found to be the best for culture establishment with immature inflorescence explants in coconut.
2. Wiping the spathe with 70 per cent ethyl alcohol before excising the immature inflorescence parts could effectively control contamination with 100 per cent culture establishment. The young inflorescence parts could survive up to 12 minutes treatment with 0.1 per cent $HgCl_2$.
3. Among the different explants tried, anthers at premeiotic stage and immature rachillae were found to be the best for callus induction and embryo formation.
4. Inflorescence of length 40 to 50 cm was found to possess male flowers at pollen mother cell stage of microsporogenesis. These inflorescence were around three to four months prior to anthesis.
5. Polyphenol interference reduced *in vitro* establishment of immature inflorescence explants to the extent of 65 per cent. Polyphenol exudation could be reduced considerably by adding PVP 0.1 per cent and activated charcoal 0.1 per cent in the culture medium.
6. Keeping the cultures under dark condition reduced the polyphenol oxidation
7. Explants cultured in Y3 medium exhibited the least polyphenol exudation while those in Blaydes medium recorded the highest phenolic interference.
8. None of the young inflorescence explants tried responded for direct organogenesis in various combinations of media tried with different concentrations of auxins, cytokinins, media additives and polyamines.
9. Higher concentration of BA (above 5 mg l^{-1}) showed inhibitory action leading to browning and death of explant

10. 2,4-D was found to be the most effective auxin for callus induction and proliferation in various explants and optimum concentration was observed to be 15 to 30 mg l^{-1}
11. Sucrose at 5.0 per cent concentration was identified as the optimum for callus induction in anthers at premeiotic stage.
12. Addition of 1 per cent mannitol increased the callus induction. Moreover it also reduced the browning of the explant and maintains the cultures fresh for longer periods (3 months).
13. As solidifying agent agar was found to be superior than phytagel.
14. The immature anther under in vitro condition responded in three different ways. In Y_3 media 40 percentage of cultures remained as such with no size enlargement and were dried with in 2 months. Thirty percentage of cultures remained viable with callus induction. Few of the anthers (30 per cent) elongated slightly and remained viable for 3 months without callusing.
15. Y_3 basal medium with 5.0 per cent sucrose and growth regulator combination of 15 mg l^{-1} 2,4-D, 0.5 mg l^{-1} picloram, 1 mg l^{-1} NAA and 0.1 mg l^{-1} TDZ was identified as the best medium for callus induction of immature anther.
16. Callus induction was observed from rachillae tissue in Y_3 medium containing 15 mg l^{-1} 2,4-D, 1 mg l^{-1} picloram, 1 mg l^{-1} IAA and 0.1 mg l^{-1} TDZ.
17. Callus initiation of female flower was observed in MS medium containing 15 mg l^{-1} 2,4-D, 0.2 mg l^{-1} picloram, 1 mg l^{-1} NAA
18. Anther like structures were formed from anther at pre meiotic stage in Y_3 medium containing 2,4-D at higher concentrations (22- 25 mg l^{-1}) along with IAA, NAA and TDZ.
19. Addition of 30 per cent tender coconut water enhanced the formation of anther like structures.
20. Addition of casein hydrolysate and glutamic acid did not favour callus induction and proliferation.
21. Pretreatment of inflorescence at 4°C for 24 hrs 30 hrs doubled the percentage of callus induction and reduced the browning of explant.

22. Use of phloroglucinol in the culture medium completely inhibited the culture establishment
23. Repeated subculturing of calli (3 months) obtained from immature anther in the same medium containing 15 mg l^{-1} 2,4-D, 0.5 mg l^{-1} picloram, 1 mg l^{-1} NAA and 0.1mg l^{-1} TDZ resulted in the formation of globular embryo.
24. The embryo formed was subcultured to various media combinations. None of the combinations attempted could support further growth and germination of embryo within 3 months. They became brown but remained alive. Embryo maturation was observed to be a very slow process.
25. Histological study of early stages of calli showed actively dividing meristematic cells forming a primary callus. At later stages the derivative cells from these parenchyma started differentiating into vascular nodules. Later some of these nodules were found to get separated from the ground parenchyma by a layer of epidermal cells forming distinct primordium. This may be shoot primordia or an embryonic unit.

