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Evaluation on the effects of media modification and hormonal concentrations on coconut (*Cocos nucifera* L.) palm explants

Nwite P.A.[1], Olanrewaju O.O.[2], Igiebor F.A.[3,5*], Ikhajiagbe B.[4,5]

[1]*Physiology Division, Nigeria Institute for Oil Palm Research (NIFOR), Benin City, Nigeria*
[2]*Plant Breeding Division, Nigeria Institute for Oil Palm Research (NIFOR), Benin City, Nigeria*
[3]*Department of Biological Sciences, College of Science and Computing, Wellspring University, Benin City, Nigeria*
[4]*Department of Plant Biology and Biotechnology, University of Benin, Benin City, Nigeria*
[5]*Environmental Biotechnology and Sustainability Research Group, University of Benin, Benin City, Nigeria*

**Corresponding author, Email address: francis.igiebor@lifesci.uniben.edu*

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Abstract: The assessment of media for invitro culture of coconut palm (*Cocos nucifera* L.) was carried out with a view to achieving possible successful *in vitro* culture of coconut palm. To optimize local environmental factors and achieve the best possible outcome with coconut culture, the research involved scouring a vast amount of literature for crop *in vitro* propagation protocols that could have been used for other resilient crops. Six (6) modified Murashige and Skoog (MS) 1962 media were identified from either literature or reconnaissance work at the Nigeria Institute for Oil Palm Research (NIFOR), Benin City. These were augmented with various organic supplements, with varied concentrations and combinations of plant growth regulators (auxins and cytokinins). Immature inflorescences and non- chlorophyllous immature leaves of coconut served as explants and were cultured onto the modified media and incubated under dark conditions in the growth room at 25 ^{o}C. Bi- weekly subcultures were carried out for eight (8) weeks to check for viable cultures. The results showed that callus was formed on the immature inflorescences explants in the modified Murashige and Skoog medium augmented with 2,4-Dichlorophenoxy acetic acid (2,4-D) and Thidiazuron (TDZ) after four (4) weeks of culture. The callus proliferated for an additional two (2) weeks before mild browning was observed. All the other inoculated explants turned brown from the 4th week of culture irrespective of the media employed.

1. Introduction

One of the most important oil crops in the tropics which plays significant roles in the socioeconomic life of the inhabitants of the tropics is the coconut (Foale, 2003). The palm tree produces a large oval brown seed with a hard woody husk bounded by fiber, crinkled with edible white meat, and containing a perfect liquid known as (coconut water) (Angus and Maurice, 2011; Gunn *et al.*, 2011). Cultivation of the coconut is majorly through sexual means, via planting of sprouted nuts. The limitation of this method of reproduction is that it is uncertain and fewer offspring are typically produced and favorable genetic might not also be passed to the offspring. Besides, propagation progress is relatively slow (Perera *et al.*, 2009). There is therefore the need to produce great yielding coconut cultivars, with different disease resistant attributes to meet the production needs of the present day.

To enhance propagation as well as conservation strategies, micropropoagation techniques including tissue culture have been adopted for most palm crops (Elzebroek and Koopwind, 2008). Invitro culture is the simplest way open for in depth asexual breeding of coconut and the improvement of a dependable *in vitro* cloning method would additionally overcome the challenges in propagation. Unfortunately, *in vitro* cultivation of coconut has always been a problem, especially as a result of phenolic browning. At NIFOR, previous attempts have been made to improve *in vitro* culture of coconut. Asemota and Eke (2009) adopted the use of Eeuwen media, supplemented with NAA + 2,4-D + BAP. They reported leaf explants callusing in 3 months, while inflorescences explants callused in 5 months. However, both calluses eventually experienced browning. Nwite et al. (2016) also used Modified Eeuwen media + NAA + 2,4-D + BAP + 2ip. They reported callusing of inflorescence explants in 4 months, while leaf explants callused in 14 weeks. Again, there was reported browning of cultures.

To improve outcomes, such that calluses are immediately harvested and transferred into media for embryo development, perhaps it would be important to use media that would increase callusing time. Earlier described methods reported successful callogenesis in 3 – 5 months (Asemota and Eke, 2009) when NAA and 2,4-D were used with combination of BAP. Nwite (2016) achieved callus after 14 weeks with the use of 2,4-D, and NAA in combination with BAP and 2ip.Notably, the longer the callusing time, the longer it would take to achieve any desired goal for successful coconut tissue culture. The target therefore was to seek out and optimize protocols towards achieving callogenesis within a targeted 8 week-period – this will be half the time achieved in 2016. The aim therefore of this study was investigate the possible media used as whole or optimized, that can ensure enhanced time of callogenesis of coconut explants.

