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Plant regeneration through organogenesis and somatic embryogenesis from plumular explants of coconut *(Cocos nucifera L.)*

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Abstract

A procedure is outlined for regeneration of complete plantlets via organogenesis from plumular tissues of coconut. Callus was induced from plumular tissues in Y3 media supplemented with either 2, 4-D (74.6 M) alone or 2, 4-D (74.6 M) in combination with TDZ (4.54 M). The frequency of callus induction increased and the browning of explants was reduced when cytokinin (TDZ) was added along with the auxin (2, 4-D) in the calus induction medium. The calli were subcultured at monthly intervals to media containing lower levels of 2, 4-D and a constant level of either cytokinins (BA and TDZ) of polyamines (spermine and putrescine). Higher percentages of embryogenic calli, somatic embryoids and meristemoids were obtained in Y3 media supplemented with either spremine of BA. Plantlets with balanced shoot and root formation were transferred to pots and established in the greenhouse. Histological studies of the differentiated tissues confirmed the development of shoot buds (organogenesis) and typical bipolar embryoids (somatic embryogenesis).

Abbreviations: BA: 6-benzyladenine; 2,4-D: 2, 4-dichlorophenoxyacetic acid; IBA:indole-3butryic acid; TDZ : 1 - phenyl - 3 (1,2,3-thiadiazol-5-yl) urea (Thidiazuron)

Key words: Coconut, plant regeneration, organogenesis, somatic embryogenesis

Coconut (*Cocos nucifera* L.) is the most important palm species cultivated in more than 90 countries in the tropics, especially on small and marginal holdings. The coconut industry at present is burdened with a number of problems such as prevalence of various pests and diseases and senility of existing plantations. Crop improvement in coconut is a difficult and time consuming programme due to its long pre-bearing age, high heterozygosity, long interval between generations and exclusively seed propagated nature. The predominantly cross-fertilized nature of coconut results in enormous variability in the seedling progenies. Though India was the first country in the world to evolve a commercial hybrid of coconut (Patel, 1937) and the country has since released 12 high yielding hybrids and varieties, current production of quality planting materials meets only about 20% of the estimated annual requirement of planting material to replace senile and disease ravaged plantations.

In vitro vegetative multiplication through somatic embryogenesis of high performance individuals thus offers the only hope for the production of homogeneous planting materials and for substantial improvement in plantation productivity. Standardization of a viable protocol for clonal propagation would open up tremendous possibilities of meeting the requirement for quality, uniform, disease resistant/tolerant planting material and of breaking down productivity barriers.

Unfortunately, coconut is a highly recalcitrant species with respect to tissue culture. Over the past few decades, many researchers have directed their efforts towards developing a method for clonal propagation of coconut. Despite this concerted effort, success in the area has been limited and only a few clonal plants have been ever established in the field (Iyer and Dhamodaran, 1994). Various problems encountered during *in vitro* propagation of coconut are intensive tissue browning (due to oxidation

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of polyphenols), slow *in vitro* response, low rate of somatic embryogenies and variation in tissue response due to heterogeneity of explants taken from different individuals. A variety of protocols have been developed using a range of explants immature inflorescence (Blake, 1991), immature and mature zygotic embryos, young tender leaflets (Raju *et al.,* 1984), leaf bases from unopened spindle and plumular tissue (see, reviews by Iyer, 1982; 1993; by Nair *et al.,* 1999; Iyer and Parthasarathy, 2000), but the protocols lack repeatability. The present investigation describes the *in vitro* regeneration of coconut plantlets using plumular tissues (which are juvenile and highly meristematic) as explants.

Materials and Methods

Plant material

Zygotic embryos were scooped out along with a portion of the endosperm using a cork borer from mature dehusked and split coconuts (11-12 months old) of the local *West Coast Tall* cultivar. The embryos were extracted from the endosperm plug using a scalpel. Under aseptic conditions, the embryos were washed in 50% chlorine-water for 20 minutes and then rinsed four times with sterile distilled water.

Conditioning of the embryos

The sterilized embryos were inoculated into plain Y3 medium (Eeuwens, 1976) containing 3.0% sucrose, 1g/1 activated charcoal and 0.55% (w/v) agar. The pH of the medium was adjusted to 5.8 with 1N NaOH or HC1 before autoclaving for 20 minutes at 1.06 kg cm^{-2}. The cultures were incubated in the dark for a month at 27±2^{0}C.