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*Orginals not seen

APPENDICES

Appendix 1.

Solubility of different growth regulators

Name of growth regulator	Solvent
Auxins	
2,4 dichlorophenoxy acetic acid	Ethanol
Indole 3 acetic acid	1N NaOH
α-Naphthalene acetic acid	1N NaOH
Dicamba	Ethanol / water
Picloram	DMSO
Cytokinins	
Benzyl adenine	1N NaOH
N-isopentenylamino purine	1N NaOH
Kinetin	1N NaOH
Zeatin	1N NaOH
TDZ	DMSO

Appendix 2.

Growth regulator combinations tried for direct organogenesis in coconut explants (Basal media: MS, Y_3, and Blaydes media)

Sl No	Growth regulator combinations (mgl^{-1})
1	BA 1
2	BA 2
3	BA 3
4	BA 4
5	BA 5
6	BA 6
7	BA 7
8	BA 8
9	BA 10
10	BA 12
11	BA 15
12	Kin 0.25
13	Kin 0.5
14	Kin 1
15	Kin 2
16	Kin 4
17	Kin 8
18	Kin 10
19	Kin 12
20	Kin 14
21	Kin 14
22	Kin 15
23	2iP 0.25
24	2iP 0.5
25	2iP 1
26	2iP 2
27	2iP 3
28	2iP 4
29	2iP 5
30	NAA 0.5 + BA 1
31	NAA 0.5 + BA 2
32	NAA 0.5 + BA 3
33	NAA 0.5 + BA 4
34	NAA 0.5 + BA 5
35	NAA 0.5 + BA 8
36	NAA 1 + BA 1
37	NAA 1 + BA 2
38	NAA 1 + BA 3
39	NAA 1 + BA 4
40	NAA 1 + BA 5
41	NAA 1 + BA 8
42	NAA 1.5 + BA 1

43	NAA 1.5+ BA 2
44	NAA 1.5 + BA 3
45	NAA 1.5 + BA 4
46	NAA 1.5 + BA 5
47	NAA 1.5 + BA 8
48	NAA 2 + BA 1
49	NAA 2 + BA 2
50	NAA 2 + BA 3
51	NAA 2 + BA 4
52	NAA 2 + BA 5
53	NAA 2 + BA 8
54	NAA 0.5 + 2ip 0.25
55	NAA 0.5 + 2ip 0.5
56	NAA 0.5+ 2ip 1
57	NAA 0.5 + 2ip 2
58	NAA 0.5 + 2ip 4
59	NAA 0.5 + 2ip 5
60	NAA 1 + 2iP 0.25
61	NAA 1 + 2iP 0.5
62	NAA 1 + 2iP 1
63	NAA 1 + 2iP 2
64	NAA 1 + 2iP 4
65	NAA 1+ 2iP 5
66	NAA 1.5 + 2iP 0.25
67	NAA 1.5 + 2iP 0.5
68	NAA 1.5 + 2iP 1
69	NAA 1.5 + 2iP 2
70	NAA 1.5 + 2iP 4
71	NAA 1.5 + 2iP 5
72	NAA 2 + 2iP 0.25
73	NAA 2 + 2iP 0.5
74	NAA 2 + 2iP 1
75	NAA 2 + 2iP 2
76	NAA 2 + 2iP 4
77	NAA 2 + 2iP 5
78	IAA 1 + Kin 0.025
79	IAA 1 + Kin 0.5
80	IAA 1 + Kin 1
81	IAA 1 + Kin 2
82	IAA 1 + Kin 3
83	IAA 1 + Kin 4
84	IAA 1 + Kin 5
85	IAA 2 + Kin 0.25