2. Methodology

2.1 Collection of plant materials

The leaf explants were obtained from coconut seedlings of about 1-2 years old. Unopened spear leaf tissues taken from the apical growing regions of the palm were used. The immature inflorescence explants used were obtained from leaf axils (frond) following the methods adopted by Steinmacher *et al.* (2007) for peach palm. The length of the external spathe was 7 - 10 cm. After collection of explants, 70% ethanol was use to spray the explants before being wrapped in aluminum foil and placed in a sampling bag (for protection) and then taken to the laboratory.

2.2 Preparation of culture medium

Experiments were carried out to assess media / protocols for the *in vitro* culture of coconut palm. Six different protocols which included Murashige and Skoog (1962) medium augmented with various growth factors or organic supplements were selected for this study. They were supplemented with different combinations of various growth regulators (Auxins and Cytokinins) leading to an experiment with eighteen treatments as shown in Tables 1- 6.

Table 1. Murashige and Skoog with B5 vitamin (1968)

Treatments	IAA (mg/l)	2,4-D (mg/l)	BAP (mg/l)
A	1.0	-	1.0
B	5.0	3.0	-
C	-	3.0	3.0

Table 2. Murashige and Skoog (1962) Medium

Treatments	2,4-D (mg/l)	KIN (mg/l)	NAA (mg/l)	2IP (mg/l)
A	3.00	0.30	-	-
B	5.00	1.00	-	-
C	-	0.30	5.00	0.30

Table 3. Murashige and Skoog with Putresine (1999)

Treatments	TDZ (mg/l)	NAA (mg/l)	2, 4-D (mg/l)
A	2.00	0.50	-
B	2.00	-	-
C	2.00	-	2.00

Table 4. Murashige and Skoog with Coconut water (1950)

Treatment	IAA (mg/l)	BAP (mg/l)	KIN (mg/l)
A	1.00	-	-
B	-	2.00	-
C	-	-	1.00

Table 5. Murashige and Skoog with Chloromphinucol and PVP (2010)

Treatments	TDZ (mg/l)	2,4-D (mg/l)	NAA (mg/l)
A	1.00	-	-
B	1.00	10.0	-
C	1.00	-	10.0

Table 6. Murashige and Skoog with Carrot juice (1964)

Treatments	PIC (mg/l)	2,4-D (mg/l)	BAP (mg/l)	2IP (mg/l)
A	1.00	1.00	-	-
B	-	-	5.00	-
C	-	5.00	-	1.00

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 added to control phenolic activities. The culture media were adjusted to pH 5.7 before autoclaving at 121 ^{o}C for 15 min. The autoclaved culture media were allowed to cool before use for inoculation of explants.

2.3 Sterilization of explants (Inflorescence and Leaves)

The inflorescence explants were washed with detergent and thoroughly rinsed with running tap water for 10-20min. The external spathes were removed under aseptic conditions. The inflorescences with the inner spathes intact were sprayed with 70 % ethanol and allowed to air dry. The leaves explants were sterilized using the method adapted by Nwite *et al.* (2016). Both explants were submerged in a mixture of potassium citrate and ascorbic acid at 1g/l each for 5 hours and later rinsed 3 times in sterile distilled water before inoculation or initiation.

2.4 Inoculation of explants

The sterilized explants were inoculated into the culture media and incubated in the growth room at 25±2 ^{o}C in the dark room for eight weeks. They were sub-cultured every two weeks in the culture room.

2.5 Data analysis

Data obtained in the study were subjected to descriptive and inferential statistical analysis and for all the treatments measurable variables were presented as a mean of 3 replicates, while observable and categorical data were presented as a modal representation of 3 observations. Means had been disjointed by the use of Duncan Multiple Range Test at $p<0.05$. Using Statistical Package for Social Science (SPSS) software version 21 as well as PAST version 2.17 was used for statistical analyses

3. Results and Discussion

3.1 Plant response

The response of leaf explants in modified Murashige and Skoog (MS) medium with B5 vitamins on callus initiation within 8 weeks is presented in Table 7. The medium was supplemented with 1.0 mg/l IAA + 1.0 mg/l BAP, of the 3 replicate cultures tubes inoculated with leaves explants, 33.3 % were contaminated by the 2nd week. 6.7 % and 20 % additional contaminated cultures were observed by the 4th and 6th week respectively. There were no traces of browning on the cultures until the 4th week. However, 53.3 % out of the 15 cultures of the MS medium augmented with 5 mg/l IAA + 3 mg/l 2,4-D were contaminated by the second week of culture with little or no browning observed. Most of the cultures however turned brown by the 8 week. The medium supplemented with 3 mg/l BAP + 3 mg/l 2, 4-D had less browning and 26.7 % of its cultures contaminated by the 6th week.

