



## ***In-vitro* development of *Nauclea diderrichii* (de Willd. & Th. Dur) Merrin liquid-M Smedia supplemented with Benzyl Amino Purine (BAP) and Naphthalene Acetic Acid (NAA)**

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**ABSTRACT:** The growth of plantlets in Temporary Immersion Bioreactor system (TIBs) relies on initial successful liquid phase transition process. The response of *N. diderrichii* explants was assessed in liquid-M Smedia with a view to mass produce its seedlings using TIBs. Seven treatments consisting (A) 0.0/0.0, (B) 0.0/0.1, (C) 0.1/0.0, (D) 0.2/0.1, (E) 0.3/0.2, (F) 0.4/0.3 and (G) 0.5/0.4mg/lBAP/NAA combinations were studied. Each group consist of seven replicates and group A without Growth Regulators (GR) serves as control. The results at 4 Weeks after Inoculation (WAI) showed that effects of the growth regulators were significant on shoot length and number of adventitious shoots while number of roots and leaves were closely related. Treatment E produced highest number of adventitious shoots (3.6) which was higher than 0.9 shoots from treatment G and closely related to others. Maximum number of leaves (16.6) was produced by treatment F followed by E (15.7) while the least (12) was obtained in treatment A. The highest number of roots (4.9) was obtained from treatments B, followed by E (4.3) with the lowest being recorded in C (2.43). Liquid MS medium supplemented with 0.3/0.2mg/lBAP/NAA shows some promise for plantlets generation for the purpose of multiple shoot production of *N. diderrichii* in TIBs.

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Applications of plant tissue culture techniques in forestry include micropropagation of tree species of economic importance. This technique aimed at rapid multiplication of plantlets, required protocol development for successful in-vitro establishment, regeneration, elongation, rooting and acclimatization as well as optimization of plantlet ex-vitro conditions. Until recently, most micropropagation processes are carried out using small culture vessels containing a semi solid culture medium as substrate for the cultured plant tissues (Loyola-Vargas *et al.*, 2008). This method has been successfully used to propagate different plants of agricultural, ornamental and forestry species (Jain and Haggman, 2007). However, large scale application of this method is limited due to its extensive manual handling, low rate of multiplication, long duration and high cost of production/plant (Etienne and Berthouly, 2002). The recent technological advancement on this existing method of micropagation welcomed the transition from the use of semi-solid media to that of liquid media in semi or fully automated system known as Temporary Immersion Bioreactor system (TIBs) (Carvalho *et al.*, 2019). TIBs allows temporary contact between plants and the liquid medium and present several advantages over the conventional method of

micropropagation. It offers a high degree of control over culture conditions (pH, aeration rate, nutrient concentration and exposure time), reduction in working space and energy while it is less labour intensive, more productive and profitable for commercial production (Etienne and Berthouly, 2002; Ayenew *et al.*, 2013). Nonetheless, prior to working with TIBs, all the parameters affecting the culture growth and productivity should be established (Nartop, 2018). An important stage during the transition from solid phase to liquid phase in TIBs is called zero shelving. This involves the gradual reduction of agar concentration in the nutrient medium from semi solid to liquid medium (Lyam *et al.*, 2012). This is usually done in order to prepare the explants for subsequent propagation and adaptation in TIBs. *N. diderrichii* is an indigenous tree species with immense economic importance in Nigeria. The tree is among the priority species for ongoing national afforestation project in the country which involves planting 25 million tree seedlings per year in a bid to mitigate climate change and stem down global warming. The species is mostly raised by seeds. Whereas, prior to planting, the seeds are exposed to bio-stresses and prone to physical damage during extraction processes which leads to low germination while young seedlings

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are fragile with low vigour. These leads to low survival rate and short supply of its seedlings. Hence, the need for alternative means of mass propagating the species. Although in-vitro propagation of *N. diderrichii* has been successfully achieved on semi-solid medium (Pitekelaou *et al.*, 2015a; 2015b), its response in liquid media has not been reported. Consequently, the effects of growth regulators in liquid MS medium on growth of *N. diderrichii* were determined in present study with a view to establish conditions necessary for its subsequent mass propagation in TIBs.

## MATERIAL AND METHODS

The study was conducted in the Biotechnology Centre of Forestry Research Institute of Nigeria located on longitude 07°23'18" to 07°23'43"N and latitude 03°51'20" to 03°23'43"E (FRIN, 2018).

