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Callogenesis of coconut (*Cocos nucifera* L.) using immature inflorescence and young leaf explants

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ABSTRACT: The study was aimed at generating callus from immature inflorescence and young leaf explants of coconut (*Cocos nucifera* L). Eeuwen's medium which was supplemented with Murashige and Skoog macro salts, 6-Benzylamino purine (BAP) and 6-y dimethylally amino purine (2iP) at concentration of 1 mg/l each, was used as the culture medium. The effect of different concentrations of growth regulators when added to the culture medium was tested on callus generation. The auxins used were 2,4-Dichlorophenoxy acetic acid (2,4-D) and Naphthalene acetic acid (NAA), at concentrations of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 mg/l. The immature inflorescence and young leaf explants were used for culture initiation. Callus production was observed between 14 – 26 weeks after culture initiation. The results obtained showed that callus generation was not possible from both explants when concentrations between 0 - 40 mg/l, 2,4-D and NAA were used for media supplementation. The growth regulator that was more effective in generating callus from both leaf and inflorescence explants was 2,4-D, with optimal concentration of 50 mg/l. In leaf culture, the optimal concentration of 2,4-D yielded 40 % callusing explant and 0.082 g fresh mean weight of callus, while the inflorescences yielded 20 % callusing explant. By a way of comparison, the leaf explant is to be preferred over the inflorescence explants for callus production in coconut.

Keywords: *Cocos nucifera,* Callogenesis, *In vitro*, BAP, 2iP, 2,4-D, NAA

Introduction

Coconut (*Cocos nucifera* L.) which belongs to the monocot family, order, Arecales, is one of the most important oil crops in the tropics. It is called "the tree of life" as it has attained an important socio-economic role in the local communities where it is produced (Persley, 1992). The tree is successfully grown in the tropic and sub-tropic areas, hence referred to as 'King of the Tropical Palms'. There are different coconut varieties and they are divided into two major groups, the 'Tall and Dwarf' cultivars. Both cultivars can hybridize to produce intermediate forms (Child, 1974). Coconut varieties include the West African tall, dwarf green, Malayan dwarf yellow, Malayan dwarf red and hybrid coconut. They are named after areas where they have been grown long enough to have developed distinctive characteristics associated with these areas of the world (Woodroof, 1970). The tall cultivars are grown for commercial purposes because they have longer life span and produce more coconut meat (by weight) than the dwarf cultivars (Thangaraj and Muthuswami, 1990). Most of the world's coconuts are grown in the Asia, Philippines, Indonesia and India being the largest producers. Over 50 million people are directly involved with its production. There is a need to produce new disease resistant, high yielding coconut cultivars to meet the production needs of the present day. The coconut palm is therefore heterozygous and because it has a long generation period of more than 10 years, breeding programme is relatively slow (Thanh-Tuyen and De Guzman, 1983). Seed production per tree is low and asexual propagation is not possible. The plant is traditionally propagated by seed (fruit) which is recalcitrant and has a short storage life. The plant is long-lived but has a very long juvenile phase. Many of these challenges in propagating coconut could be surmounted by *in vitro* culture technique.

In vitro culture is one of the channels opened for intensive vegetative propagation of coconut and the development of a reliable *in vitro* cloning technique (Davis, 1969). Vegetative multiplication of elite palms is a promising possibility for producing uniform planting materials of high quality. This would not only improve the productivity in coconut lands but also produce uniform parent materials for breeding programmes. This can also pave the way to genetic transformation of coconut by transfer of foreign genes that confer desirable characters (Assy, 1986). The study therefore was aimed at generating callus from coconut leaf and immature inflorescence explants, the first step towards plant regeneration.

Materials and Methods

Sources of explants

Coconut tissues from two different sources, young leaves and immature inflorescences, were obtained from Dwarf Green coconut palms from chemistry field of the Nigerian Institute for Oil Palm Research (NIFOR). The leaf explants were obtained from coconut seedlings of about 2-3 years old. Unopened spear leaf tissues taken from the apical growing regions of the palm were used for callus production. The immature inflorescence explants were obtained from leaf axils (frond). Following the methods adopted by Steinmacher *et al.* (2007) for peach palm, the inflorescences used were very young. The length of the external spathe was 8-10 cm. After collection of explants, 70% ethanol was used to spray the explant before being wrapped in aluminum foil and put in a sampling bag for protection and then taken to the laboratory.

