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EFFECT OF PLANT GROWTH REGULATORS ON CALLUS INDUCTION IN *Dennettia tripetala* Bak. F. SHOOT EXPLANT

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ABSTRACT

Dennettia tripetala high consumption pattern and variety of uses, combined with its low germination and slow seedling growth, pose a serious threat to the extinction of this vital tree crop. Therefore, this experiment was undertaken to optimize a reproducible protocol for callus induction on shoot explants of Dennettia tripetala using different concentrations of plant growth regulators (PGRs). Shoot segments served as explants and cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of Auxins, Indo-3-Acetic Acid (IAA) (0, 0.5, 1.0, 1.5. 2.0 and 2.5 mg/l) and cytokinins, Kinetin and 6-Benzyl amino purine (BAP) (0, 0.1 and 1.0 mg/l) alone and in combinations for callus induction. Among the MS media supplemented with either IAA/BAP or Kinetin, it was observed that the media supplemented with BAP did not produce callus. The callus induced in media supplemented with combinations of Plant Growth Regulators (PGRs) at varied concentrations varied in weight of callus, days of callus formation, colour and nature of callus. In combining PGRs in MS media, maximum callus of 0.63 g was produced in a media supplemented with IAA at 2.5 mg/l and kinetin at 0.1 mg/l 21 while media containing IAA at 2.5 mg/l with BAP at 1.0 mg/l generated maximum callus weight of 0.48 g. First day of callus formation for IAA with kinetin combinations and IAA with BAP combinations was 21 and 27 days respectively. MS media supplemented with 0.5 mg/l IAA+ 0.1 mg/l kinetin, 2.5 mg/l IAA+ 0.1 mg/l kinetin, 1.5 mg/l IAA + 1.0 mg/l kinetin and 1.5 mg/l IAA + 0.1 mg/l BAP gave early callogenesis at 21 and 27 days respectively. The result showed in this study indicated that high level of auxins (IAA) and moderate to high level of cytokinins was required for inducing optimum callus weight on cultured shoot of D. tripetala. Also, for inducing early callogenesis, IAA at 2.5 mg/l with kinetin at 0.5 mg/l is recommended.

Keyword: Callus induction, callogenesis, Indo-3-Acetic Acid, Kinetin, 6-Benzyl amino purine

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INTRODUCTION

Plant Growth Regulators are synthetic chemicals that play significant role in the development and growth of cells or tissues in cultured media and are needed at very low concentration. In tissue culture, it is an important media component which determines the development and developmental pathway of plant cells. These regulators used singly or in combination with different hormones in growth media enable the maintenance of specific and balanced inorganic content in the growing tissues that lead in the induction of callus, development of shoot and/or root or death (Ikram and Muhammad, 2007). The growth regulators are used in small amount to bring about cell differentiation. Auxins and cytokinins are the most important plant hormones for regulating growth and morphogenesis in plant

tissue and organ culture (Machakova *et al.*, 2008). Auxins, primarily control growth through cell enlargement and stimulate apical dominance, and on certain occasion induce cell division. It promotes the growth of calli, cell suspensions, and organs, primarily in conjunction with cytokinins, and also affects the direction of morphogenesis (Machakova *et al*., 2008). Cytokinins are a type of plant growth regulators that promote cell division in plant roots and shoot. They are primarily added to cultured media for the proliferation of growth of bud and the formation of shoot. The most widely used auxins are IAA (Indole-3-acetic acid), NAA (Napthalene acetic acid), 2, 4-D (2, 4-Dichlorophenoxyacetic acid) and IBA (Indole butyric acid). Cytokinins that is widely used in tissues culture include, kinetin, zeatin and BAP (6-Benzyl amino purine) (Dubey, 2010). The concentration and combination of auxins and cytokinins in growth medium is an important factor which determines successful regeneration of plant.