The study involved the use of Murashige & Skoog (MS) liquid media supplemented with GR: Benzyl Amino Purine (BAP) and  $\alpha$ -Naphthalene Acetic Acid (NAA) in order of 0.0/0.0, 0.0/0.1, 0.1/0.0, 0.2/0.1, 0.3/0.2, 0.4/0.3 and 0.5/0.4mg/l BAP/NAA. The treatment were set up with 7 replicate and laid out in completely randomised design. MS powder of 34.43 g/l was used to prepare the basal media. The media was divided into the number of treatments while GR were added based on individual group treatment. The media pH was adjusted to 5.8, dispensed at 2 ml/tube and sterilized at 121°C and 15 psi for 15 min. Forceps, scalpels, petri-dishes and filter papers used for inoculation were also sterilized at the same condition. About 1.5 cm shoot tips and nodal segments of seed-raised plantlets were slant-cut and inoculated at one explant/tube. The tubes were capped, sealed with

parafilm and place in the growth room under 16/8 hrs light and dark photoperiod.

Shoot Length (SL), Number of Leaves (NL), Number of Roots (NR) and Adventitious Shoots (AS) were measured at two-week interval; starting from 2<sup>nd</sup> Weeks after Inoculation (WAI). The data were subjected to ANOVA and significantly different means were separated with DMRT at  $p \leq 0.05$

## RESULTS AND DISCUSSION

The effects of BAP and NAA in liquid MS media on the development of *N. diderrichii* explants were assessed. The results of the analysis of variance of the data collected showed that treatment effects was significant ( $p \leq 0.05$ ) for shoot length and number of adventitious shoots while that of number of leaves and roots were not significant ( $p > 0.05$ ) at 2 and 4 weeks after inoculation (WAI). The medium without GR gave the highest shoot length of 2.86 cm and was significantly higher than other treatments at 2 WAI (Table 1). Similarly, by 4 WAI, the same medium devoid of GR produced 4.0 cm shoot length which was comparable to 3.14 cm from medium added 0.4/0.3 mg/l BAP/NAA while higher than others (Table 1 and Plate 1). In addition, media with 0.0/0.1 and 0.3/0.2 mg/l BAP/NAA produced comparable average number of adventitious shoots of 2.1 each at 2WAI (Table 1). This value was significantly higher than 0.4 shoots produced by medium added 0.5/0.4mg/l BAP/NAA when compared to others treatments. At 4 WAI, highest adventitious shoots of 3.6 was produced by 0.3/0.2 mg/l BAP/NAA medium, significantly higher than 0.9 shoots from 0.5/0.4mg/l BAP/NAA medium while it was closely related to others (Table 1 and Plate 1).

**Table 1.** Effects of BAP/NAA in liquid medium on shoot length and number of adventitious shoots of *N. diderrichii*

Treatments (BAP/NAA)	Shoot length (cm)		Number of adventitious shoots	
	2 WAI	4 WAI	2 WAI	4 WAI
0.0/0.0 mg/l	2.86a	4.00a	1.3ab	2.1ab
0.0/0.1 mg/l	1.86b	2.86b	2.1a	2.9a
0.1/0.0 mg/l	2.29b	3.00b	1.9ab	2.7a
0.2/0.1 mg/l	2.14b	2.71b	1.6ab	2.7a
0.3/0.2 mg/l	2.00b	2.29b	2.1a	3.6a
0.4/0.3 mg/l	2.00b	3.14ab	1.0ab	2.3ab
0.5/0.4 mg/l	1.86b	2.71b	0.4b	0.9b

Means in the same column followed by different alphabet are significantly different at  $p \leq 0.05$  by DMRT.

The results of number of leaves produced by the plantlets as affected by GR at 2 WAI showed that highest average of 11.3 number of leaves was obtained from medium added 0.0/0.1 mg/l BAP/NAA followed by 11.1 from medium added 0.3/0.2 mg/l BAP/NAA while the least (7.7) was from control (Figure 1). Conversely, by 4 WAI, highest number of leaves (16.6) was obtained from 0.4/0.3 mg/l BAP/NAA medium followed by 15.7 from 0.3/0.2 mg/l BAP/NAA