Preparation of culture medium

Eeuwens (1976) medium which was modified with Murashige and Skoog macro salts (1962) was used as the basal stock. The basal medium was supplemented with growth regulators, 2,4-dichlorophenoxy acetic acid (2,4-D) and 1-naphthalene acetic acid (NAA) at different concentrations ranging from 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 mg/l. The medium was also supplemented with 6-Benzyl amino purine (BAP) and 6-y dimethylallyl amino purine (2iP) at concentration of 1 mg/l each. Sucrose at 30 g/l was used as carbon source, agar at 8 g/l was used to solidify the medium and 2.5 g/l activated charcoal was added to control phenolic activities. The culture media were adjusted to pH 5.7 before autoclaving at 121^{o}C for 15-20 min. The autoclaved culture media were allowed to cool before use for inoculation.

Sterilization of explants

The inflorescence explants were washed with detergent and thoroughly rinsed with running tap water for 10-20 min. The external spathes were removed under aseptic conditions. The inflorescences with the inner spathes intact was sprayed with 70 % ethanol and allowed to air dry. Both explants were submerged in a disinfectant (2.5 % sodium hypochlorite) solution and gently agitated for 3-5 min. A wetting agent Tween 20 was added to the disinfectant to reduce tension and improve contact between disinfectant and tissue. The disinfectant solution was discarded and explants were rinsed three times with sterile distilled water.

Inoculation of explants

The sterilized explants were inoculated into the culture media. The inoculated cultures were incubated in growth room at 25±2 ^{o}C and subcultured every 4-8 weeks in the culture room. The cultures were exposed under dark and light photoperiod, 12-14 and 8-10 hours respectively, and were maintained in the dark during the first 3-5 months for callus induction.

Monthly subculture

Monthly subculture was carried out every 4 weeks and the numbers of callus formed were counted and recorded. The cultures that were contaminated were removed at the end of each week. In each sub culture a fresh media were used.

Results

One week after culture initiation, the explants (young leaf and immature inflorescence) gradually enlarged to 2 to 3 times the original size. After 6 weeks in culture, some changes were observed around the cut edges of the explants. There was a change in explant coloration from white to yellowish-brown. The results obtained for percentage (%) callusing leaf explant of *Cocos nucifera* cultured on modified Eeuwens medium supplemented with 2,4-D are shown in Table 1**.** Callus formation was not observed in the 0 – 40 mg/l treatments. Callus induction was observed between 14-18 weeks in the 2,4-D (45-55 mg/l) supplemented medium. Of the treatments tested, 45 mg/l produced 26.6 % callus, 50 mg/l produced 40% callus, and 55 mg/l produced 26.6% callus, while 40 mg/l and 60 mg/l produced no callus. Callus was first observed in the 50 mg/l, 2,4-D treatment at 14 weeks (Plate 1).

Table 1: Percentage callusing leaf explant of *Cocos nucifera* in modified Eeuwens medium supplemented with 2,4-D

Concentration of growth regulator			Time of callus formation (Week)	Percentage callusing explants
2,4-D (mg/l)	**2iP (mg/l)**	**BAP (mg/l)**		
00	0.0	0.0	-	0.00
00	0.0	1.0	-	0.00
00	1.0	0.0	-	0.00
05	1.0	1.0	-	0.00
10	1.0	1.0	-	0.00
15	1.0	1.0	-	0.00
20	1.0	1.0	-	0.00
25	1.0	1.0	-	0.00
30	1.0	1.0	-	0.00
35	1.0	1.0	-	0.00
40	1.0	1.0	-	0.00
45	1.0	1.0	14	26.6
50	1.0	1.0	16	40.0
55	1.0	1.0	18	26.6
60	1.0	1.0	-	0.0

2,4-D: 2,4-dichlorophenoxy acetic acid; BAP: Benzylamino purine; 2iP: 6-Y-dimethylallyl amino purine

Plate 1: Morphogenetic response of coconut leaf explant on modified Eeuwens medium supplemented with 50 mg/l 2,4-D.

Table 2 shows the results obtained for callus initiation from leaf explant when 2,4-D at various concentrations were used in media supplementation. Callus intensity was greatest at 50mg/l 2,4-D. The yield of fresh callus by mean weight in treatment 45 mg/l, 50 mg/l and 55 mg/l were 0.038 g, 0.082 g and 0.035 g, respectively.