86	IAA 2 + Kin 0.5
87	IAA 2 + Kin 1
88	IAA 2 + Kin 2
89	IAA 2 + Kin 3
90	IAA 2 + Kin 4
91	IAA 2 + Kin 5
92	IAA 3+ Kin 0.25
93	IAA 3+ Kin 0.5
94	IAA 3+ Kin 1
95	IAA 3+ Kin 2
96	IAA 3+ Kin 3
97	IAA 3+ Kin 4
98	IAA 3+ Kin 5
99	IAA 4+ Kin 0.25
100	IAA 4+ Kin 0.5
101	IAA 4+ Kin 1
102	IAA 4+ Kin 2
103	IAA 4+ Kin 3
104	IAA 4+ Kin 4
105	IAA 4+ Kin 5

Appendix 3.

Growth regulator combinations tried for callus induction (Basal media: MS, Y_3 and Blaydes media)

Sl No	Growth regulator combinations (mgl^{-1})
1	2,4-D 0.25
2	2,4-D 0.5
3	2,4-D 1
4	2,4-D 1.5
5	2,4-D 2
6	2,4-D 2.5
7	2,4-D 3
8	2,4-D 5
9	2,4-D 10
10	2,4-D 15
11	2,4-D 20
12	2,4-D 25
13	2,4-D 30
14	2,4-D 35
15	2,4-D 40
16	2,4-D 45
17	2,4-D 50

18	NAA 0.5
19	NAA 1
20	NAA 1.5
21	NAA 2
22	NAA 3
23	NAA 5
24	IAA 0.5
25	IAA 1
26	IAA 2
27	IAA 3
28	IAA 4
29	2,4-D 0.25 + Kin 0.25
30	2,4-D 0.25 + Kin 0.5
31	2,4-D 0.25 + Kin 1
32	2,4-D 0.25 + Kin 1.5
33	2,4-D 0.25 + Kin 2
34	2,4-D 0.25 + Kin 2.5
35	2,4-D 0.5 + Kin 0.25
36	2,4-D 0.5 + Kin 0.5
37	2,4-D 0.5 + Kin 1
38	2,4-D 0.5 + Kin 1.5
39	2,4-D 0.5 + Kin 2
40	2,4-D 0.5 + Kin 2.5
41	2,4-D 1 + Kin 0.25
42	2,4-D 1 + Kin 0.5
43	2,4-D 1 + Kin 1
44	2,4-D 1 + Kin 1.5
45	2,4-D 1 + Kin 2
46	2,4-D 1 + Kin 2.5
47	2,4-D 1.5 + Kin 0.25
48	2,4-D 1.5 + Kin 0.5
49	2,4-D 1.5 + Kin 1
50	2,4-D 1.5 + Kin 1.5
51	2,4-D 1.5 + Kin 2
52	2,4-D 1.5 + Kin 2.5
53	2,4-D 3.5 + Kin 0.25
54	2,4-D 3.5 + Kin 0.5
55	2,4-D 3.5 + Kin 1
56	2,4-D 3.5 + Kin 1.5
57	2,4-D 3.5 + Kin 2
58	2,4-D 3.5 + Kin 2.5
59	IAA 1+ NAA 0.5 + BA 1
60	IAA 1+ NAA 0.5 + BA 2
61	IAA 1+ NAA 0.5 + BA 3
62	IAA 1+ NAA 0.5 + BA 4
63	IAA 1+ NAA 1 + BA 1
64	IAA 1+ NAA 1 + BA 2
65	IAA 1+ NAA 1 + BA 3