Callus induction and maintenance

After a month in the conditioning medium, the plumular ends were sliced out from the embryos and inoculated into Y3 medium supplemented with either 2,3-D (74.6 M) alone (Callus Induction Medium) or 2,4-D (74.6 M) in combination with TDZ (4.54 M) (Callus Induction Medium II). The media also contained 3.0% sucrose, 1g/1 activated charcoal and was solidified with 0.55% (w/v) agar. The pH of the media was adjusted to 5.8 prior to adding agar and autoclaving. The cultures were incubated in dark at 27±2^{0}C for two months.

Induction of somatic embryoids / meristemoids

The plumular calli were transferred to media containing 2,4-D (45.25 M), charcoal (1g/1) and one of the following growth regulators at different concentrations: BA (2.22 and 4.44 M), TDZ (2.27 and 4.54 M), spermine (25, 50, 75 and 100 M) and putrescine, (1, 5, 7.5 and 10mM). Initially, a small-scale experiment was conducted with the above treatments. Based on the results obtained, BA (4.44 M), TDZ (4.54 M), spermine (100 M) and putrescine (1mM) were selected for further experiments. The charcoal concentration was 0.75mg/1 at this stage. At each monthly subculture, the 2,4-D concentration was reduced to 22.62 M, 4.52 M and 0.45 M. The charcoal concentration was also reduced to 0.5 mg/1, 0.25mg/1 and 0.1mg/1 with the reduction in 2,4-D concentration. Plain Y3 minerals without any growth regulators served as control. All the above media were supplemented with 3.0% sucrose and 0.55% (w/v) agar and the pH was adjusted to 5.8 prior to autoclaving. The cultures were initially incubated in dark at 27±2^{0}C. As soon as somatic embryoid/meristemoid formation was noticed, the cultures were transferred under warm-white fluorescent light with a 16-hour photoperiod. Observations regarding the percentage of embryogenic calli, somatic embryoids, meristemoids and plantlets were taken at regular intervals.

Plantlet regenerations and maintenance

Somatic embryoids and meristemoids, when formed, were transferred initially to plain liquid Y3 medium without any growth regulators and later to a medium containing BA (17.76 M). Plantlets derived from meristemoids were transferred to a rooting medium (liquid Y3 medium supplemented with 9.8 M IBA).

Plant acclimatization

Plantlets with 3-5 leaves and adequate rooting were removed individually from the culture tubes and washed with sterile water. Before transferring to pots, the plantlets were treated with carbendazim 91%) and thereafter with IBA solution (4.9mM) for an hour. The potting mixture consisted of sterilized soil, sand and coir dust in equal proportions. Initially, the plantlets were covered with polythene bag for two weeks. Gradually, the bags were perforated to reduce humidity and later the bags were removed during the night. After 4 weeks, the bags were removed completely.

Statistical analysis

The experiments were replicated thrice with 20 explants per replication. Analysis of variance (one way ANOVA) was used to test if there are significant differences between means obtained with different treatments at the 5% level of significance (Snedecor and Cochran, 1975). For percentage data, angular transformation was used (sin^{-1}p where p is the proportion of the particular character recorded).

Specimens for histological studies were fixed in Carnoy's B fixative (60% absolute ethanol, 30%

chloroform, 10% glacial acetic acid) for 24 hours and were dehydrated in alcohol-butanol series before embedding in paraffin wax. Serial sections of 10 M thickness were taken using a rotary microtome. After deparaffinization, they were stained with 0.1% toluidine blue, the excess stain was washed off, dehydrated, cleared and mounted in DPX.

Results

Callus initiation was noticed within 4-5 weeks of culture from the perivascular strands of the leaf primordia. Callus formation was obtained with or without cytokinin (TDZ), but required an auxin, namely 2,4-D at a concentration of 74.6 M. The percentage of callus formation ranged from 74.3% to 88% (Fig. 1). The frequency of callus induction was increased and the browning of explants reduced when cytokinin (TDZ) was added along with auxin in the callus induction medium.