Table 2: Effect of 2,4-D in modified Eeuwens medium on callus generation from *Cocos nucifera* leaf explant

Concentration of growth regulator			Callus intensity	Fresh mean weight of callus (g)
2,4-D (mg/l)	2iP (mg/l)	BAP (mg/l)		
0	0	0	-	0.000
40	1.0	1.0	-	0.000
45	1.0	1.0	++	0.038
50	1.0	1.0	++++	0.082
55	1.0	1.0	++	0.035
60	1.0	1.0	-	0.000

- : No callus formation; +: Not profuse; ++: Slightly profuse; +++: Profuse; ++++: Very profuse;2,4-D: 2,4-Dichlorophenoxy acetic acid; BAP: Benzylamino purine; 2iP: 6-Y-dimethy amino purine

When coconut leaf explant was cultured on modified Eeuwens medium supplemented with NAA (Table 3), there was no callus formation between 0-40 mg/l treatments. Callus induction was first observed between 20-22 weeks after culture initiation in the various treatments. Of the treatments tested, 45 mg/l NAA produced 6.6% callus; 55 mg/l produced 6.6% callus, while callus formation was not observed in other treatments. The earliest callus formation was observed in the 55 mg/l NAA treatment after 20 weeks after culture initiation.

Table 3: Percentage callusing leaf explant of *Cocos nucifera* in modified Eeuwens medium supplemented with NAA

Concentration of growth regulator			**Time of callus formation (Week)**	**Percentage callusing explants**
NAA (mg/l)	**2iP (mg/l)**	**BAP (mg/l)**		
00	0.0	0.0	-	0.0
00	0.0	1.0	-	0.0
00	1.0	0.0	-	0.0
05	1.0	1.0	-	0.0
10	1.0	1.0	-	0.0
15	1.0	1.0	-	0.0
20	1.0	1.0	-	0.0
25	1.0	1.0	-	0.0
30	1.0	1.0	-	0.0
35	1.0	1.0	-	0.0
40	1.0	1.0	-	0.0
45	1.0	1.0	22	6.6
50	1.0	1.0	-	0.0
55	1.0	1.0	20	6.6
60	1.0	1.0	-	0.0

NAA: Naphthalene acetic acid; BAP: Benzylamino purine; 2ip: 6-Y-dimethylallyl amino purine

The results obtained for the weight of callus and callus intensity when leaf explants of *Cocos nucifera* was cultured on modified Eeuwens medium supplemented with NAA (Table 4) showed that callus was initiated at the various

concentrations of 40– 60 mg/l. Callus formation intensity was greatest at 55 mg/l concentration. The mean weights of fresh callus in 45 mg/l and 55 mg/l NAA treatments were 0.04 g and 0.06 g, respectively.

Table 4: Effect of NAA in modified Eeuwens medium on callus generation from *Cocos nucifera* leaf explant

Concentration of growth regulator			**Callus intensity**	**Fresh mean weight of callus (g)**
NAA (mg/l)	**2iP (mg/l)**	**BAP (mg/l)**		
0	0	0	-	0.00
40	1.0	1.0	-	0.00
45	1.0	1.0	++	0.04
50	1.0	1.0	-	0.0
55	1.0	1.0	+++	0.06
60	1.0	1.0	-	0.00

- : No callus formation; +: Not profuse; ++: Slightly profuse; +++: Profuse; 2,4-D: 2,4-dichlorophenoxy acetic acid; BAP: Benzylamino purine; 2iP: 6-Y-dimethylallyl amino purine

In the Percentage callusing inflorescence explants of *Cocos nucifera* (Table 5), callus induction occurred in the 50 mg/l (20 %; Plate 2) and 55 mg/l (6.6 %) 2,4-D treatments at 22 and 26 weeks after culture initiation, respectively. No visible callus was observed in other treatments.

Table 5: Percentage callusing inflorescence explant of *Cocos nucifera* in modified Eeuwens medium supplemented with 2,4-D

Concentration of growth regulator			**Time of callus formation (Week)**	**Percentage callusing explant**
2,4-D (mg/l)	**2iP (mg/l)**	**BAP (mg/l)**		
00	0.0	0.0	-	0.0
00	0.0	1.0	-	0.0
00	1.0	0.0	-	0.0
05	1.0	1.0	-	0.0
10	1.0	1.0	-	0.0
15	1.0	1.0	--	0.0
20	1.0	1.0	-	0.0
25	1.0	1.0	-	0.0
30	1.0	1.0	-	0.0
35	1.0	1.0	-	0.0
40	1.0	1.0	-	0.0
45	1.0	1.0	-	0.0
50	1.0	1.0	22	20.0
55	1.0	1.0	26	6.6
60	1.0	1.0	-	0.0

