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Evaluation of *In-vitro* Response of *Dennettia tripetala* (Pepper Fruit) Bak. f. Seedlings as Shoot Explants to a Combination of Plant Growth Regulators

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ABSTRACT: *Dennettia tripetala* (Pepper fruit) an important tree crop deeply ingrained in the socio-cultural life of the people of Nigeria, faces the threat of extinction due to inconsistent fruiting, poor seed germination and slow seedling growth. Hence, the objective of this paper was to evaluate the *in-vitro* response of *Dennettia tripetala* (pepper fruit) seedlings as shoot explants to growth regulators using standard procedures by culturing 10 weeks old seedlings of the plant as shoot explant in three (3) levels of Naphthalene acetic acid (NAA) and six (6) levels of 6-Benzyl amino purine (BAP) or Kinetin supplemented in Murashige and Skoog (MS) medium. Data collected included the weight of callus, period of callogenensis, texture and colour of callus. From the results obtained, *D. tripetala* shoot explants cultured in Murashige and Skoog (MS) medium supplemented with various levels of NAA in combination with BAP/Kinetin at different levels effected callus formation at varying degrees. Generally, the nature of the callus observed was either friable or compact with the former occurring more. The colour of the callus was either white or brown. The highest callus intensity was observed in the media containing NAA at 0.5mg/*l* in combination with Kinetin at 1mg/*l* and also in the media containing 2.5 mg/*l* NAA in combination with 1 mg/*l* BAP. MS media supplemented with 1.5 mg/*l* NAA+0.1 mg/*l* BAP and 2.5 mg/*l* NAA + 1 mg/*l* BAP were optimum for early callogenesis in NAA+BAP combinations while 2.0 mg/*l* NAA + 1.0 mg/*l* Kinetin was optimum for early callogenesis in NAA + Kinetin combinations. The result shows that shoot explants of the plant are amenable to tissue culture when subjected to appropriate hormonal combination. Further work is needed in the area of embryogenensis, plantlet production and subsequent regeneration of the tree crop.

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Dennettia tripetela Bak. f. belongs to the family of Annonaceae. It is a small- medium sized tree whose range extends throughout the tropical rainforest zone of Nigeria and sometimes in Savanna areas (Okwu *et al*., 2005). This tree grows up to 15 m height and 0.6 m in girth, with a dense compact crown. The plant flowers from October to November and fruits from March to May. The flowers are light brown outside, reddish inside and usually in small clusters on the young or older woods (cauliflorous); stalks up to 0.6 cm long, hairy, with very small bracts; small broad sepals dark brown and shortly hairy; petals ovate, about 1 m long, leathery, shortly hairy (Keay, 1989). The fruits are green at first but eventually reddish pink

with finger-like carpel constricted between the seeds. The number of seeds in each fruit can be as low as one and the highest number can be as much as 10 seeds.
The plant is an important tree crop in the socio-cultural life of the Nigerian people. The fruits, seeds, leaves, roots and young shoots are highly consumed by humans due to the medicinal value they provide. Animal also browse the leaves, young shoots and eat up falling fruit from the tree. The peppery fruit is an important ingredient in the diet of pregnant and nursing mothers as it aids uterine contraction. Also, it is served to guests in cultural events as a form of entertainment most especially in Southern part of Nigeria. As a result of this high consumption and utilization placed on the plant and its products there is a negative impact in the regeneration of this fruit tree and subsequent threat to extinction. The plant has inconsistent fruiting, poor seed germination and slow seedling growth (Osaigbovo *et al*, 2010). IUCN (2014) categorizes the plant as threatened as a result of habitat loss to agricultural expansion, population growth and bush fire. The fruit is in high demand due to the various benefits derived from its consumption. These factors put together have largely contributed to the decline of the wild population of this tree species.

In vitro culture techniques represent a viable tool for mass production of superior stock plant and conserving their natural population for reducing risk of extinction. Explants, when cultured on appropriate medium, supplemented with auxin and cytokinin, produce unorganized, growing and dividing mass of cells. Usually the first step in *in vitro* plant culture is callus induction. Callus is an amorphous tissue consisting of dedifferentiated, unorganized cell masses (George *et al.*, 2008). *In vitro* culture is a technique use to propagate plants whose seed has germination problem, slow growth rate and infrequent fruiting. There is paucity of information relating to *in vitro* propagation of this plant. Based on this fact; the objective of this paper was to evaluate the *in-vitro* response of *Dennettia tripetala* (Pepper Fruit) Bak. f. seedlings as shoot explants to a combination of plant growth regulators.