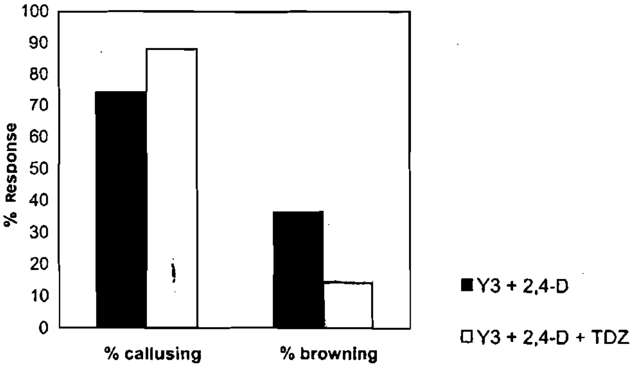

Fig. 1. Percentage callus induction and browning of explants using the two callus induction media

After two months of culture in the callus induction medium, the calli were transferred to media containing (i) either cytokinins (BA or TDZ) or polyamines (spermine and putrescine) in combination with 2,4-D, (ii) to a medium containing 2,4-D alone or (iii) a medium free of any growth regulators. Within four weeks of culture, the calli could be recognized as embryogenic, non-embryogenic or rhizogenic portions were removed during monthly intervals to media containing lower levels of 2,4-D. The non-embryogenic and rhizogenic portions were removed during the monthly subcultures, while the embryogenic portions were retained. This identification and selection of the whitish-yellow and friable embryogenic callus (Fig. 2A) clumps was found to be essential for regeneration.

The embryogenic calli exhibited globular structures within 2-3 weeks of the second subculture (Fig. 3A). The embryoids germinated (Fig. 2B) and plantlets with both root and shoot axes were formed during subsequent subculture (Fig. 2C, 2D). Light microscopic observations revealed organized bipolar structure with coleoptile-coleorhizal axes (Fig. 3B).

Some of the calli also produced protuberances on the surface, which formed green bud spots in light. These gradually grew into well defined leafy shoots (Fig. 2E, 2F). Histological analysis of the organogenesis induction and development revealed the initiation of numerous meristematic regions in the calli and the formation of hump-shaped shoot bud primordia through the formation of shoot apical meristems (Fig. 3C). Plantlets with 3-5 leaves and adequate rooting were removed individually from the culture tubes and transferred to pots for acclimatization (Fig. 2G).

For the percentage of embryogenic calli, the difference due to the callus media, regeneration media and their interactions were found significant (Table 1.) On an average, significantly higher percentage of embryogenic callus was obtained from Callus Induction Medium II (containing 2,4-D and TDZ) than Callus Induction Medium I (containing 2,4-D). Among the different growth regulators tried for regeneration, significantly higher percentages of embryogenic calli were obtained in Y3 media supplemented with either spermine or BA (Table 1). About 26% of the cultures in spermine-supplemented medium and 21% of the cultures in BA-supplemented medium (derived from Callus Induction Medium II) produced somatic embryoids. Cultures derived from Callus Induction Medium II produced more somatic embryoids compared to Callus Induction Medium I. Significantly higher percentage of shoot meristemoids was produced in TDZ and BA supplemented media 0% each) compared to others. Significantly higher percentage of rhizogenesis was recorded when the calli were derived from Callus Induction Medium I. Also, calli maintained in a medium containing 2,4-D alone were found to the highest average percentage of rhizogenesis (44.02%). The tendency to turn rhizogenic decreased with the supplementation in the media of either a polyamine (spermine) or cytokinins (BA and TDZ). However, average percentage rhizogenesis was also found to be high in calli maintained in medium supplemented with putrescine (40.66%). The lowest rhizogenesis percentage was observed in BA and spermine-supplemented medium. Maximum number of complete plantlets (30-31%) was formed from cultures (derived from Callus Induction Medium II) maintained in media containing spermine and BA.

There was no response for any of the parameters recorded in medium without any growth regulator supplementation (which served as control). Hence it was excluded from the analysis.