66	IAA 1+ NAA 1 + BA 4
67	IAA 1+ NAA 1.5 + BA 1
68	IAA 1+ NAA 1.5 + BA 2
69	IAA 1+ NAA 1.5 + BA 3
70	IAA 1+ NAA 1.5 + BA 4
71	IAA 1+ NAA 2 + BA 1
72	IAA 1+ NAA 2 + BA 2
73	IAA 1+ NAA 2 + BA 3
74	IAA 1+ NAA 2 + BA 4
75	IAA 2+ NAA 0.5 + BA 1
76	IAA 2+ NAA 0.5 + BA 2
77	IAA 2+ NAA 0.5 + BA 3
78	IAA 2+ NAA 0.5 + BA 4
79	IAA 2+ NAA 1 + BA 1
80	IAA 2+ NAA 1 + BA 2
81	IAA 2+ NAA 1 + BA 3
82	IAA 2+ NAA 1 + BA 4
83	IAA 2+ NAA 1.5 + BA 1
84	IAA 2+ NAA 1.5 + BA 2
85	IAA 2+ NAA 1.5 + BA 3
86	IAA 2+ NAA 1.5 + BA 4
87	IAA 2+ NAA 2 + BA 1
88	IAA 2+ NAA 2 + BA 2
89	IAA 2+ NAA 2 + BA 3
90	IAA 2+ NAA 2 + BA 4
91	IAA 3+ NAA 0.5 + BA 1
92	IAA 3+ NAA 0.5 + BA 2
93	IAA 3+ NAA 0.5 + BA 3
94	IAA 3+ NAA 0.5 + BA 4
95	IAA 3+ NAA 1+ BA 1
96	IAA 3+ NAA 1+ BA 2
97	IAA 3+ NAA 1+ BA 3
98	IAA 3+ NAA 1+ BA 4
99	IAA 3+ NAA 1.5 + BA 1
100	IAA 3+ NAA 1.5 + BA 2
101	IAA 3+ NAA 1.5 + BA 3
102	IAA 3+ NAA 1.5 + BA 4
103	IAA 3+ NAA 2 + BA 1
104	IAA 3+ NAA 2 + BA 2
105	IAA 3+ NAA 2 + BA 3
106	IAA 3+ NAA 2 + BA 4
107	IAA 4+ NAA 0.5 + BA 1
108	IAA 4+ NAA 0.5 + BA 2
109	IAA 4+ NAA 0.5 + BA 3
110	IAA 4+ NAA 0.5 + BA 4
111	IAA 4+ NAA 1+ BA 1
112	IAA 4+ NAA 1+ BA 2
113	IAA 4+ NAA 1+ BA 3

114	IAA 4+ NAA 1+ BA 4
115	IAA 4+ NAA 1.5+ BA 1
116	IAA 4+ NAA 1.5+ BA 2
117	IAA 4+ NAA 1.5+ BA 3
118	IAA 4+ NAA 1.5+ BA 4
119	IAA 4+ NAA 2+ BA 1
121	IAA 4+ NAA 2+ BA 2
122	IAA 4+ NAA 2+ BA 3
123	IAA 4+ NAA 2+ BA 4
124	IAA 1 + Kin 0.25
125	IAA 1 + Kin 0.5
126	IAA 1 + Kin 1
127	IAA 1 + Kin 2
128	IAA 1 + Kin 4
129	IAA 2 + Kin 0.25
130	IAA 2 + Kin 0.5
131	IAA 2 + Kin 1
132	IAA 2 + Kin 2
133	IAA 2 + Kin 4
134	IAA 3 + Kin 0.25
135	IAA 3 + Kin 0.5
136	IAA 3 + Kin 1
137	IAA 3 + Kin 2
138	IAA 3 + Kin 4
139	IAA 4 + Kin 0.25
140	IAA 4 + Kin 0.5
141	IAA 4 + Kin 1
142	IAA 4 + Kin 2
143	IAA 4 + Kin 4
144	NAA 0.5 + IAA 1 + 2iP 0.25
145	NAA 0.5 + IAA 1 + 2iP 0.5
146	NAA 0.5 + IAA 1 + 2iP 1
147	NAA 0.5 + IAA 1 + 2iP 2
148	NAA 0.5 + IAA 2 + 2iP 0.25
149	NAA 0.5 + IAA 2 + 2iP 0.5
150	NAA 0.5 + IAA 2 + 2iP 1
151	NAA 0.5 + IAA 2 + 2iP 2
152	NAA 0.5 + IAA 3 + 2iP 0.25
153	NAA 0.5 + IAA 3 + 2iP 0.5
154	NAA 0.5 + IAA 3 + 2iP 1
155	NAA 0.5 + IAA 3 + 2iP 2
156	NAA 0.5 + IAA 4 + 2iP 0.25
157	NAA 0.5 + IAA 4 + 2iP 0.5
158	NAA 0.5 + IAA 4 + 2iP1
159	NAA 0.5 + IAA 4 + 2iP 2
160	NAA 1 + IAA 1 + 2iP 0.25
161	NAA 1 + IAA 1 + 2iP 0.5
162	NAA 1 + IAA 1 + 2iP 1