MATERIALS AND METHODS

Plant materials: Shoots were collected from 10 weeks old seedlings of *Dennettia tripetala* located 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.

Media preparation and sterilization of equipment: The study was carried out using the facilities in the Biotechnology Laboratory of Nigerian Institute for Oil Palm Research (NIFOR), Benin City, Edo State. Murashige and Skoog (MS) medium that consists of macro and micro nutrients, vitamins, iron and other supplements was used. The prepared medium was based on the laboratory protocol of Murashige and Skoog (1962). Phytohormones at varying levels and combinations were added to the media and dispensed into culture test tubes of 25 × 150 mm and the pH was adjusted to 5.6 using 0.1N NaOH or 0.1 N HCl. The test tubes containing the media, forceps, petri dishes, wash bottle, surgical knife and foil paper were wrapped with foil paper and sterilized by autoclaving at 121^{o}C, 15 psi for 25 minutes. Following autoclaving, the media were transferred to the cooling room for cooling.

Fig 1. Fruits of *Dennettia tripetela*

Fig 2. Seeds of *Dennettia tripetela*

Sterilization and inoculation of explants: The shoot explants were surface sterilized by dipping in sterilized bottle containing 0.02 % Mercury chloride for 5 minutes, followed by rinsing with distilled water in order to prevent reaction before dipping into another bottle containing 3.5 % w/v Sodium hypochlorite for another 5 minutes. The surface sterilization procedure was carried out in order to remove any fungal and

bacterial contaminant inherent in the mother plant. This was followed by flooding with distilled water 3 to 5 times to remove any trace of sterilant due to their toxic effect on cells of the explants. After sterilization, the shoots were placed on a petri dish of 100 mm × 15 mm using a sterile forceps and cut into 3 cm using a surgical knife. The surface sterilised explants were implanted in Murashige and Skoog (MS) medium according to Murashige and Skoog (1962). All these procedures were done under the laminar flow hood. The *in vitro* cultures were inoculated in the dark growth room at 25±2ºC and 50 - 60% relative humidity until callus formation was induced.

Maintenance of in vitro culture: The cultured shoots were maintained by regular subcultures at 4 weeks intervals on fresh medium with the same nutrient composition and phytohormones to avoid accumulation of toxic metabolites and use up of the media nutrient.

Experimental Design: The experimental design was a Completely Randomized Design (CRD) with four replicates to determine the effect of Napthalene acetic acid (NAA) in six concentrations (0, 0.5, 1, 1.5, 2, & 2.5 mg/*l*) in combination with Benzyl amino purine (BAP) or Kinetin in three concentrations (0, 0.1 & 1 mg/*l*) to give eighteen NAA × BAP combinations and eighteen NAA × Kinetin combinations.

RESULTS AND DISCUSSION

The result obtained when *D. tripetala* shoot explants were cultured in MS medium supplemented with NAA, BAP alone and in combinations (Table 1) shows that the initiated explants started callusing on the 21st day at the edges where cut was made, gradually spread along the whole cut surface and ended on the 40th day. No callus was observed in MS medium free from NAA and BAP. MS medium supplemented with NAA alone at a level of 0.5, 1.5 and 2 mg/*l* gave callus weight that ranged between 0.11 to 0.12 g. The time of callus formation at the levels of 0.5, 1.5 and 2 mg/l NAA alone ranged between 24 – 40 days. Callus was not observed in MS medium supplemented with 0 mg/*l* NAA+0.1 mg/*l* BAP and 0 mg/*l* NAA+1mg/*l* BAP. MS medium supplemented with NAA at levels of 0.5, 1.5, 2, and 2.5 mg/*l* in combination with BAP at level 0.1 mg/*l* gave callus fresh weight that ranged between 0.23 to 0.49 g. The period of callus induction at these levels ranged between 21 -32 days. MS medium supplemented with 1mg/*l* BAP alone and that supplemented with 1mg/*l* NAA in combination with 1mg/*l* BAP did not produce callus. NAA at levels of 0.5, 1.5, 2 and 2.5 in combination with BAP at 1mg/*l* gave callus weight that ranged between 0.13 – 0.38 g. The period taken to achieve callogenesis at these levels ranged between 21 – 33 days. Therefore, MS basal medium supplemented with 2.5mg/*l* NAA in combination with 0.1mg/l BAP enabled the achievement of the highest weight of callus. The earliest period of callus induction from the date of initiation was 21 days and was observed in a basal medium containing 1.5mg/*l* NAA in combination with 0.1mg/*l* BAP; and 2.5mg/l NAA in combination with 1mg/*l* BAP. Its calli were mostly friable while a few were compact. Their colours are either white or brown. A fully grown friable callus obtained in MS medium supplemented with NAA at 1.5 mg/*l* NAA with BAP free is shown in Figure 3. Highly profuse callus was observed in the medium supplemented with 2.5 mg/*l* NAA+0.1 mg/*l* BAP. Physically, the friable calli were very soft, brittle and watery. The compact calli on the other hand, were hard and required some pressure application to break.