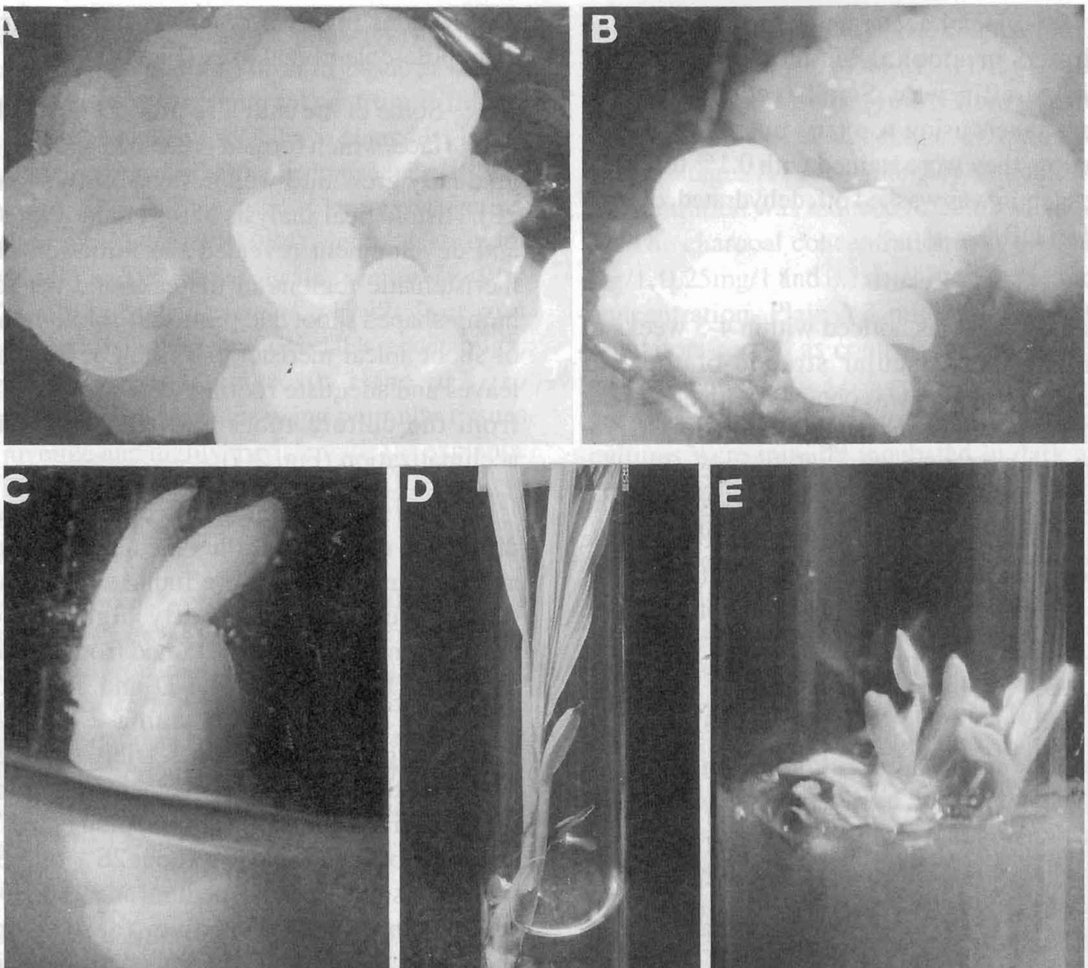

Figure 2. Regeneration through somatic embryogenesis / organogenesis from plumular explants of coconut