163	NAA 1 + IAA 1 + 2iP 2
164	NAA 1 + IAA 2 + 2iP 0.25
165	NAA 1 + IAA 2 + 2iP 0.5
166	NAA 1 + IAA 2 + 2iP 1
167	NAA 1 + IAA 2 + 2iP 2
168	NAA 1 + IAA 3 + 2iP 0.25
169	NAA 1 + IAA 3 + 2iP 0.5
170	NAA 1 + IAA 3 + 2iP 1
171	NAA 1 + IAA 3 + 2iP 2
172	NAA 1 + IAA 4 + 2iP 0.25
173	NAA 1 + IAA 4 + 2iP 0.5
174	NAA 1 + IAA 4 + 2iP 1
175	NAA 1 + IAA 4 + 2iP 2
176	NAA 1.5 + IAA 1 + 2iP 0.25
177	NAA 1.5 + IAA 1 + 2iP 0.5
178	NAA 1.5 + IAA 1 + 2iP1
179	NAA 1.5 + IAA 1 + 2iP 2
180	NAA 1.5 + IAA 2 + 2iP 0.25
181	NAA 1.5 + IAA 2 + 2iP 0.5
182	NAA 1.5 + IAA 2 + 2iP 1
183	NAA 1.5 + IAA 2 + 2iP 2
184	NAA 1.5 + IAA 3 + 2iP 0.25
185	NAA 1.5 + IAA 3 + 2iP 0.5
186	NAA 1.5 + IAA 3 + 2iP 1
187	NAA 1.5 + IAA 3 + 2iP 2
188	NAA 1.5 + IAA 4 + 2iP 0.25
189	NAA 1.5 + IAA 4 + 2iP 0.5
190	NAA 1.5 + IAA 4 + 2iP 1
191	NAA 1.5 + IAA 4 + 2iP 2
192	NAA 2 + IAA 1 + 2iP 0.25
193	NAA 2 + IAA 1 + 2iP 0.5
194	NAA 2 + IAA 1 + 2iP 1
195	NAA 2 + IAA 1 + 2iP 2
196	NAA 2 + IAA 2 + 2iP 0.25
197	NAA 2 + IAA 2 + 2iP 0.5
198	NAA 2 + IAA 2 + 2iP 1
199	NAA 2 + IAA 2 + 2iP 2
200	NAA 2 + IAA 3 + 2iP 0.25
201	NAA 2 + IAA 3 + 2iP 0.5
202	NAA 2 + IAA 3 + 2iP 1
203	NAA 2 + IAA 3 + 2iP 2
204	NAA 2 + IAA 4 + 2iP 0.25
205	NAA 2 + IAA 4 + 2iP 0.5
206	NAA 2 + IAA 4 + 2iP 1
207	NAA 2 + IAA 4 + 2ip 2
207	2,4-D 15 + Picloram 0.25
208	2,4-D 15 + Picloram 0.5