The response of inflorescence explants in Murashige and Skoog (MS) medium with B5vitamin has been presented in Table 8. Using the different growth regulators, medium supplemented with1.0 mg/lIAA+1.0 mg/l BAP, 16.7 % inflorescence cultures out of 12 test tubes were contaminated with traces of browning at the 2nd week. Browning was obvious at 4th week and 33.3 % cultures were contaminated by 6th week, following the dark coloration of the cultures. However, less contaminated cultures were observed in the medium containing 5.0 mg/lIAA + 3.0 mg/l 2,4-D between 2nd and 4th week. By the 6th week 41.7 % cultures were contaminated and browning was high. Further, there was no contaminated culture in the medium augmented with 3.0 mg/lBAP + 3.0 mg/l 2, 4-D between 2nd and 4th week of inoculation. Browning was noticed at the 2nd week, and 16.7 % cultures were contaminated by 6th week. There was no callus obtained from inflorescence explants within 8 weeks. However, most of the cultures turned dark brown (Necrosis).

Table 9 shows leaf explants in MS medium. In the medium supplemented with 3.0 mg/l 2,4-D + 0.3 mg/l KIN, 5 leaf cultures out of 41.7 % tubes cultures inoculated were contaminated by the 2nd week following inoculation. Cultures were free from browning, between 2nd and 4th week. Whereas, MS medium with 5.0 mg/l2, 4-D + 1.0 mg/lKin, contaminated cultures at 2nd week were 50.0 % out of 12, with light browning following the other weeks of inoculation. There was no browning in cultures supplemented with 0.3 mg/l + 2ip 0.3 mg/l +NAA 5.0 mg/l, although 50.0 % cultures were contaminated out of 12 test tubes inoculated by 2nd week. No callus induction from leaf explants within 8 weeks was observed. However, by 8th week some showed brown colorations.

Table 7. The effects of modified MS medium with B5 vitamin supplemented with IAA, 2, 4-D alone or each separately combined with BAP on callus formation, explant colour and % contamination from leaf explants.

Treatments	Growth Regulators And Concentrations (mg/L)			RESPONSE OF LEAF EXPLANTS AT DIFFERENT BI-WEEKKLY REGIMES											
				2nd Week			4th Week			6th Week			8th Week		
	IAA	24D	BAP	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.
1	1.0	-	1.0	NIL	Normal	33.3	NIL	Normal	6.7	NIL	Light brown	20.0	NIL	Light brown	-
2	5.0	3.0	-	NIL	Normal	53.3	NIL	Normal	-	NIL	Light brown	40.0	NIL	Browning	-
3	-	3.0	3.0	NIL	Normal	40.0	NIL	Light brown	13.3	NIL	Browning	26.7	NIL	Browning	-

IAA:- Indole-3 Acetic acid, 2,4-D:- 2,4 Dichlorophenoxy Acetic acid, BAP:- 6 - Benzylamino purine, Callus F.:- Callus formation, Explant C.:- Explant colour, % Con.:- Percentage contamination

Table 8. The effects of modified MS medium with B5 vitamin supplemented with IAA, 2, 4-D alone or each separately combined with BAP on callus formation, explant colour and % contamination from inflorescence explants

Treatments	Growth Regulators and Concentrations (mg/L)			RESPONSE OF INFLORESCENCE EXPLANTS AT DIFFERENT BI-WEEKKLY REGIMES											
				2nd Week			4th Week			6th Week			8th Week		
	IAA	24D	BAP	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.
1	1.0	-	1.0	NIL	Light brown	16.2	NIL	Brown	8.3	NIL	Dark brown	33.3	NIL	Dark brown	8,3
2	5.0	3.0	-	NIL	Brown	16.7	NIL	Brown	-	NIL	Brown	41.7	NIL	Dark brown	-
3	-	3.0	3.0	NIL	Brown	-	NIL	Brown	-	NIL	Brown	16.7	NIL	Dark brown	-

IAA:- Indole-3 Acetic acid, 2,4-D:- 2,4 Dichlorophenoxy Acetic acid, BAP:- 6 - Benzylamino purine, Callus F.:- Callus formation, Explant C.:- Explant colour, % Con.:- Percentage contamination