A. Fribale, embryogenic callus

B. Germination of embryoids on the surface of the callus

C. Germinating embryoid with bipolar axes

D. Development of a normal plantlet from an embryoid

E. F. Develpment of shoot buds through organogenesis

G. Established plantlets in pots.

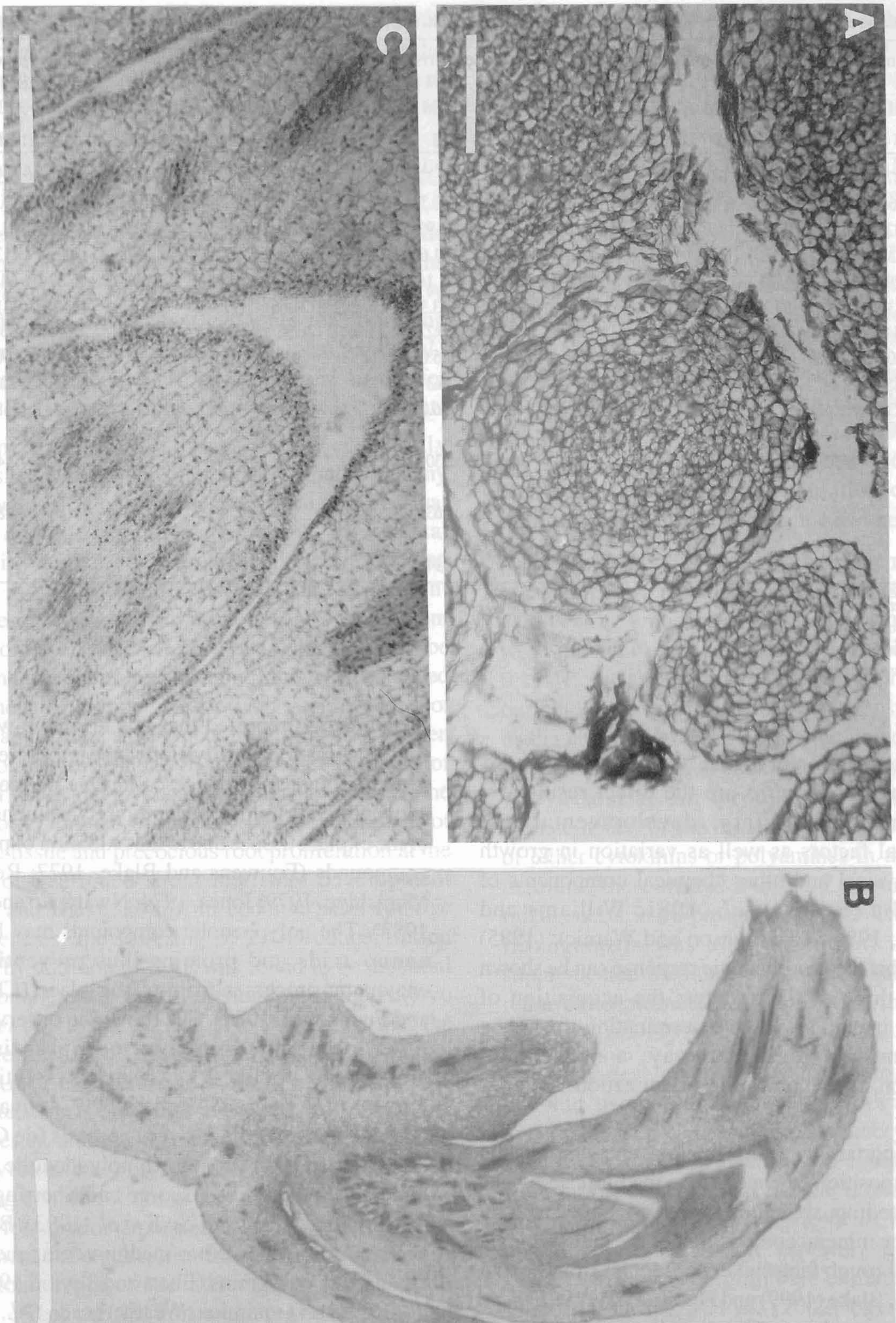

Figure 3. Histological studies of different stages of coconut regeneration
A. Globular structures seen on the surface of the embryogenic callus (bar=100m)
B. Germinating somatic embryoid showing coleoptile-coleorhizal axes (bar=400m)
C. Initial stage of meristemoid development (bar=250m)

Table 1. Effect of different growth regulators on the induction of embryogenic calli, somatic embryogenesis, organogenesis and plantlet formation