while control also gave the least (12) (Figure 1 and Plate 1). Moreover, the number of roots produced by the plantlets were comparable among the treatments at both periods of observation. Highest average number of roots of 3.7 and 4.9 were obtained from medium added 0.0/0.1 mg/l BAP/NAA at 2 and 4 WAI respectively (Figure 2). This was followed by average of 4.3 roots from 0.3/0.2 mg/l BAP/NAA medium while the least number of roots (2.4) was obtained

from 0.1/0.0 mg/l BAP/NAA medium at 4 WAI (Figure 2 and Plate 1). The observed higher shoot length of *N. diderrichii* plantlets from growth regulators (GRs) free medium compared with others at 4 WAI (Table 1 and Plate 1A) could be attributed to the presence of endogenous cytokinins in the plant species and the opposing effects of NAA on stem elongation in media having the hormone. NAA belong to Auxins group which stimulates plant cells division and root initiation but can suppress morphogenesis at high concentrations (Smith, 2013). The result indicated that exogenous GRs are not an absolute requirement for shoot regeneration of *N. diderrichii* in-vitro. Whereas, the similarity observed in the shoot length from medium without GRs and the medium

added 0.4/0.3 mg/l BAP/NAA (Table 1) indicated that these hormone levels are optimum among others for the species shoot elongation in-vitro. This also explained the highest average number of leaves (16.6) produced by the *N. diderrichii* plantlets at 4 WAI from the same medium containing 0.4/0.3 mg/l BAP/NAA which was similar to other media and control (Figure 1 and Plate 1F). These results could be related to that of Pitekellabou *et al.*, (2015b), when *N. diderrichii* plantlets were successfully regenerated from nodal segments though on solid Woody Plant Medium devoid of GRs. It also correlated that of Hung and Trueman (2011) who obtained comparable *Khaya senegalensis* shoot length between control and medium having BA and NAA.

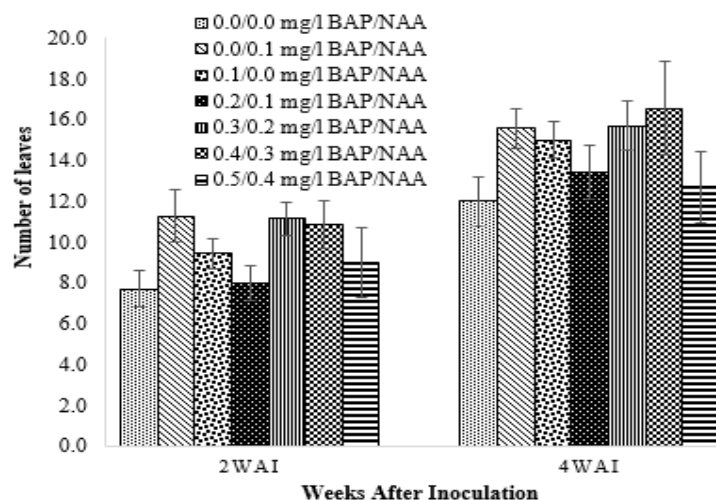


Fig 1. Effects of BAP/NAA in liquid medium on number of leaves of *N. diderrichii*; WAI: Weeks after inoculation. BAP/NAA: Benzyl Amino Purine/Naphthalene Acetic Acid.

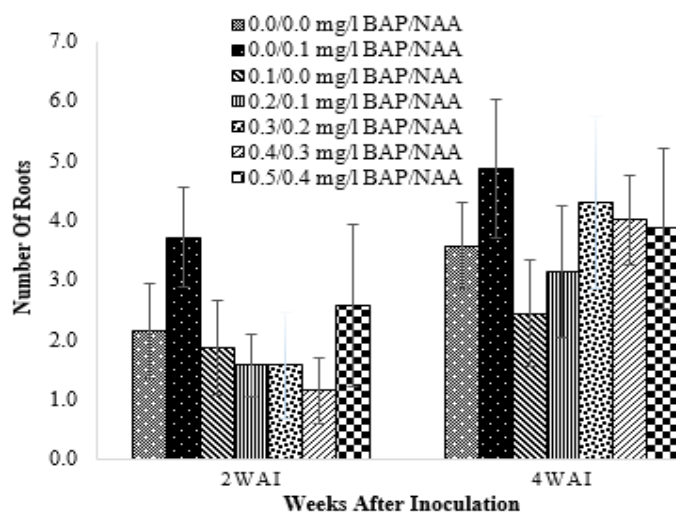


Fig 2. Effects of BAP/NAA in liquid medium on number of roots of *N. diderrichii*