Dennettia tripetala Baker f., a member of the family of Annonaceae is a small tree that spreads throughout the rainforest zone of Africa. It is found in the tropical rainforest region of Nigeria and sometimes in Savanna areas. The plant and its products including fruits, young shoots, and leaves are useful to man and animal in various ways. The fruit is used as masticators, which when chewed produces unique peppery effect (Keay, 1989). The leaves are used in combination with other herbs for the treatment of cough, asthma, toothache, diarrhoea and rheumatism. The peppery fruits of this tree crop are applied to the food meant for pregnant women and are important in the diets of postpartum women, during which time it is claimed that spices or herbs aid uterine contraction (Achinewhu *et al*., 1995; Okwu and Morah, 2004). Pharmacologically, the oil extracted from the fruit of *D. tripetala* is used in the manufacture of mouth wash (Nwinuka and Nwiloh, 2009). Poor seed germination and slow seedling growth has been reported on *Dennettia tripetala* by Osaigbovo *et al*. (2010). Considering the high consumption pattern and the diversity of uses of *D. tripetala* and coupled with its low germination and slow seedling growth, there is heightened threat of extinction of this tree crop. Also, no work has been carried out on this high value crop in the area of tissue culture and micropropagation. Hence, it becomes pertinent to undertake into the effect of IAA and Kinetin/BAP on callus induction of *Dennettia tripetala* shoot explants in order to boost its natural population.

MATERIALS AND METHODS

Plant Materials

Shoot explants were collected from 12 weeks old seedlings of *Dennettia tripetala* raised in the Nursery unit of the Department of Forest Resources and Wildlife Management, Faculty of agriculture, University of Benin, Benin City. The central point of the nursery is located at latitude 06° 24' 0.38''N and longitude 005° 37' 24.0''E and altitude of 106 m (GPS, location). Shoots of 3 cm were used as source of explants.

Culture Medium Preparation

Murashige and Skoog (MS) medium (1962) was prepared and supplemented with plant growth regulators at different concentrations of IAA at 0, 0.1, 1 mg/*l* in combination with kinetin/BAP at 0, 0.5, 1, 1.5, 2, 2.5 mg/*l*. Prior to autoclaving at 121^0 C for 30 minutes, the pH was adjusted to 5.8 and the medium dispensed into test tubes and Mc-Cartney bottles. After autoclaving, the media were taken to the cooling room and allowed to cool. The experiment was carried out in Plant Tissue Culture Laboratory of the Nigerian Institute for Oil Palm Research (NIFOR), Benin City, Edo State.

Experimental Design

Factorial experiment of 3×6 was set in a Completely Randomized Design with four replicates to determine the effect of Indole -3-acetic acid (IAA) in 3 concentrations: 0, 0.1, 1 mg/*l* in combination with Kinetin or Benzyl amino purine (BAP) in 6 concentrations: 0, 0.5, 1, 1.5, 2, 2.5 mg/*l* to give 18 each of IAA× Kinetin and IAA × BAP combinations.

Disinfection and Inoculation of Explants, and Incubation of Culture

Explants of 12-week-old were disinfected by immersing them first in 3.5 % Sodium hypochlorite for 5 minutes and the Sodium hypochlorite disinfected explants were rinse with distilled water before further disinfection was carried out using 0.02 % Mercury chloride for 5

minutes. After the sterilization, the explants were rinsed thoroughly with sterile distilled water 4 times to remove any trace of the sterilant that is injurious to the cells of the explants. The disinfected shoot explants were inoculated vertically on the surface of semi-solid MS media supplemented with Plant Growth Regulators (PGRs) at different concentrations. All these procedures were carried out under a laminar flow hood. The cultures were incubated in a dark growth room at 25±2 ^{0} C and relative humidity of 50 to 60 %.

Maintenance of *In Vitro* culture

The cultured shoots were maintained by regular subcultures at 4-week interval on fresh medium with the same nutrient composition and phytohormones to avoid accumulation of toxic metabolites and used up of the media nutrient.

Data Collection

Variables measured were time of callogenesis and weight of callus. Observation was made on a daily basis and weight of callus was measured using electronic weighing balance. The period (days) of callus induction was also noted.