Table 10 shows the response of inflorescence explants in MS medium within 8 weeks. Medium augmented with 3.0mg/l 2, 4-D + 0.3mg/l Kin, 8.3 % inflorescence culture out of 12 test tubes got contaminated and cultures were free from browning in the 2nd week. By the 4th week, there was no contaminated culture, although slight browning was observed. Whereas by the 8th week, there were no calluses, 41.7 % cultures were contaminated and browning was high. 25.0 % contaminated cultures and necrosis was noticed in the 4th week, in medium with 5.0mg/l 2, 4-D + 1.0mg/l Kin. Further, cultures turned dark brown by the 8th week. However, medium augmented with 0.3mg/l Kin + 0.3mg/l 2ip + 5.0mg/l NAA, culture showed necrosis by 8th week.

The assessment of leaf explants in modified MS medium with Thidiazuron (TDZ) has been presented in Table 11. Fourteen (14) test tubes were inoculated in each of the treatment. However, there were no calluses obtained within the first 8 weeks. By 2nd week, contaminated cultures were 42.9 % in medium supplemented with 2.0mg/lTDZ +NAA 0.5mg/l than the other media. Whereas, at 4th week, there were light browning in medium augmented with 2.0mg/l TDZ + 2,4- D 2.0mg/l,andat the 8th week some showed greenish and light brown colorations, while few cultures were still clean.

Callusing response of inflorescence explants in Modified MS medium has been presented in Table 12. In modified MS medium complemented with 2.0mg/l TDZ +2.0mg/l Put, 58.3 % inflorescence cultures out of 12 inoculated test tubes were contaminated and traces of browning were observed in the 2nd week. By the 4th week, the browning was obvious and became dark brown by the 8th week. However, medium with 2.0mg/l TDZ + 2.0mg/l NAA, browning of cultures was observed and 33.3 % cultures were contaminated at week 2, as a result of phenolic compound. Further, by the 8th week, cultures turned dark brown without callusing. Whereas, in medium supplemented with 2.0mg/l TDZ + 2.0mg/l 2, 4-D, contaminated cultures were 16.7 % out of 12, with no browning effect at 2 weeks. Callus was observed at the 4th week of inoculation (Figure 1). By the 8th week, most of the cultures turned brown with 58.3% contamination while the callus greatly proliferated.

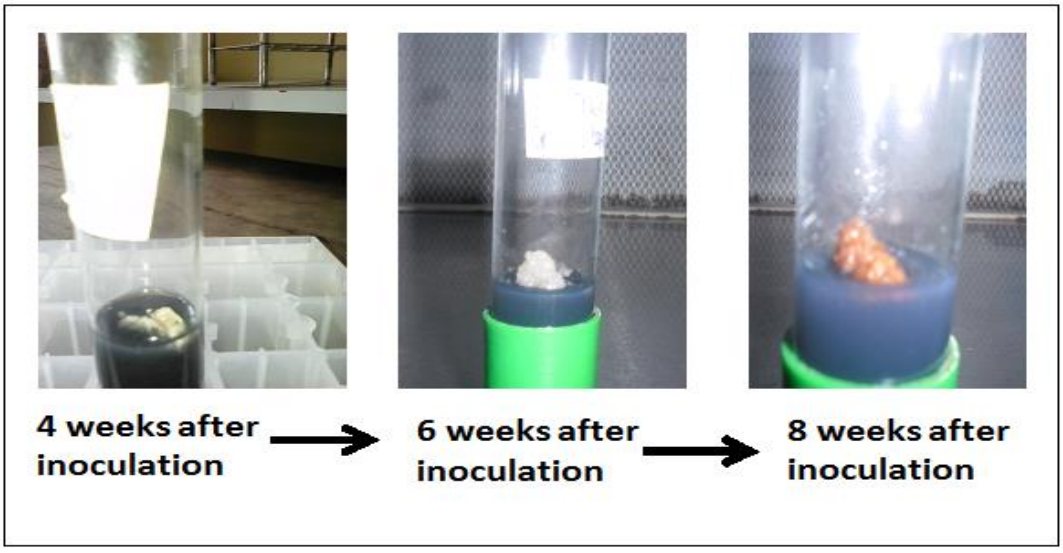

Figure 1. Callus formation from inflorescence explants after 4 to 8 weeks

Table 13 shows the response of leaf explants in MS medium with Coconut water (CW). Of the inoculated leaf cultured on 1.0 mg/l IAA, 30 % were contaminated at the 2nd week. Furthermore, browning and 40 % contaminated cultures were present in the medium with 2.0 mg/l BAP, by the week

6th. However, medium supplemented with 1.0 mg/l KIN, 55.0 % cultures had contamination, no trace of browning and there were increase in the size of the cultures at 2nd week.