Character	Embryogenic calli (%)			Somatic embryoids (%)			Shoot meristemoids (%)			Rhizogenesis (%)			Cultures producing plantlets (%)		
Media	CIM I	CIM II	Mean	CIM I	CIM II	Mean	CIM I	CIM II	Mean	CIM I	CIM II	Mean	CIM I	CIM II	Mean
2,4-alone	19.87	25.29	22.58	0.00	8.61	4.30	4.30	21.32	12.81	47.86	40.18	44.02	0.00	4.305	2.15
	(11.66)	(18.33)	(15.00)	(0.00)	(3.33)	(1.66)	(1.66)	(13.33)	(7.50)	(55.00)	(41.66)	(48.33)	(0.00)	(1.66)	(0.83)
2,4-D+TDZ	21.32	16.59	18.95	4.30	4.30	4.30	32.12	45.93	39.03	36.22	23.21	35.22	14.75	21.32	18.04
	(13.33)	(8.33)	(10.83)	(1.66)	(1.66)	(1.66)	(28.33)	(51.66)	(40.00)	(35.00)	(31.66)	(33.33)	(6.66)	(13.33)	(10.00)
2,4-D=BAP	25.29	33.19	29.24	21.32	27.69	24.51	37.24	41.14	39.19	27.69	21.32	24.51	25.29	34.21	29.75
	(18.33)	(30.00)	(24.16)	(13.33)	(21.66)	(17.50)	(36.66)	(43.33)	(40.00)	(21.66)	(913.33)	(17.50)	(18.33)	(31.66)	(25.00)
2,4-D +	26.44	37.24	31.84	24.03	31.05	27.54	27.69	36.22	31.96	26.55	24.03	25.29	28.24	33.19	31.02
Spermine	(20.00)	(36.660	(28.33)	(16.66)	(26.66)	(21.66)	(21.66)	(35.00)	(28.33)	(20.00)	(16.66)	(18.33)	(23.33)	(30.00)	(26.66)
Mean	22.85	28.44		11.65	18.6		26.48	36.37		35.89	31.98		17.09	22.87	
	(15.33)	(23.66)		(7.00)	(13.33)		(23.00)	(36.00)		(35.00)	(29.00)		(11.33)	(18.00)	
CD (p=0.05) for CIM		1.932			3.883			2.30			1.57			2.428	
CD (p=0.05) for RM		3.055			6.140			3.638			2.49			3.83	
CD (p=0.05) for CIM x RM		4.321			-			5.144			3.524			-	

CIM I : Callus Induction Medium I
CIM II : Callus Induction Medium II
RM : Regeneration media
(Actual percentage are given in parentheses)

Discussion

Both somatic embryogenesis and organogenesis of tissues cultured *in vitro* are the direct result of a combination of hereditary, developmental and environmental factors as well as variation in growth regulator, physical and other chemical components of culture media (Evans *et al.,* 1981; Williams and Maheswaran, 1986). Christianson and Warnick (1985) have shown that the morphogenic response can be shown to occur as three distinct stages: the acquisition of competence; induction or the determination towards a particular morphogenic pathway; a subsequent differentiation, in which tissue organization becomes apparent.

An important factor for in *vitro* regenerations the nutrient composition of the culture medium. Eeuwens (1976) tried to improve callus formation and growth by optimizing the mineral composition of the culture media for coconut through factorial experiments. Branton and Blake (1984), Blake (1999) and Dublin *et al.* (1991) have suggested hormonal imbalance as a reason for the low percentage of callus formation and the development of abnormal plantlets.

Micropropagation of coconut from any tissue requires a strong auxin stimulus and 2,4-D is the most commonly used auxin for collagenosis and somatic embryogenesis (Hronug, 1995). Verdiel and Buffard-Morel (1995) reported that this hormone was essential for the activation and division of the undifferentiated cells in the explant to produce calli. One of the difficulties encountered in palm regeneration is the intense browning of the explant tissues due to the oxidation of polyphenolic compounds (Eeuwens and Blake, 1977; Reynolds and Murashige, 1979; Jones, 1974; Nwankwo and Kirkorian, 1983). The polyphenolic compounds may bind up key amino acids and proteins thus preventing critical enzymatic processes from taking place (Chalker-Scott and Fuchigami, 1989). The browning observed could be linked to the high sensitivity of the explant tissue to plant growth regulator like 2,4-D, which is essential for callus formation (Hronug and Verdiel, 1999). Activated charcoal is added to the media to avoid necrosis (de Guzman and del Rosario, 1964), to adsorb polyphenolic compounds secreted by the tissue (George and Sherrington, 1984). and ethylene (Mensuali-Sodi *et al.,* 1993). But activated charcoal also binds other medium components such as auxins and cytokinins (Ebert and Taylor, 1990; Ebert *et al.,* 1993), and vitamins (Weatherhead *et al.,* 1978), thus altering the ratios of medium components. Blake (1990) reported that the formation of pro-embryoids from coconut calloids was generally observed in the presence of activated charcoal and a high level of 2,4-D. The effect of activated charcoal on culture establishment has been reported in various species. Since no response of explants was observed in the control medium (without any growth regulators) in this investigation, it can be considered that activated charcoal may be involved in normalizing the development/regeneration in the presence of growth regulators by its adsorption of inhibitory substances in the culture medium. In this study, the proportion of activated charcoal in the media was reduced with the reduction in the concentration of auxin.