Statistical Analysis

Statistical analysis of weight and period of callus formation was carried out with the use of Genstat Statistical Package 12th edition. Data collected were subjected to analysis of variance (ANOVA) procedure of a completely randomized design and variables that showed significant difference were separated using Duncan's New Multiple Range Test at 5 % probability.

RESULTS

When IAA alone was used in media supplementation of *D. tripetala* cultures, the callogenesis response, period of response, mean weight, colour, nature and intensity of the callus generated are shown in Table 1. The callus obtained had a friable nature and was white in colour. No callus was induced during the culture period in the control (basal medium without growth regulator) and that supplemented with IAA at 0.5 mg/*l*. MS medium containing IAA alone at levels of 1, 1.5 2 and 2.5 mg/*l* effected callus formation that ranged between 0.14-0.18 g and the period for callus formation at these levels ranged between 21-31 days. The earliest day of callogenesis was 21 days attributed to the medium containing 2.5 mg/*l* IAA. The highest callus intensity of weight 0.18 g was in the medium supplemented with 1.5 mg/*l* IAA.

No callus was induced in MS medium free from kinetin and that supplemented with 1.0 mg/*l* Kinetin but callus was induced in MS medium containing 0.1 mg/*l* kinetin (Table 2). The callus obtained from kinetin is white and friable in nature. No callus was induced in MS media supplemented with BAP alone.

Table 3 presents the results obtained for the measured variables when IAA and kinetin were combined in the media supplementation. At levels of 0, 0.5, 1.0, 1.5, 2 and 2.5 mg/*l* IAA + 0.1 mg/*l* Kinetin respectively, callus was induced between 21 - 38 days and mean weight of callus ranged between 0.22 - 0.63 g. Callus was also induced at 0.5, 1.0, and 1.5 mg/*l* IAA in combination with 1mg/*l* Kinetin between 21 and 32 days and the weight of callus formed ranged between 0.12 - 0.18 g while 0, 2 and 2.5 mg/*l* IAA in combination with 1mg/*l* kinetin gave no callus formation. The highest callus fresh weight of 0.63 g (Figure 1) was obtained at 2.5 mg/*l* IAA+0.1 mg/*l* of Kinetin. The earliest time of callogenesis of 21days was observed in media containing 0.5 mg/*l* IAA+0.1 mg/*l* Kinetin (0.41 g), 1 mg/*l* IAA+0.1 mg/*l* kinetin (0.21 g), 2.5 mg/*l* IAA+0.1 mg/*l* kinetin (0.63 g), and 1.5 mg/*l* IAA+0.1 mg/*l* kinetin (0.18 g).

MS medium supplemented with 0.5, 1.5, 2 and 2.5 mg/*l* IAA in combination with 0.1 mg/*l* of BAP, enabled callus formation between 27 - 40 days; and the mean weight of callus ranged between 0.20 - 0.12 g (Table 4). Callus formation was also obtained at 0.5, 1.5, 2, and 2.5 mg/*l* IAA in combination with 1 mg/*l* BAP between 33 - 42 days, and mean weight of the callus obtained at these levels was between 0.48 - 0.14 g. The highest fresh weight (0.48 g) of callus was produced in a basal medium supplemented with 2.5 mg/*l* IAA in combination with 1mg/*l* BAP. The earliest time of callogenesis which was 27 days was found in the media supplemented with 1.5 mg/*l* IAA+0.1 mg/*l* BAP.

Table 1: Effect of IAA in MS media supplementation on period of callogenesis and callus weight of shoot explants of *Dennettia tripetala*.

Concentration of IAA (mg/*l*)	Type of Response	Period (days) of Callogenesis	Mean Weight±SD (g) n=4	Colour	Nature	Intensity
0.0	-	-	-	-	-	-
0.5	-	-	-	-	-	-
1.0	Callus	29.25^{b}	0.16 ± 0.02^{b}	White	Friable	++
1.5	Callus	28.50^{bc}	0.18 ± 0.03^{bc}	White	Friable	++
2.0	Callus	30.50^{b}	0.16 ± 0.03^{ef}	White	Friable	++
2.5	Callus	21.00^{e}	0.14 ± 0.03^{ef}	White	Friable	++

-: No callus formation; ++: Slightly profuse; +++: Profuse MS: Murashige and Skoog; IAA: Indo-3- acetic acid

Table 2: Effect of Kinetin in MS media supplementation on period of callogenesis and callus weight of shoot explants of *Dennettia tripetala*.