The outcome of inflorescences explants in MS medium with coconut water (Table14) 41.7 % cultures had contamination with traces of browning in the medium supplemented with 1.0 mg/l IAA, at the 2nd week. However, 16.7 % contaminated cultures were present in CW medium with 2.0mg/l BAP. While, medium with 1.0 mg/l KIN, contaminated cultures were 33.3 % at 2 weeks. By the 8th week, most of the cultures showed dark brown without callusing.

When MS medium with carrot juice (CJ) were used the results (Table15) reveal that leaf explants cultures in medium augmented with 1.0 mg/l Picloram + 1.0 mg/l 2, 4-D, had 20.0 % contaminated cultures by the 2nd week, and by the 8th week 30.0 %. However, by 4th week 30.0 % contaminated cultures were notice in the medium with 5.0 mg/l BAP. Although, medium supplemented with 5.0 mg/l 2, 4-D + 1 mg/l 2ip, by the 2nd week had 10 % contamination 35 and 20 % cultures were contaminated by the 4th and 6th week respectively.

A similar trend was observed for the inflorescence explants (Table 16). Murashige and skoog (MS) medium with carrot juice was used for the initiation of callus. Medium supplemented with 1.0mg/l Picloram + 1mg/l 2,4-D, recorded 41.7 % contamination within the 2 weeks. Medium containing 5.0mg/l BAP had 16.7 % contaminated cultures at 2 weeks, by the 6th week cultures turns dark brown. 25.0 % contaminated cultures were observed in the medium supplemented with 5.0mg/l 2, 4-D + 1mg/l 2ip, without callus induction between 2nd and 4th weeks.

Table 9. The effects of Murashige and Skoog (MS) medium supplemented with auxins and cytokinins on callus formation explant colour and % contamination from leaf explants

Treatments	Growth Regulators And Concentrations (mg/L)				RESPONSE OF LEAF EXPLANTS AT DIFFERENT BI-WEEKKLY REGIMES											
					2nd Week			4th Week			6th Week			8th Week		
	24D	KIN	2iP	NAA	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.
1	3.0	0.3	-	-	NIL	Normal	41.7	NIL	Light brown	-	NIL	Browning	25.0	NIL	Browning	-
2	5.0	1.0	-	-	NIL	Light brown	50.0	NIL	Light brown	25.0	NIL	Browning	-	NIL	Dark brown	-
3	-	0.3	0.3	5.0	NIL	Normal	50.0	NIL	Light brown	16.7	NIL	Light brown	33.3	NIL	Light brown	-

2,4-D:- 2,4 Dichlorophenoxy Acetic acid, KIN:- Kinetin, 2iP:- , 6 - γ,y -Dimethylallylamino purine, NAA:- 1-Naphthaleneacetic acid, Callus F.:- Callus formation, Explant C.:- Explant colour, % Con.:- Percentage contamination

Table 10. The effects of Murashige and Skoog (MS) medium supplemented with auxins and cytokinins on callus formation explant colour and % contamination from inflorescence explants

Treatments	Growth Regulators and Concentrations (mg/L)				RESPONSE OF INFLORESCENCE EXPLANTS AT DIFFERENT BI-WEEKKLY REGIMES											
					2nd Week			4th Week			6th Week			8th Week		
	24D	KIN	2iP	NAA	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.
1	3.0	0.3	-	-	NIL	Normal	8.3	NIL	Light brown	-	NIL	Brown	-	NIL	Brown	41.7
2	5.0	1.0	-	-	NIL	Light brown	16.7	NIL	Brown	25.0	NIL	Brown	-	NIL	Dark brown	-
3	-	0.3	0.3	5.0	NIL	Normal	50.0	NIL	Light brown	16.7	NIL	Brown	25.0	NIL	Light brown	-

2,4-D:- 2,4 Dichlorophenoxy Acetic acid, KIN:- Kinetin, 2iP:-, 6 - γ,y -Dimethylallylamino purine, NAA:- 1-Naphthaleneacetic acid, Callus F.:- Calformation, Explant C.:- Explant colour, % Con.:- Percentage contamination

Table 11. The effects of Murashige and Skoog (MS) medium augmented with cytokinins and auxins on callus formation explant colour and % contamination from leaf explants

