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Direct *in vitro* shoot development from immature rachilla explants of Coconut (*Cocos nucifera* L.)

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Abstract

Young inflorescences extracted from the axil of the just opening leaf measuring about 12 cm was the source of explant. The rachilla cut into bits of 5 to 7 mm were conditioned for two spells of 16 weeks each on half strength MS medium with 200 mg NaH2PO4, 1.0 mg Calcium pantothenate 0.05 mg Biotin and 0.01 mg folic acid fortified with 41.41 µM picloram and 4.54 µM thidiazuron, 30 g sucrose, 1g/l activated charcoal and 5.5 g/l agar and incubated in the dark. They were maintained for a third spell of 16 weeks on modified ½ MS with 20.71µM picloram and 4.54 µM TDZ followed by a change to modified ½ MS medium with 8.28 µM and 4.54 µM TDZ, maintained in diffused light showed the emergence of shoots from the floral primordia. They on transfer to basal media without hormones and incubated in light developed into plantlets. The presence of picloram, TDZ, biotin, calcium pantothenate and folic acid were crucial for the shoot development.

Abbreviations: 2,4-D: 2,4-Dichlorophenoxyaceticacid, P: Picloram (4-Amino-3,5,6-trichloropicolinic acid), TDZ: Thiadiazuron [1-phenyl-3 (1,2,3-thiadiazol-5yl)urea]

Key words: Coconut, *in vitro* shoot development, Floral primordia, Picloram, Thiadiazuron.

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Introduction

Coconut the monotypic genus has only limited variability. Very often they are grown under small holdings. The crop also faces other problems due to the various diseases like cadang-cadang in Asia (Hanold and Randles, 1991); lethal yellowing in America (Arellano and Oropeza, 1995); root (wilt) in Kerala (Solomon *et al.*, 1983) besides a large number of pests. The average yield of coconut is low mainly due to the poor genotypes under cultivation.

There are exceptionally high yielding genotypes occasionally seen in the coconut plantations. These genotypes cannot be fixed as the plant is not amenable to any of the conventional methods of vegetative propagation. Utilization of such genotypes for breeding for developing new variety with all its characteristics is impractical. Tissue culture with the multifaceted aspects can be a boon to the coconut industry if the *in vitro* propagation using tissue culture is made a reality.

Coconut is a highly recalcitrant material for tissue culture. The tissue culture techniques have been tried for the past several decades using different explants tender leaf (Branton and Blake, 1983; Raju *et al.*, 1984; Buffard-Morel *et al.*, 1992), immature inflorescence explants (Branton and Blake, 1986; Verdeil *et al.*, 1994; Blake and Hornung, 1995), embryos and plumular tissues (Karunaratne and Periyapperuma, 1989; Hornung, 1995; Chan *et al.*, 1998; Rajesh *et al.*, 2005), endosperm (Prakash *et al.*, 1985) with limited success. The inconsistency in the results obtained so far is beyond the scope of commercialization. Any progress in achieving the clonal propagation of this highly recalcitrant species will be of great advantage to the improvement of this crop and the well being of the coconut farmers world wide. This paper reports the formation of plantlets from the floral primordia by switching the floral meristem to a vegetative pattern of growth.

Materials and methods

The young inflorescences collected form the axil of the first opening leaf of 15 to 20 year old West Coast Tall palms were extracted without sacrificing the tree. The inner spathe of the inflorescences measuring 10 to 18 cm in length depending on the number of leaves on the crown as well as the spatial arrangement of the leaves on the crown. They were surface sterilized in 0.1% mercuric chloride solution for 10 minutes followed by washing in six changes of sterile water. In an earlier set of experiments, where the rachilla explants of 2 to 12 mm sizes were used as the explant, the best response was obtained from 5 to 7 mm sized bits. So after removing the spathe the rachillae were cut into 5 to 7 mm long bits and cultured on to the different nutrientmedia.