Concentration of Kinetin (mg/*l*)	Type of Response	Period (days) of Callogenesis	Mean Weight±SD (g) n=4	Colour	Nature	Intensity
0.0	-	-	-	-	-	-
0.1	Callus	20.50^{e}	0.27 ± 0.02^{c}	White	Friable	+++
1.0	-	-	-	-	-	-

-: No callus formation; +++ : Profuse; MS: Murashige and Skoog

Table 3: Effect of IAA in combination with Kinetin in MS media supplementation on period of callogenesis and callus weight of shoot explants of *Dennettia tripetala*.

Concentration of growth regulator		Type of Response	Period (days) of Callogenesis	Mean Weight±SD (g) n=4	Colour	Nature	Intensity
IAA (mg/*l*)	KIN (mg/*l*)						
0.0	-	-	-	-	-	-	-
0.5	0.1	Callus	21.00^{e}	0.41 ± 0.05^{b}	White	Friable	++++
1.0		Callus	22.00^{de}	0.21 ± 0.05^{d}	White	Friable	+++
1.5		Callus	37.50^{a}	0.34 ± 0.04^{c}	White	Friable	+++
2.0		Callus	27.75^{bc}	0.43 ± 0.03^{b}	White	Friable	++++
2.5		Callus	21.25^{e}	0.63 ± 0.02^{a}	White	Friable	+++++
0.5	1.0	Callus	25.00^{cd}	0.15 ± 0.03^{ef}	White	Compact	++
1.0		Callus	31.50^{b}	0.12 ± 0.02^{f}	Brown	Friable	++
1.5		Callus	20.50^{e}	0.18 ± 0.05^{de}	White	Compact	++
2.0		-	-	-	-	-	-
2.5		-	-	-	-	-	-

Key: - : No callus formation; (++): Slightly profuse; (+++): Profuse; (++++): Very profuse; (+++++): Highly profuse; MS: Murashige and Skoog; IAA : Indo -3- acetic acid; KIN : Kinetin

Table 4: Effect of IAA in combination with BAP in MS media supplementation on period of callogenesis and callus weight of shoot explants of *Dennettia tripetala*.

Concentration of growth regulator		Type of Response	Period (days) of Callogenesis	Mean Weight±SD (g) n=4	Colour	Nature	Intensity
IAA (mg/*l*)	BAP (mg/*l*)						
0.0	0.0	-	-	-	-	-	-
0.5	0.1	Callus	30.50^{a}	0.19±0.03^{e}	White	Compact	++
1.0		-	-	-	-	-	-
1.5		Callus	27.00^{e}	0.16±0.02^{fg}	White	Friable	++
2.0		Callus	28.00^{e}	0.20±0.01^{e}	Brown	Friable	+++
2.5		Callus	32.00^{cd}	0.12±0.02^{h}	White	Compact	++
0.5	1.0	Callus	40.75^{a}	0.21±0.03^{e}	Brown	Friable	+++
1.0		-	-	-	-	-	-
1.5		Callus	42.00^{a}	0.14±0.02^{gh}	White	Friable	++
2.0		Callus	32.50^{c}	0.38±0.03^{b}	White	Compact	++++
2.5		Callus	41.25^{a}	0.48±0.03^{a}	White	Friable	+++++

Key: - : No callus formation; (++): Slightly profuse; (+++): Profuse; (++++) : Very profuse; (+++++) : Highly profuse; MS : Murashige and Skoog; IAA : Indo acetic acid; BAP : 6-benzyl amino purine