Treatments	Growth Regulators and Concentrations (mg/L)				RESPONSE OF LEAF EXPLANTS AT DIFFERENT BI-WEEKKLY REGIMES											
					2nd Week			4th Week			6th Week			8th Week		
	TDZ	PUT	NAA	24D	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con	Callus F.	Explant C.	% Con	Callus F.	Explant C.	% Con
1	2.0	0.5	-	-	NIL	Normal	14.3	NIL	Normal	-	NIL	Light brown	14.3	NIL	Light brown	-
2	2.0	-	0.5	-	NIL	Normal	42.9	NIL	Normal	21.4	NIL	Light brown	-	NIL	Light brown	-
3	2.0	-	-	2.0	NIL	Normal	28.6	NIL	Light brown	14.3	NIL	Light brown	14.3	NIL	Greenish	-

TDZ: -Thidiazuron, PUT:- Putrescine, NAA:- 1-Naphthaleneacetic acid, 2,4-D:- 2,4 Dichlorophenoxy Acetic acid, Callus F.:- Callus formation, Explant C.:- Explantcolour, % Con.:- Percentage contamination

Table 12. The effects of Murashige and Skoog (MS) medium augmented with cytokinins and auxins on callus formation explant colour and % contamination from inflorescence explants

Treatments	**Growth Regulators and Concentrations (mg/L)**				**RESPONSE OF INFLORESCENCEEXPLANTS AT DIFFERENT BI-WEEKKLY REGIMES**											
					2nd Week			**4th Week**			**6th Week**			**8th Week**		
	TDZ	PUT	NAA	24D	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.
1	2.0	0.5	-	-	NIL	Light brown	58.3	NIL	Brown	-	NIL	Dark brown	16.7	NIL	Dark brown	-
2	2.0	-	0.5	-	NIL	Light brown	33.3	NIL	Light brown	8.3	NIL	Brown	16.7	NIL	Dark brown	-
3	2.0	-	-	2.0	NIL	Normal	16.7	Callus formation	Light brown	-	NIL	Brown	16.7	NIL	Brown	58.3

TDZ:-Thidiazuron, PUT:- Putrescine, NAA:- 1-Naphthaleneacetic acid, 2,4-D:- 2,4 Dichlorophenoxy Acetic acid, Callus F.:- Callus formation, Explant C.:- Explant colour, % Con.:- Percentage

Table 13: The effects of Modified MS medium augmented with coconut water with combination of cytokinins and auxinson callus formation explant color and % contamination from leaf explants

Treatment	Growth Regulators And Concentrations (mg/L)			Response of Leaf Explants at Different Week											
				2nd Week			4th Week			6th Week			8th Week		
	IAA	BAP	KIN	Callus Formation	Explant Color	% Contamination.	Callus Formation	Explant Color	% Contamination.	Callus Formation	Explant Color	% Contamination	Callus Formation	Explant Color	% Contamination
1	1.0	-	-	None	Light brown	30,0	None	Light brown	20.0	None	Brown	20.0	None	Brown	5.0
2	-	2.0	-	None	Light brown	20.0	None	Light brown	15.0	None	Brown	40.0	None	Brown	-
3	-	-	1.0	None	White	55,0	None	White	20,0	None	Light brown	15.0	None	Brown	-

IAA:- Indole-3 acetic acid, BAP:- 6 - Benzylamino purine, KIN:- Kinetin, , %.:- Percentage

Table 14: The effects of MS medium augmented with coconut water with combination of cytokinins and auxinson callus formation explant color and % contamination from inflorescence explants

Treatment	Growth Regulators And Concentrations (mg/L)			Response of In florescence Explants At Different Week											
				2nd Week			4th Week			6th Week			8th Week		
	IAA	BAP	KIN	Callus Formation	Explant Color	% Contamination	Callus Formation	Explant Color	% Contamination	Callus Formation	Explant Color	% Contamination	Callus Formation	Explant Color	% Contamination
1	1.0	-	-	None	Light brown	41.7	None	Brown	-	None	Brown	16.7	None	Dark brown	8.3
2	-	2.0	-	None	Light brown	16.7	None	Brown	16.7	None	Light brown	8.3	None	Dark brown	8.3
3	-	-	1.0	None	Light brown	33.3	None	Brown	-	None	Brown	25.0	None	Dark brown	-

IAA:- Indole-3 acetic acid, BAP:- 6 - Benzylamino purine, KIN:- Kinetin, % - Percentage

Table 15: The effects of modified MS medium with carrot juice supplemented with cytokinins and auxinson callus formation explant color and % contamination from leaf explants