Table 1: Period (days) of callogenesis and mean weight (g) of callus grown in MS basal medium supplemented with combinations of NAA and BAP (mg/*l*).

NAA (mg/*l*)	BAP (mg/*l*)	Response	Period (days) of callogenesis	Mean weight ± SD (g): n=4	Colour	Nature	Intensity
0	0	-	-	-	-	-	-
0.5		Callus	37	0.11±0.01	White	Compact	++
1		-	-	-	-	-	
1.5		Callus	40	0.12±0.01	Brown	Friable	++
2		Callus	24	0.12±0.03	White	Friable	++
2.5		-	-	-	-	-	-
0	0.1	-	-	-	-	-	-
0.5		Callus	32	0.29±0.03	Brown	Friable	+++
1		-	-	-	-	-	-
1.5		Callus	21	0.23±0.03	White	Compact	+++
2		Callus	28	0.36±0.03	White	Friable	++++
2.5		Callus	30	0.49±0.03	White	Friable	+++++
0	1	-	-	-	-	-	-
0.5		Callus	28	0.13±0.02	White	Friable	++
1		-	-	-	-	-	-
1.5		Callus	33	0.19±0.03	White	Friable	++
2		Callus	33	0.26±0.03	White	Friable	+++
2.5		Callus	21	0.38±0.03	White	Friable	++++

Key :(-) = No callus formation; (++) = slightly profuse; (+++) = Profuse; (++++) = Very profuse; (+++++) = highly profuse

Fig 3: Fully grown friable callus of *Dennettia tripetela* cultured in MS medium supplemented in 1.5 mg/l NAA alone 40 days after initiation

When the shoots were cultured in MS medium supplemented with NAA and Kinetin alone and in combination, it was observed (Table 2) that the explants formed callus within 23 and 25 days at 0.5 – 2 mg/*l* NAA in culture medium and weight of callus ranged between 0.15 - 0.43g. Callus was induced in 0.1 mg/*l* Kinetin in 30 days and the weight of callus at this concentration was 0.13 g. Also, 1 mg/*l* Kinetin gave 0.14 g of callus in 30 days. NAA concentrations at 0.5, 1, 1.5 and 2.5 in combination with 0.1 mg/*l* kinetin respectively formed callus that ranged between 0.13 - 0.19g. The period of callus formation at these levels ranged between 29 – 40 days. At concentration 0.5, 1, 1.5, 2 and 2.5 mg/*l* in combination with 1 mg/*l* kinetin, callus was induced between 23 - 39 days and the weight of callus ranged between 0.15 to 0.56 g. It was indicated that 0.5 mg/*l* NAA+1 mg/*l* kinetin produced the biggest callus fresh weight of 0.56 g among the various concentrations of NAA+Kinetin used. The earliest period of callus formation which was 23 days was in a medium supplemented with 2 mg/*l* NAA +1 mg/*l* kinetin. Its calli were mostly friable while a few were brown. The main colours were white with few having brown colour. A newly developed callus obtained in MS medium supplemented with 0.5 mg/l NAA + 1 mg/l kinetin is shown in figure 4.

Table 2: Period (days) of callogenesis and callus weight (g) of callus grown in MS basal medium supplemented with combinations of NAA and Kinetin (mg/*l*).