The obtained highest multiple shoots of 3.6/plantlet obtained from medium added 0.3/0.2 mg/l BAP/NAA compared with others having GRs and control at 4 WAI (Table 1 and Plate 1E) indicated that the combination of BAP and NAA below or above these levels are not the best for *N. diderrichii* multiple shoot induction in liquid medium. This was evident in the lower multiple shoots results obtained from the concentrations below and above 0.3/0.2 mg/l BAP/NAA at 4 WAI. Meanwhile the endogenous cytokinins in this case was inadequate for better *N. diderrichii* shoot induction as observed in shoot length compared with when GRs were added but for the lowest shoots (0.9) obtained from the highest BAP/NAA combination (0.5/.04 mg/l respectively)

(Plate 1G). Previous reports on cultured *N. diderrichii* in liquid medium is not available. Hence, the formation of multiple shoots in the present study could be related to that of Mroginski *et al.*, (2003) who observed occurrence of shoot on nodal segments of *Toonia ciliate* cultured on quarter MS basal medium without plant growth regulators while stating that the addition of BAP alone or in combination with any of the auxins tested resulted in an increased number of shoots produced. Similarly, the results corresponded that of Hung and Trueman (2011) on *Khaya senegalensis* when highest numbers of shoots which ranged from  $2.3 \pm 0.1$ – $2.6 \pm 0.2$  per explant were obtained from media supplemented with 8.9  $\mu$ M BA alone, or 4.4 or 8.9  $\mu$ M BA in combination with 0.3  $\mu$ M NAA.

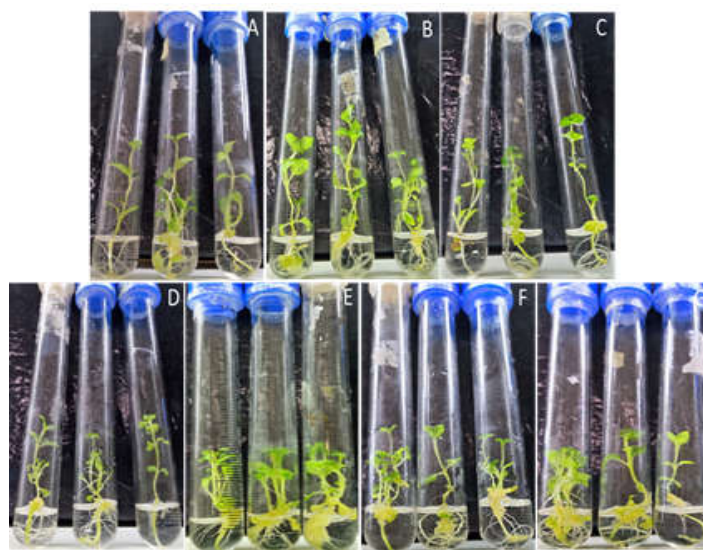


Plate 1. Effects of BAP/NAA in liquid medium on the growth of *N. diderrichii* plantlets at 4 WAI.

Furthermore, the non-significant results observed on rooting of *N. diderrichii* plantlets at 4 WAI (Figure 2) indicated that any of the treatments including control can be used for the species in-vitro root induction in liquid medium. Nonetheless, highest number of roots obtained from the lowest NAA concentration only (0.1 mg/l) compared to others in this study underscored the effectiveness of Auxins such as NAA at low level for better root induction.

GR elicited no significant root growth in *Vitellaria paradoxa* (Afolabi *et al.*, 2020). High level application of Auxins is said to inhibit root elongation or acts as herbicides (Read and Preece, 2014). Hence, the use of 0.1 mg/l NAA only in this study could be adjudged to be efficient concentration which allowed *N. diderrichii* root induction without being inhibitory to its elongation in liquid MS medium.

**Conclusion:** This study envisaged the in-vitro development of *Nauclea diderrichii* in liquid media using MS medium supplemented with BAP and NAA. The results indicated that medium added 0.3/0.2 BAP/NAA mg/l (Treatment E) produced highest number of adventitious shoots (3.6/plantlet) at 4 WAI. Hence, for the purpose of mass production and scaling up in Temporary immersion bioreactor, using liquid MS medium supplemented with 0.3/0.2 BAP/NAA mg/l is best and hereby recommended for in-vitro propagation of *N. diderrichii*.

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