209	2,4-D 15 + Picloram 1
210	2,4-D 18 + Picloram 0.25
211	2,4-D 18 + Picloram 0.5
212	2,4-D 18 + Picloram1
213	2,4-D 22 + Picloram 0.25
214	2,4-D 22 + Picloram 0.5
215	2,4-D 22 + Picloram 1
216	2,4-D 25 + Picloram 0.25
217	2,4-D 25 + Picloram 0.5
218	2,4-D 25+ Picloram 1
219	2,4-D 15 + TDZ 0.025
220	2,4-D 15 + TDZ 0.5
221	2,4-D 15 + TDZ 0.075
222	2,4-D 15 + TDZ 0.1
223	2,4-D 15 + TDZ 0.5
224	2,4-D 15 + TDZ 1.5
225	2,4-D 15 + TDZ 2
226	2,4-D 20 + TDZ 0.25
227	2,4-D 20 + TDZ 0.5
228	2,4-D 20 + TDZ 0.075
229	2,4-D 20 + TDZ 0.1
230	2,4-D 20 + TDZ 0.5
231	2,4-D 20 + TDZ 1.5
232	2,4-D 20 + TDZ 2
233	2,4-D 22 + TDZ 0.025
234	2,4-D 22 + TDZ 0.5
235	2,4-D 25 + TDZ 0.075
236	2,4-D 25 + TDZ 0.1
237	2,4-D 25 + TDZ 0.5
238	2,4-D 25 + TDZ 1.5
239	2,4-D 25 + TDZ 2
240	2,4-D 15 + Picloram 0.5 + TDZ 0.5
241	2,4-D 15 + Picloram 0.5 + TDZ 0.1
242	2,4-D 15 + Picloram 0.5 + TDZ 1.5
243	2,4-D 15 + Picloram 0.5 + TDZ 2
244	2,4-D 15 + Picloram 1 + TDZ 0.5
245	2,4-D 15 + Picloram 1 + TDZ 0.1
246	2,4-D 15 + Picloram 1 + TDZ 1.5
247	2,4-D 15 + Picloram 1 + TDZ 2
248	2,4-D 15 + Picloram 2 + TDZ 0.1
249	2,4-D 15 + Picloram 2 + TDZ 0.5
250	2,4-D 15 + Picloram 2 + TDZ 1.5
251	2,4-D 15 + Picloram 2 + TDZ 2
252	2,4-D 15 + NAA 1
253	2,4-D 15 + NAA 2
254	2,4-D 18 + NAA 1
255	2,4-D 18 + NAA 2

256	2,4-D 22 + NAA 1
257	2,4-D 22 + NAA 2
258	2,4-D 25 + NAA 1
259	2,4-D 25 + NAA 2
260	2,4-D 15 + Picloram 0.25 + NAA 1
261	2,4-D 15 + Picloram 0.5 + NAA 1
262	2,4-D 18 + Picloram 0.5 + NAA 1
263	2,4-D 18 + Picloram 0.2 + NAA 1
264	2,4-D 22 + Picloram 0.5 + NAA 1
265	2,4-D 25 + Picloram 0.5 + NAA 1
266	2,4-D 15 + IAA 1 + NAA 1
267	2,4-D 18 + IAA 1 + NAA 1
268	2,4-D 22 + IAA 1 + NAA 1
269	2,4-D 25 + IAA 1 + NAA 1
270	2,4-D 15 + IAA 1
271	2,4-D 15 + IAA 2
272	2,4-D 18 + IAA 1
273	2,4-D 18 + IAA 2
274	2,4-D 22 + IAA 1
275	2,4-D 22 + IAA 2
276	2,4-D 25 + IAA 1
277	2,4-D 25 + IAA 2
288	2,4-D 15 + 2ip 1
299	2,4-D 15 + 2ip 1
300	2,4-D 15 + NAA 1 + TDZ 0.1
301	2,4-D 15 + NAA 1 + TDZ 0.075
302	2,4-D 15 + NAA 2 + TDZ 0.1
303	2,4-D 15 + NAA 2 + TDZ 0.075
304	2,4-D 18 + IAA 0.5 + TDZ 0.1
305	2,4-D 18 + IAA 0.5 + TDZ 0.075
306	2,4-D 18 + IAA 1 + TDZ 0.075
307	2,4-D 18 + IAA 1 + TDZ 0.1
308	2,4-D 18 + IAA 2 + TDZ 0.1
309	2,4-D 18 + IAA 2 + TDZ 0.075
310	2,4-D 22 + IAA 1 + TDZ 0.1
311	2,4-D 22 + IAA 1 + TDZ 0.075
312	2,4-D 22 + IAA 2 + TDZ 0.075
313	2,4-D 22 + IAA 2 + TDZ 0.1
314	2,4-D 20 + IAA 1 + TDZ 0.1
315	2,4-D 25 + IAA 1 + TDZ 0.1
316	2,4-D 25 + IAA 0.5 + TDZ 0.075
317	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1
318	2,4-D 15 + Picloram 1 + NAA 1 + TDZ 0.1
319	2,4-D 20 + Picloram 0.5 + NAA 1 + TDZ 0.1
320	2,4-D 20 + Picloram 1 + NAA 1 + TDZ 0.1
321	2,4-D 20 + Picloram 0.5 + IAA 1 + TDZ 0.1
322	2,4-D 20 + Picloram 1 + NAA 1 + TDZ 0.1