NAA (mg/*l*)	Kinetin (mg/*l*)	Type of response	Period (days) of callogenesis	Mean weight ± SD (g) n=4	Colour	Nature	Intensity
0	0	-	-	-	-	-	-
0.5		Callus	25	0.37±0.02	White	Friable	++++
1		Callus	25	0.15±0.03	Brown	Friable	++
1.5		Callus	23	0.17±0.02	White	Compact	++
2		Callus	25	0.43±0.03	White	Friable	++++
2.5		-	-	-	-	-	-
0	0.1	Callus	35	0.13±0.01	White	Friable	++
0.5		Callus	40	0.14±0.03	White	Friable	++
1		Callus	39	0.19±0.03	White	Friable	++
1.5		Callus	29	0.15±0.03	White	Friable	++
2		-	-	-	-	-	-
2.5		Callus	39	0.16±0.03	White	Compact	++
0	1	Callus	30	0.14±0.01	White	Friable	++
0.5		Callus	25	0.56±0.03	White	Friable	+++++
1		Callus	33	0.25±0.03	White	Friable	+++
1.5		Callus	26	0.17±0.03	Brown	Compact	++
2		Callus	23	0.28±0.03	Brown	Friable	+++
2.5		Callus	35	0.15±0.03	White	Friable	++

Key: (-) = No callus formation; (++) = slightly profuse; (+++) = Profuse; (++++) = Very profuse; (+++++) = highly profuse

In this investigation, the phytohormones used effected callus on the treated shoot explants of *D. tripetala.* Various levels of NAA in combination with different levels of BAP gave callus of highest weight than when NAA is used alone. This agrees with the findings of (Igbinosa and Oboho, 2021) that a combination of appropriate levels of auxins and cytokinins is needed for high callus intensity of the plant. Among the growth regulators under investigation, MS medium supplemented with 2.5 mg/*l* NAA +0.1 mg/*l* BAP was optimal for the production of higher fresh weight of callus compared to other combinations of NAA+BAP. This is in line with Kataria *et al* (2013) that internodal segment of *Albizia lebbeck* on MS medium supplemented with 0.5 mg/*l* NAA+2.0 mg/*l* BAP gave fresh callus of high intensity. Also, this is in conformity with the findings of (Singh &Lal, 2007) that MS media supplemented with BAP in combination with NAA supported the highest percentage of callus induction for *Albizia lebbeck.* Also, this is in conformity with (Yadav & Singh, 2011) that MS medium containing BAP and NAA gave the

highest percentage of callus for *Leucaena leucocephala.* High amount of NAA alone stimulated callogenesis. This was in conformity with the report of Gaba (2005) that a greater than optimum concentration of auxin often causes callus production. The findings of Hussain *et al.,* (2012) also confirmed that high amount of auxin in MS medium induced callogenesis. MS medium containing 0.5 mg/*l* NAA + 1mg/*l* Kinetin was optimal for the production of higher fresh weight of callus compared to other combinations. This was in conformity with the report of Oselebe and Ene-Obong (2007) that highest callus fresh weight was obtained when stem explants of *Dioscoreophyllum cumminsii* was cultured in MS medium supplemented with NAA and Kinetin. Plant growth regulator free MS medium did not induce callus on the shoot explant of D. *tripetela*. This is in conformity with the findings of (Igbinosa and Oboho, 2012) that shoot explant of the plant cultured in hormone free MS medium do not yield callus.

Fig 4: Newly developed callus of *Dennettia tripetala* in MS medium supplemented with 0.5 mg/*l* NAA+ 1 mg/*l* Kinetin 25 days of intiation.

*Conclusion***:** The study indicated that shoot explant of *Dennettia tripetala* effected callus when cultured *in vitro* in Murashige and Skoog's medium supplemented with appropriate levels of plant growth regulators combination. The study recommends that a high level of auxins and low levels of cytokinins in appropriate amount is needed for producing callus on shoot explant of *D. tripetela* culture *in-vitro* in MS medium. Therefore, the protocol developed offers itself not only as a highly efficient method for mass *in vitro* callus induction on the shoot of *D. tripetala* but also for its conservation. There is still need for further work in the area of embryogenesis, plantlet production and subsequent regeneration of the plant.

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