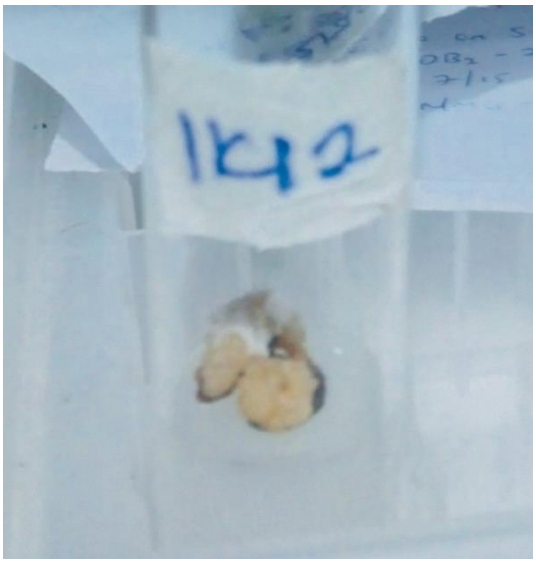

Figure 1: Friable Callus of *Dennettia tripetala* shoot explant in MS medium supplemented with 2.5 mg/*l* IAA + 0.1 mg/*l* Kinetin 46 days after Inoculation.

DISCUSSION

The result indicated that callus generated varied in weight and time of callogenesis according to the plant growth regulator used. Moderate to high level of IAA with moderate to high level of Kinetin gave rise to callus formation. When IAA alone was used in media supplementation, low intensity of callus was generated. But in media supplemented with both IAA and kinetin, there were increases in callus intensity. MS medium supplemented with 2.5 mg/*l* IAA + 0.1 mg/*l* Kinetin 46 days after Inoculation gave the highiest fresh weight of 0.63 g of callus as compared with other treatments of IAA+Kinetin. This medium was optimal for early callogensis. The fndings shows that higher levels of IAA with lower levels of Kinetin were needed for high intensity of callus induction. This observation

was similar with the findings of Wang *et al.* (2005) that the presence of auxins and cytokinins in the culture medium regulates various aspects of dedifferentiation and differentiation at cellular levels in a particular dose.

It was studied that BAP in combination with IAA played key role in callus induction on the shoot of *Dennettia tripetala*. However, the medium having only BAP did not show any callus induction response. The results obtained show that weight of callus induced increase with the levels of IAA and BAP. This opposed the findings of Rashmi (2011) when he cultured pointed gourd (*Trichosanthes dioica* Roxb.) in higher concentrations (above 2.5 mg/*l*) of BAP. It was also observed in the study that high levels of IAA with low levels of BAP were needed for high intensity of callus induction on shoot explants of *D. tripetala*. MS media fortified with 2.5 mg/*l* IAA and 1.0 mg/*l* BAP gave the highest callus fresh weight of 0.48 g. This is in consonant with the observations of Plevens *et al.* (2006) who reported maximum callus induction on the explants of tomato when cultured *in-vitro* in MS media supplemented with 2.0 mg/*l* IAA and 1.0 mg/*l* BAP. Shah *et al.* (2015) reported maximum callus induction on the hypocotyls explants of *Solanum lycopersicum* when cultured in 2.0 mg/*l* IAA and 2.5 mg/*l* BAP which was in opposition to our findings. Findings from this study clearly showed that high intensity of callus could be achieved with higher levels of auxins than that of cytokinins.

CONCLUSION

The study showed that *D. tripetala* shoot explants is responsive to callogenesis when cultured in Murashige and Skoog's medium containing plant growth regulators. Considering the plant growth regulators under investigation, it was recorded that IAA in combination with kinetin treatments were more effective than IAA in combination with BAP treatments in inducing callogenesis in *D. tripetala* as higher callus intensity was recorded in the former. IAA at 2.5 mg/*l* in combination with 0.1 mg/*l* Kinetin gave the best treatment. Therefore, IAA at 2.5 mg/*l* in combination with Kinetin at 0.1 mg/*l* is recommended as the best hormonal combination required for callus induction on shoot explants of *D. tripetala.* More work is recommended to able to ascertain the optimum condition for the culture and maintenance of *D. tripetala* as well as determine other hormonal regulator combinations to stimulate callusing and hence advance steps in plantlet production.

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