Y3 (Eeuwens, 1976), half strength MS (Murashige and Skoog, 1962) and half strength MS medium with 200 mg NaH_2PO_4, 1.0 mg Calcium pantothenate, 0.05 mg Biotin and 0.01 mg folic acid (hereafter mentioned as modified ½ MS) were used as the basal media for culturing. All media had 30g/l sucrose, 1g/l activated charcoal and 5.5g/l agar. pH of the media was adjusted to 5.8 with 1N NaOH or 1N HCl and sterilized by autoclaving at 1.06 kg cm^2 for 15 minutes. The cultures were incubated in the dark for the first three spells of 16 weeks each and later transferred to the light.

Culture initiation:

In the culture initiation stage all the three basal media supplemented with three levels of picloram (41.41, 20.71 and 8.28 µM) and three levels of 2,4-D (100 µM, 50 µM, 25 µM) alone and in combination with three levels of TDZ (9.08, 4.54 and 2.27 µM) were used for inoculation of the fresh explants. The cultures were incubated in the dark at 27 ± 2°C. Each treatment had 10 cultures and four replications. The responses were checked at intervals of 8 weeks.

Subculture:

After 16 weeks of incubation, the cultures which were remaining healthy without browning, signs of flower development or callus formation were transferred to modified ½ MS medium with P 41.41µM + TDZ 4.54µM, P 41.41 µM + TDZ 9.08 µM and P 20.71 µM+ TDZ 4.54µM which gave maximum healthy cultures and incubated in the dark at 27 ± 2°C for 16 weeks. They were observed at intervals of 8 weeks.

The cultures after the second spell of 16 weeks incubation were then transferred to the corresponding media with half the level of picloram and were again incubated in the dark at 27 ± 2°C.

Statistical analysis:

The data recorded on the number of cultures showing callus formation, remaining fresh and which were turning brown or developing into flowers was analysed by a two factorial design. The two factors considered were six levels of auxins, P1 – P3 (44.41, 20.71 and 8.28 µM picloram), D1 to D3 (100,50 and 25µM 2,4-D) and the four levels of cytokinin TDZ (0.0, 9.08, 4.54 and 2.27 µM). As the values were the count of the cultures, a square root transformation (Gomez and Gomez 1984) was done before the analysis.

Shoot induction:

The cultures which had survived these three cycles of incubation without callusing, floral development or browning were then transferred to the respective media were the concentration of picloram was reduced to half and incubated in diffused light (less than 100 lux) for 16 weeks at 27±2°C.

Plantlet development:

The cultures which had shoot initiation were then transferred to the modified ½ MS medium without any growth regulators after separating into individual ones or as bunches of shoots (without separating individually). All these cultures were incubated in the light from white fluorescent lamps of 6800°K adjusted to 16 hour light photoperiod at 27 ± 2°C.

Results and discussion

The spadices measuring 10-12 cm in length only responded to shoot induction, whereas the smaller ones (inner spathe less than 10 cm in length) showed callus formation and the older ones (spathes of more than 13 cm in length) developed into floral structures. Pooled response of the explants on the three basal media is presented in Figure 1.

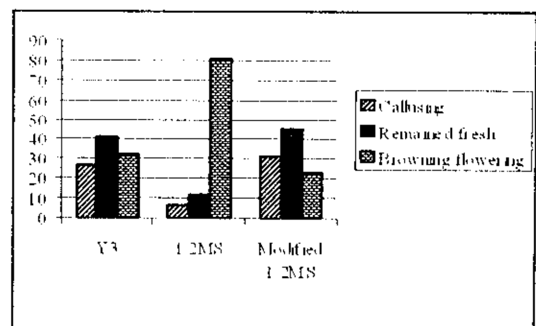

Fig. 1. General response of rachilla explants to the basal media; pooled data (in %) from all hormone combinations: modified ½ MS retaining the explants fresh during the conditioning period

The percentage response of floral primordia to different media are given in Table 1. The influence of Y_3 medium as well as the modified media on the rachilla explants was almost similar. Callusing was higher (31%) in modified ½ MS, compared to 27 % in Y_3 medium; at the same time, the browning was only 23% in modified ½ MS medium and 32 % in Y_3 medium. Three types of response were seen in the cultures at the end of two culture cycles of 16 weeks duration each: remaining fresh with no appreciable growth, callus formation and browning. The mean of the values along with the actual values (in parenthesis) and the CD are given in Tables 1, 2 and 3 respectively for the three response types. The best combination was 44.41 µM picloram

along with 4.54 µM TDZ followed by 44.41 µM picloram and 9.08 µM TDZ for preconditioning the rachilla explants for shoot development.