Treatment	Growth Regulators and Concentrations (mg/L)				Response of leaf Explants At Different Week											
					2nd Week			4th Week			6th Week			8th Week		
	PIC	24D	BAP	2IP	Callus Formation	Explant Color	% Contamination	Callus Formation	Explant Color	% Contamination	Callus Formation	Explant Color	% Contamination	Callus Formation	Explant Color	% Contamination
1	1.0	1.0	-	-	None	Light brown	20.0	None	Light brown	10.0	None	Light brown	10.0	None	Brown	30.0
2	-	-	5.0	-	None	Light brown	5.0	None	Brown	30.0	None	Brown	20.0	None	Dark brown	-
3	-	5.0	-	1.0	None	Light brown	10.0	None	Brown	35.0	None	Brown	20.0	None	Brown	10.0

BAP:- 6 - Benzylamino purine, PIC:- Picloram , 2,4-D:- 2,4 Dichlorophenoxy acetic acid, 2iP:- 6- γ, y-Dimethylallylamino purine, % - Percentage

Table 16: The effects of modified MS medium with carrot juice supplemented with cytokinins and auxinson callus formation explant color and % contamination from inflorescence explants

Treatment	Growth Regulators and Concentrations (mg/L)				Response of Inflorescence Explants At Different Week											
					2nd Week			4th Week			6th Week			8th Week		
	PIC	24D	BAP	2IP	Callus Formation	Explant Color	% Contamination	Callus Formation	Explant Color	% Contamination.	Callus Formation	Explant Color	% Contamination	Callus Formation	Explant Color	% Contamination
1	1.0	1.0	-	-	None	Light brown	41.7	None	Brown	-	None	Dark brown	8.3	None	Dark brown	25.0
2	-	-	5.0	-	None	Brown	25.0	None	Brown	25.0	None	Dark brown	-	None	Dark brown	16.7
3	-	5.0	-	1.0	None	Light brown	25.0	None	Brown	16.7	None	Brown	8.3	None	Brown	-

BAP:- 6 - Benzylamino purine, PIC:- Picloram , 2,4-D:- 2,4 Dichlorophenoxy acetic acid, 2iP:- 6- γ, y-Dimethylallylamino purine, %.:- Percentage

MS medium with high growth hormones was presented in (Table 17). MS medium augmented with 100mg/l 2, 4-D + 3.0 mg/l 2ip + 0.05 mg/l Biotin was used for initiation of leaf explants. By the 2nd week there were no contaminations and browning in the cultures. Though, traces of browning were observed by the 4th week with 16.7 % contaminated cultures, and by the 6th week 11.1 % additional cultures had becomes contaminated. Furthermore, the cultures inoculated into the medium with 50mg/l Kin + 3.0 mg/l 2ip + 0.05 mg/l Biotin showed no browning at 2nd to the 6th week of inoculation. However, 11.1 % contaminated cultures were seen at the 4th week. In the medium supplemented with 50mg/l 2,4-D + 3.0 mg/l 2ip + 0.05mg/l Biotin, 11.1 % and 27.8 % leaf cultures were contaminated between 4th and 6th week respectively. Generally, there was no induction of callus but there was increment in size of the culture. Response of inflorescence explants in MS medium (Table 18). Cultures were free from browning and contamination in the medium augmented with 100mg/l 2, 4-D + 3.0mg/l 2ip + 0.05mg/l Biotin, at 2 week. Traces of browning and 33.3 % contaminated cultures were observed at 4th week. Browning was obvious on the 6th week and became dark brown by the 8th week. Nevertheless, medium supplemented with 50mg/l Kin + 3.0mg/l 2ip + 0.05mg/l Biotin, had 33.3%, contaminated cultures at 2 weeks. 6th weeks, most of the cultures turned dark brown, with 16.7 % contaminated cultures. Whereas, there were no contaminated cultures, in the medium accompanied with 50mg/l 2, 4-D + 3.0mg/l 2ip + 0.05mg/l Biotin, in the 2nd week, until the 4th week, traces of browning were noticed with 25.0 % contaminated cultures. However, by the 8th week, most of the culture turned dark brown (Necrosis).