The use of TDZ in association with 2,4-D in the callus induction medium was effective for enhancing the rate of regeneration. Huetteman and Preece (1993) have reported that TDZ is a potent cytokinin for promoting callus formation when used at 0.1M. Neuman *et al.*, (1998) used 2,4-D and TDZ in the primary media for immature cotyledonary tissue cultures of black walnut.

Somatic embryo maturation is commonly accomplished with growth regulator-free media in many crops. However, in coconut, workes have reported that once the nodular calloids were formed, the gradual reduction in the auxin concentration with a corresponding increase in the cytokinin level in the subsequent subculture was necessary for initiation and development of embryoids. The reduction in auxin level has to be gradual and in a controlled manner to achieve a balanced development of plantlet with a well organized shoot meristem and normal adventitious root formation. Sudden removal of auxins resulted in massive development of haustorial tissue and precocious root proliferation at the expense of continued shoot meristem development of haustorial tissue and precocious root proliferation at the expense of continued shoot meristem development (Branton and Blake, 1983). In coconut palm somatic embryogenesis, the lowering of 2,4-D concentration followed by addition of BAP was found to be essential for the complete bipolar differentiation of the embryo (Verdeil *et al.*, 1994). Merkle (1995) reported that the supplementation of cytokinins during the histo-differentiation phase could compensate for the detrimental effects of auxins on meristem development. In the present study, exogenous supplementation with either cytokinins /polyamines, in the presence of decreasing concentrations of an auxin, was found to be essential for somatic embryo maturation/meristemoidal development and regeneration into normal plantlets. The auxin-cytokinin/polyamine interaction might have had a decisive effect on the hormonal regime of explant proliferation and eventual embryogenesis/organogenesis in coconut. Ammirato (1987) pointed out that normal development of somatic embryos required a fine temporal and spatial regulation of cell division, enlargement and differentiation. Growth regulators may exert multiple effects in these processes depending on the concentration or on the embryo stage at the time of application.

The frequency of induction of embryogenic calli, somatic embryoids and production of plantlets was highest in Y3 medium supplemented with spermine. Exogenously supplied polyamines have been known to induce somatic embryogenesis and plant regeneration in many species (Galston and Flors, 1991; Martin-Tanguy and Carrie, 1993; Adkins *et al.*, 1998; Sargent *et al.*, 1998 Rajesh *et al.*, 2003). The mechanism of polyamine functions in living cells is probably linked to their chemical and physical interactions with nucleic acids, proteins and phospholipids, and is due to their cationic nature (Bagni *et al.*, 1982; Smith, 1985). Polyamines play a stabilizing role on nucleic acids by binding to phosphate groups, and in particular, they form complexes with DNA (Liquori *et al.*, 1967; Zhurkin *et al.*, 1980). There are also reports that polyamines could be involved as second messengers on hormonal regulation of growth and development (Galston and Kaur-Sawhney, 1990).

Chan *et al.*, (1998) had earlier reported that a greater proportion of the plumule explants developed into calli bearing embryogenic structures when the cultures were maintained undisturbed in a medium containing 0.1mM 2,4-D and no subculturing was practiced. But, in our study, we have seen that monthly subcultures into media containing lower concentrations of 2,4-D with a constant concentration of either polyamines or cytokinins was essential for production of embryoids or meristemoids for the plumular explants. In the absence of either cytokinins or polyamines in the regeneration medium, the cultures exhibited a tendency towards rhizogenesis in the presence of 2,4-D. This shift towards rhizogenesis was also prominent in putrescine-supplemented medium. Polyamine treatment has been reported to promote *in vitro* rooting in olive cultives (Rugini *et al.*, 1992; Grigoriadou *et al.*, 2002).

The development of an efficient method of cloning coconut using plumular explants (with more rapid development of calli and somatic embryos and greater frequencies of plant regeneration compared with calli from inflorescence or leaf tissues) offers a potential for the development of a long-term *in vitro* means of conserving significant coconut germplasm by cryopreservation of plumular explants (Hornutg *et al.*,2001). Oropeza *et al.* (2002) have suggested the possibility of massive multiplication of elite palms selected on the basis of resistance to lethal yellowing disease of coconut using plumular explants. Plumule cultures can be used for rapid multiplication of proven coconut hybrids. Also, the results of this study can form a model for future regeneration studies from adult tissues of coconut.

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