Table 1. The mean number of cultures which remained fresh at the end of two cycles of 16 weeks incubation in the dark on media with different hormone levels

	0.0 TDZ	9.08 µM TDZ	4.54 µM TDZ	2.27 µM TDZ	
44.41 µM Picloram	0.707 (0.000)	2.246 (4.600)	2.622 (6.400)	1.448 (2.000)	1.756 (3.250)
20.71 µM Picloram	0.985 (0.600)	1.667 (2.600)	1.572 (2.200)	1.497 (1.800)	1.430 (1.800)
8.28 µM Picloram	0.811 (0.200)	1.232 (1.200)	1.089 (0.800)	0.707 (0.000)	0.960 (0.550)
100 µM 2,4-D	0.707 (0.000)	0.707 (0.000)	0.811 (0.200)	0.707 (0.000)	0.733 (0.050)
50 µM 2,4-D	0.707 (0.000)	0.707 (0.000)	0.914 (0.400)	0.707 (0.000)	0.759 (0.100)
25 µM 2,4-D	0.811 (0.200)	0.707 (0.000)	0.914 (0.400)	1.018 (0.600)	0.862 (0.200)
	0.788 (0.167)	1.211 (1.400)	1.320 (1.733)	0.014 (0.733)	1.083 (1.008)

CD for TDZ - 0.194 ** significant at 1%
CD for Auxins - 0.389 ** significant at 1%
CD for interaction - 0.389 ** significant at 1%

Table 2. The mean number of cultures developing callus at the end of two cycles of 16 weeks incubation in the dark on media with different hormone levels

	No TDZ	9.08 µM TDZ	4.54 µM TDZ	2.27 µM TDZ	
44.41 µM Picloram	2.066 (3.800)	1.497 (1.800)	1.510 (1.800)	1.605 (2.200)	1.669 (2.400)
20.71 µM Picloram	1.770 (3.000)	1.147 (1.000)	1.115 (1.000)	1.160 (1.000)	1.298 (1.500)
8.28 µM Picloram	1.115 (1.000)	0.914 (0.400)	0.914 (0.400)	0.914 (0.400)	0.964 (0.500)
100 µM 2,4-D	1.907 (3.400)	1.695 (2.800)	1.501 (2.000)	1.393 (1.600)	1.624 (2.450)
50 µM 2,4-D	1.675 *(2.600)	1.887 (3.200)	1.637 (2.600)	1.268 (1.400)	1.617 (2.450)
25 µM 2,4-D	1.160 (1.000)	1.568 (2.000)	1.335 (1.400)	0.882 (0.400)	1.236 (1.200)
	1.616 (2.467)	1.451 (1.867)	1.335 (1.533)	1.204 (1.167)	1.401 (1.758)

CD for first factor (TDZ) - 0.298
CD for second factor (Auxin) - 0.243
CD for interaction - Not Significant

Cultures forming callus, flowers and those turning brown were not sub-cultured in this set of experiments. Cultures which remained fresh after 32 weeks incubation in the dark alone were sub-cultured for further development. They were whitish and remained without any visible callusing from any part of the explant (Fig. 2a). The subtending bracts alone showed some expansion but no callusing.

Table 3. The mean number of cultures turning brown and developing into flowers at the end of two cycles of 16 weeks incubation in the dark on media with different hormone levels

	No TDZ	9.08 µM TDZ	4.54 µM TDZ	2.27 µM TDZ	
44.41 µM Picloram	2.584 (6.200)	1.999 (3.600)	1.497 (1.800)	2.506 (5.800)	2.146 (4.350)
20.71 µM Picloram	2.614 (6.400)	2.613 (6.400)	2.657 (6.800)	2.760 (7.200)	2.661 (6.700)
8.28 µM Picloram	3.043 (8.800)	2.978 (8.400)	3.043 (8.800)	3.177 (9.600)	3.061 (8.900)
100 µM 2,4-D	2.631 (6.600)	2.746 (7.200)	2.867 (7.800)	2.978 (8.400)	2.806 (7.500)
50 µM 2,4-D	2.796 (7.400)	2.691 (6.800)	2.691 (7.000)	3.006 (8.600)	2.796 (7.450)
25 µM 2,4-D	3.047 (8.800)	2.913 (8.000)	2.945 (8.200)	3.077 (9.000)	2.996 (8.500)
	2.786 (7.367)	2.657 (6.733)	2.617 (6.733)	2.917 (8.100)	2.744 (7.233)