Table 17. The effects of modified MS medium with Biotin supplement and high auxins and cytokinins on callus formation explant colour and % contamination from leaf explants

Treatments	Growth Regulators and Concentrations (mg/L)				RESPONSE OF LEAF EXPLANTS AT DIFFERENT BI-WEEKKLY REGIMES											
					2nd Week			4th Week			6th Week			**8th Week**		
	BIO	24D	KIN	2IP	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.
1	0.05	100	-	3.0	NIL	Normal	-	NIL	Light brown	16.7	NIL	Brown	11.1	NIL	Brown	30.0
2	0.05	-	50.0	3.0	NIL	Normal	-	NIL	Normal	-	NIL	Normal	11.1	NIL	Light brown	-
3	0.05	50.0	-	3.0	NIL	Normal	-	NIL	Normal	11.1	NIL	Light brown	27.8	NIL	Light brown	10.0

BIO:- Biotin, 2,4-D:- 2,4 Dichlorophenoxy Acetic acid, KIN:- Kinetin,2iP:- 6- γ, y-Dimethylallylamino purine , Callus F.:- Callus formation, Explant C.:- Explant colour, % Con.:- Percentage contamination

Table 18. The effects of modified MS medium with Biotin supplement and high auxins and cytokininson callus formation explant colour and % contamination from inflorescence explants

Treatments	Growth Regulators and Concentrations (mg/L)				RESPONSE OF INFLORESCENCE EXPLANTS AT DIFFERENT BI-WEEKKLY REGIMES											
					2nd Week			4th Week			6th Week			**8th Week**		
	BIO	24D	KIN	2IP	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.	Callus F.	Explant C.	% Con.
1	0.05	100	-	3.0	NIL	Normal	-	NIL	Light brown	33.3	NIL	Brown	8.3	NIL	Brown	16.7
2	0.05	-	50.0	3.0	NIL	Light brown	33.3	NIL	Brown	-	NIL	Dark brown	16.7	NIL	Dark brown	-
3	0.05	50.0	-	3.0	NIL	Normal	-	NIL	Light brown	25.0	NIL	Dark brown	16.7	NIL	Dark brown	25.0

BIO:- Biotin, 2,4-D:- 2,4 Dichlorophenoxy Acetic acid, KIN:- Kinetin,2iP:- 6- γ, y-Dimethylallylamino purine , Callus F.:- Callus formation, Explant C.:- Explant colour, % Con.: - Percentage contamination

Discussion

This study focused on investigating the possible culture media used that can ensure a shorter period for callogenesis of coconut explants. Coconut palm culture is a long process and can be very challenging (Fernando *et al*., 2010). This is due mainly to lethal browning of cultures and microbial contaminations both of which delay callus establishment.

Given the difficulties in the plantlet growth, an attempt was made to determine the best procedure to establish a culture for early callus generation on the explants. As a result, in order to find an appropriate callus-initiating protocol, a series of experiments involving six (6) separate /treatments media were conducted. With respect to these six modified media used, callus was not initiated on the leaf explants of coconut in any of the treatments used. This is consistent with the work of many workers. Karunaratne *et al.* (1991), reported that embryonic potential of coconut leaf explants is very low (<10%). Furthermore, the embryogenesis of the leaf explants is of short duration. All these limits the use of coconut leaf for clonal propagation. The induction of callus and formation of roots and shoots in plant tissue cultures also appear to be regulated basically by balances of two hormonal substances, auxins and cytokinins as proposed by Cutter and Wilson (2020). However, callus initiation and growth were markedly stimulated by the combination of auxins 2,4-Dichlorophenoxy acetic acid (2,4-D) and cytokinins (Thidiazuron) within 4 weeks of culture of immature inflorescence in the medium of modified Murashige and Skoog. The callus induction rate in this experiment though low (5 %,) was however expected since immature inflorescence explants were used. Belt (2019) had reported that immature inflorescence explants are more promising explants than leaves as they bear numerous meristematic points. An interesting observation made in this study was that the degree of browning in inflorescence explants was higher than those of the leaf explants. This probably accounted for the inhibition of callus esterblishment of the explants in almost all the protocols used. However, it was concluded that the highest callusing so far reported was 30 %. The implication of this earliness in callogenesis on MS medium containing 2,4-D and TDZ is that for early plantlet generation, this medium should be used. Blake (1983) proposed that for the propagation of all palms through tissue culture, the aim must be to initiate callus as quickly as possible and allow rapid determination of embryogenic cells so that embryogenic development can occur when transfer is made to an embryogenesis medium. It can therefore be claimed that the short duration of 4 weeks to initiate callus in the medium adequately satisfies these criteria.

Conclusion

This work was done to assess 6 modified media for in-vitro culture of coconut palm using leaf and inflorescence explants. Most of the cultures remained viable and callus was induced on the immature inflorescence, from one of the modified media in this study. The used of inflorescence as explants source has the advantage of being non- destructive in which case the mother palm could be sampled successively

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