2,4-D: 2,4-dichlorophenoxy acetic acid; BAP: Benzylamino purine; 2iP: 6-Y-dimethylallyl amino purine

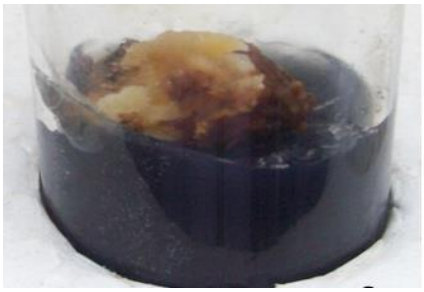

Plate 2: Morphogenetic response of coconut inflorescence explant on modified Eeuwens medium supplemented with 50 mg/l 2,4-D.

Table 6 shows the Percentage callusing inflorescence explants of *Cocos nucifera* in NAA supplemented medium. Only 60 mg/l treatment produced callus, with percentage callusing explant of 13.3. Callus was first observed between 22 - 24 weeks after culture initiation in the various treatments.

Table 6: Percentage callusing inflorescence explant of *Cocos nucifera* in modified Eeuwens medium supplemented with NAA

Concentration of growth regulator			**Time of callus formation (Week)**	**Percentage callusing explants**
NAA (mg/l)	**2 iP (mg/l)**	**BAP (mg/l)**		
00	0.0	0.0	-	0.0
00	0.0	1.0	-	0.0
00	1.0	0.0	-	0.0
05	1.0	1.0	-	0.0
10	1.0	1.0	-	0.0
15	1.0	1.0	-	0.0
20	1.0	1.0	-	0.0
25	1.0	1.0	-	0.0
30	1.0	1.0	--	0.0
35	1.0	1.0	-	0.0
40	1.0	1.0	-	0.0
45	1.0	1.0	-	0.0
50	1.0	1.0	-	0.0
55	1.0	1.0	-	0.0
60	1.0	1.0	24	13.3

NAA: Naphthalene acetic acid; BAP: Benzylamino purine; 2iP: 6-Y-dimethylallyl amino purine

Discussion

One week after culture initiation, the explants (young leaf and immature inflorescence) gradually enlarged to 2 to 3 times the original size. After 6 weeks in culture, some changes were observed around the cut edges of the explants. This study focused on callus induction from coconut young leaf and immature inflorescence explants. Callus can be obtained from different parts of a plant including leaf, shoot and pollen (Chan *et al.,* 1998) and zygotic embryos (Gupta *et al.,* 1995). In the present study, callus was obtained between 14 -26 weeks after culture initiation. The earliest callus was observed in 2,4-D at 50 mg/l for leaf explant, while the earliest callus was generated from inflorescence explant at 55 mg/l. This observation was similar with the finding of Thomas *et al*. (1995), where he reported that callus induction was observed in coconut leaf and inflorescence explants, 16 weeks after culture initiation. In date palm the duration of callusing is shorter than that of coconut explant, even in low concentration of 2-4,D. It took 3- 4 weeks for formation of callus in leaf, while inflorescence produced callus 6 -8 weeks after culture initiation (Zaid, 1981).

Calli obtained from this study were whitish brown, whitish yellow and highly friable. A similar report has been made where a friable callus was generated from shoot tip and inflorescence explants of date palm (Abul-Soad, 2011). Callogenesis occurred more in cultures made from modified Eeuwen's medium containing 2,4-D than those containing NAA for both leaf and inflorescence explants. In the medium containing 2,4-D, the highest amount of callus was generated. Various studies on the behavior of coconut explant with respect to auxin, showed that 2,4-D produced favorable callus than NAA (Blake and Eeuwens, 1982). Concentrations from 0-40 mg/l did not result in callus formation. High amount of 2,4-D from 45-60 mg/l were necessary for callogenesis. A similar observation was reported by Odewale *et al.* (1996) in callus formation of oil palm, where high level of auxin promoted callus induction. However, the culture containing NAA required long period for callus induction while 2,4-D required shorter period for callus induction (George and Sherrington, 1984). The optimum concentration that yielded callus, was 50 mg/l of 2, 4 - D.

Conclusion

This study indicated that callus can be generated from inflorescence and leaf explants of coconut, using modified Eeuwens medium supplemented with NAA and 2,4-D, in combination with BAP and 2iP. The earliest time of callus formation from coconut explant was 14 weeks after initiation. The auxin, 2,4-D was more suitable than NAA at optimum concentration of 50 mg/l for media supplementation. Also by a way of comparison callus obtained from leaf explant was preferred to that generated from the inflorescence.

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