CD for first factor (TDZ) - 0.191
CD for second factor (Auxin) - 0.156
CD for interaction - 0.382
*(Significant at 5% level)

The cultures in media containing 2,4-D developed more callus or turned brown in contrast to those on picloram. The callus formed from the floral primordia in modified ½ MS medium with 100 µM 2,4-D and 2.27 µM TDZ was soft and highly friable with an off-white colouration (Fig. 2b). These did not survive on subculture in the same medium as well as in those with several modifications of hormone and vitamin concentrations. The staminodes and thalamus from the inflorescences measuring over 15 cm produced calloids. The floral primordia developed roots after a brief callusing on Y_3 medium with 100 µM 2,4-D and 2.27 µM TDZ (Fig. 2c).

During the third cycle of maintenance under diffused light, the elongation of the floral meristem and formation of vegetative shoot could be seen (Fig. 2d,e). At the end of this cycle of incubation, the shoots were quite distinct and could be separated individually or

sub-cultured as small groups. The number of shoots developing per rachilla explant varied from 1 to 14. Many of them had developed roots at the base of the shoots (Fig. 2f).

The floral primordia located towards the base of rachilla which normally develop into female flowers instead produced very odd structures (Fig. 2g), or in some cases developed into a basal globular button-like structure with 3 to 4 stigmatic lobes (Fig. 2h).

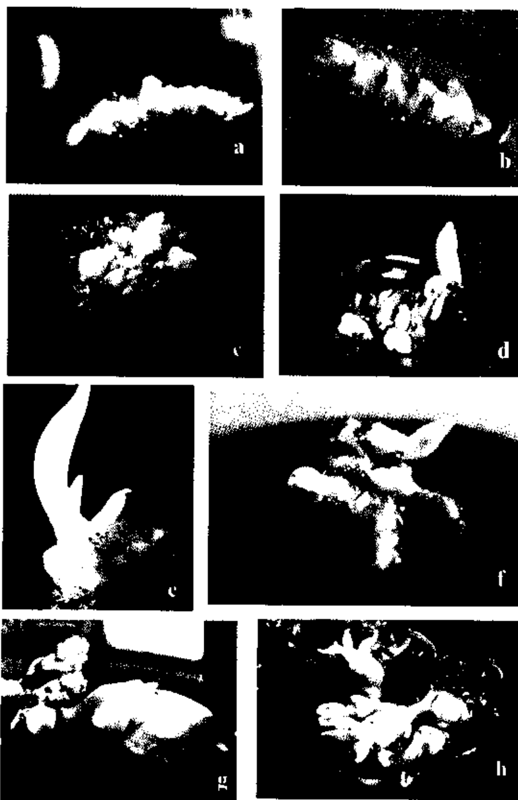

Fig. 2. The responses of the rachilla explants to different media in vitro: (a) explant remaining fresh after 32 weeks in media containing P 44.41 □M + 4.54 □M TDZ, (b) Rachilla, (c) rachilla bits on Y3 with 100□M 2,4-D and 2.27 □M TDZ developing callus followed by rooting, (d) third subculture which received diffused light developed into shoots, (e) shoots formed in total darkness which lack chlorophyll, (f) developing primordia transferred to light developed green shoots and roots, (g-h) female flowers developing into weird structures some with multiple stigmatic lobes

Transfer of the vegetative shoots into plain basal MS media without any hormones resulted in the emergence of green shoots (Fig. 3a). The shoots that separated too early produced profuse roots and shoot growth was eventually inhibited (Fig. 3b). The emerging shoots when transferred as small groups developed faster than as individuals. When they were transferred from diffused to normal light, they developed chlorophyll and started growing faster after an initial two weeks (Fig. 3c, d). After 15-16 weeks incubation in normal light, one out of the four shoots grew to about 15 cm in height (Fig. 3e). The plants obtained in this manner and are yet to be planted in the potting medium.