333	2,4-D 15 + Picloram 0.5 + IAA 1 + TDZ 0.1
334	2,4-D 15 + Picloram 0.1 + IAA 1 + TDZ 0.1
335	2,4-D 25 + IAA 1 + NAA 1 + TDZ 0.1
336	2,4-D 20 + IAA 1 + NAA 1 + TDZ 0.1
337	2,4-D 22 + IAA 1 + NAA 1 + TDZ 0.1
338	2,4-D 15 + IAA 1 + NAA 1 + TDZ 0.1
339	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 + Dicamba 0.25
340	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 + Dicamba 0.5
341	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 + Dicamba 1
342	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 + Dicamba 1.5
343	2,4-D 1 + NAA 10 + IAA 1 + TDZ 0.1
344	2,4-D 1 + NAA 15 + IAA 1 + TDZ 0.1
345	2,4-D 1 + NAA 20 + IAA 1 + TDZ 0.1
346	2,4-D 1 + IAA 5 + NAA 1 + TDZ 0.1
347	2,4-D 1 + IAA 10 + NAA 1 + TDZ 0.1
348	2,4-D 1 + IAA 15 + NAA 1 + TDZ 0.1
349	2,4-D 15 + Picloram 0.5 + IAA 1
350	2,4-D 15 + Picloram 1 + IAA 1

Appendix 4.

Growth regulator combinations tried for callus regeneration (Basal media: Y_3)

Sl No	Growth regulator combinations (mgl^{-1})
1	2,4-D 5 + Picloram 0.2 + IAA 0.25 + TDZ 0.2 + 2iP 0.5
2	2,4-D 5 + Picloram 0.2 + IAA 0.25 + TDZ 0.2 + 2iP 1
3	2,4-D 5 + Picloram 0.2 + IAA 0.25 + TDZ 0.2 + 2iP 2
4	2,4-D 5 + Picloram 0.2 + IAA 0.25 + TDZ 0.2 + 2iP 3
5	2,4-D 5 + Picloram 0.2 + IAA 0.25 + TDZ 0.2 + 2iP 4
6	2,4-D 5 + Picloram 0.2 + IAA 0.25 + TDZ 0.1 + Zeatin 0.5
7	2,4-D 5 + Picloram 0.2 + IAA 0.25 + TDZ 0.1 + Zeatin 0.25
8	2,4-D 5 + Picloram 0.2 + IAA 0.25 + TDZ 0.1 + Zeatin 1
9	2,4-D 5 + Picloram 0.2 + IAA 0.25 + TDZ 0.1 + Zeatin 2
10	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 + Spermine 1µM
11	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 + Spermine 2µM
12	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 + Putrescine 5mM
13	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 + Putrescine 10mM
14	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 +2iP 0.5
15	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 +2iP 1
16	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 +2iP 2
17	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 +2iP 3
18	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 +2iP4
19	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 +2iP 5
20	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 + Zeatin 0.25
21	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 + Zeatin 0.5
22	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 + Zeatin 1
23	2,4-D 15 + Picloram 0.5 + NAA 1 + TDZ 0.1 + Zeatin 2

RESPONSE OF IMMATURE INFLORESCENCE FOR *IN VITRO* REGENERATION IN COCONUT (*Cocos nucifera* L.)

By
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ABSTRACT OF THE THESIS

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ABSTRACT

Investigations on 'Response of immature inflorescence for *in vitro* regeneration in coconut (*Cocos nucifera* L.)' were carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during 2005 to 2006.

Y_3 medium was found to be the best basal medium for *in vitro* culture of immature inflorescence of coconut. Inflorescence of length 40 to 50 cm was found to posses male flowers at pollen mother cell stage of microsporogenesis. Wiping the spathe with 70 per cent ethyl alcohol before excising the immature inflorescence parts could effectively control contamination with 100 per cent culture establishment. The young inflorescence parts could survive up to 12 minutes treatment with 0.1 per cent $HgCl_2$. Among the different explants tried, anthers at premeiotic stage and immature rachillae were found to be the best for callus induction and embryo formation. When the explants were inoculated the exudation of polyphenols from the explants adversely affected their survival. Polyphenol exudation was checked by incorporating PVP 0.1 per cent and activated charcoal 0.2 per cent in the medium and by incubation under dark condition.

2,4-D at 15 to 30 mgl^{-1} was found to be the most effective auxin for callus induction and proliferation. Y_3 basal medium with growth regulator combinations of 15 mg l^{-1} 2,4-D, 0.5 mg l^{-1} picloram, 1mg l^{-1} NAA and 0.1 mg l^{-1} TDZ was identified as the best medium for callus induction and embryogenesis of immature anther. Sucrose at 5 per cent concentration was identified as the optimum concentration for callus induction. Pretreatment of inflorescence at 4°C for 24 hrs or 30 hrs doubled the callus induction and reduced the browning of explant. Callus induction was observed from rachillae tissue in Y_3 medium containing15 mg l^{-1} 2,4-D, 1 mg l^{-1} picloram, 1 mg l^{-1} IAA and 0.1 mg l^{-1} TDZ.

Addition of 1 per cent mannitol increased the callus induction. Moreover it also reduced the browning of explant and retained the cultures fresh for longer periods. The immature anther under *in vitro* condition responded in three different ways. In Y_3 media 40 percentage of cultures remained as such with no size enlargement and were dried within 2 months. Thirty percent of cultures remained viable with callus induction. Few of the anthers (30 per cent) elongated slightly and remained viable for 3 months without callusing. Anther like structures were formed from immature anther in Y3 medium containing 2,4-D 25 mg l^{-1}, IAA 1 mg l^{-1}, TDZ 0.075 mg l^{-1}. Addition of 30 per cent coconut water enhanced the formation of anther like structure. Histological study of early stages of calli showed actively dividing meristematic cells forming a primary callus. At later stages the derivative cells from these parenchyma started differentiating into vascular nodules. Later some of these nodules were found to get separated from the ground parenchyma by a layer of epidermal cells forming distinct primordium. This may be shoot primordia or an embryonic unit. Somatic embryo was induced in Y_3 medium containing 2,4-D 15 mgl^{-1}, picloram 0.5 mg l^{-1}, NAA 1 mg l^{-1} and TDZ 0.1 mg l^{-1} from the immature anther derived callus. The embryo formed from the immature anther derived callus was subcultured to various media combinations. None of the combinations attempted could support further growth and germination of embryo. They became brown but remained alive. Embryo maturation was observed to be a very slow process. A prolonged growth period has been reported for the development of zygotic embryo in coconut (11 to 12 months). Such a delay can be expected in the development of somatic embryo also. Further long-term studies are required to obtain viable mature embryo from proembryos in coconut.