The rachilla explants which responded to shoot induction trials with minimal browning were those measuring around 5-7 mm in length. Some of the explants showed browning at the extreme ends but most of them remained healthy without callus formation, progressing into floral development or browning. This is in contrast to 1 - 1.5 mm rachilla segments used for calloid induction (Verdeil *et al.*, 1994). The cultures in media with 2,4-D normally formed callus, but to induce direct switching of floral meristems into vegetative phase, 2,4-D was avoided in later stages.(How much later ?) Smaller explants measuring less than 5 mm did not show any shoot formation in any of the media. Probably the larger proportion of injured tissues in a smaller explant may be adversely affecting the further organized development of the floral primordia.

Inclusion of thidiazuron has been essential for the transformation of the floral meristem into vegetative shoot. Huetteman and Preece (1993) demonstrated the use of this compound for induction of shoot regeneration in several recalcitrant woody plant species like *Acer rubrum*, *Acer saccharinum*, *Acanthopanax*, *Gymnocladus dioicus*, *Cercis*, *Hedera canariensis*, *Hibiscus rosa-sinensis* etc Since then its efficacy has been demonstrated in several plant species (Michelangeli *et al.*, 2002 in annato; Yang Yuan *et al.*, 2003 in Anthurium leaf culture; Purohit *et al.*, 2004 in sapota leaf

culture; Kim *et al.*, 2004 in *Centella*; Franklin *et al.*, 2004 in eggplant root culture; and Rajesh *et al.*, 2005 in coconut plumule culture).

Picloram, a synthetic pyridine herbicide, has turned out to be a very handy auxin for inducing somatic embryogenesis and regeneration in a number of plants such as, the Pejibaye palm (*Bactris gasipaes* H.B.K.) (Valverde *et al.*, 1987), *Areca catechu* (Karun *et al.*, 2004), *Musa* (Filippi *et al.*, 2001), the highly recalcitrant rattan species, *Calamus merrillii* and *C. subinermis* (Goh *et al.*, 2001), chickpea (Kumar *et al.*, 1995) and *Tulipa gesneriana* (Bach *et al.*, 2001). It also showed a synergistic effect with TDZ in several plants on somatic embryogenesis and plant regeneration (Sharma *et al.*, 2005 in wheat shoots; Gill and Saxena, 1993 in tobacco leaf discs). Chan *et al.* (1998) and Rajesh *et al.* (2005) reported plantlet regeneration through somatic embryogenesis from plumule culture with 2,4-D, whereas in the present study with immature rachilla explants, only picloram in combination with TDZ was effective in inducing direct plantlet development.

The young inflorescences which can be extracted in a non-destructive manner has proved to be a good source of meristematic tissue for the clonal propagation of coconut palm. From the rachilla of comparable maturity, Verdeil *et al.* (1994) have demonstrated callus induction followed by somatic embryogenesis. The callus phase is often associated with the possibility of somaclonal variation and hence the present procedure of direct shoot regeneration from floral primordia can be a surer means for clonal propagation without the risk of somaclonal variation.

Production of bulbil shoots as a rare, spontaneous, recessive mutation has been recorded in coconut by Davis (1948), as also in oil palm (Davis, 1959), and in *Borassus flabellifer* L. (Davis and Basu, 1969). Attempts to propagate these bulbil shoots of mutant coconut by air-layering were successful (Sudasrip, Kaat and Davis, 1978) although the resulting clonal palms continued to produce only bulbils and no nuts. The present attempt has demonstrated the distinct possibility of inducing such bulbil shoots *in vitro* under appropriate culture conditions. Using the right developmental stage of inflorescence rachilla explants, it has been possible to convert the floral meristems into vegetative shoots directly without the intervention of a callus-phase, prior to the onset of the morphogenetic signal to form a flower. Hence, the present success of inducing direct shoot formation from the would-be floral primordia, holds promise of evolving a viable technology for the large-scale clonal multiplication of coconut *via* the bulbil-pathway.

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