A bi-annual journal published by the Faculty of Science, University of Lagos, Nigeria
http://jsrd.unilag.edu.ng/index.php/jsrd

Optimization of α-naphthalene acetic acid and 6-benzylaminopurine Concentrations for the in vitro micropropagation of *Discorea bulbifera* Linn

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(*Received 18 January 2026 /Revised 24 April 2026 /Accepted 12 May 2026)*

Abstract

One of the 600 species of the Dioscoreaceae family that have been used in traditional medicine all over the world is *Dioscorea bulbifera.* It is known as bitter yam and is indigenous to Asia, tropical Africa, America, and northern Australia. This herbaceous climbing plant has been used to treat various diseases including diabetes and ulcers. In order to improve and conserve this important medicinal plant, growth responses of micropropagated *Dioscorea bulbifera* was observed on Murashige and Skoog medium. The effects of two plant growth regulators; 6-benzylaminopurine, BAP (0.5, 1.0, 2.0 and 3.0) mg/l and α-naphthalene acetic acid, NAA (1.0, 2.0, 3.0 and 4.0) mg/l was used singly and in combination. Culture was maintained at 25 ± 2°C with 16/8-light/ dark photoperiod and a light intensity of 40 lmol m-2 s-1 provided by cool white fluorescent tubes. Results showed that treatment with 2 mg/L of NAA in shoot length 4.01±0.33 was remarkably high and the root length 2.28±0.64 was statistically significant. Also, 2 mg/L of BAP produced the highest shoot length 2.92±1.16 significantly. The combination of 1BAP+2NAA had the highest of proliferation of 3.10±0.46 followed by 2BAP+3NAA that produced 2.41±0.25. In this present study, the synergistic and advantageous effects of BAP and NAA on *D. bulbifera* culture boosted shoot induction and proliferation.

Keywords: 6-benzylaminopurine BAP; *Dioscorea bulbifera*; Micropropagation; Murashige and Skoog (MS); Plant growth regulators

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Introduction

The monocotyledonous *Dioscorea* which is known as yam was termed after the ancient Greek physician and Botanist Dioscorides. This genus consists of about 600 Species which has significant economic value (Ayensu, 1972; Supriya, *et al.,* 2013). *Dioscorea* species possess great cultural value; especially for people living in tropical regions (Obidiegwu et al., 2020). Yams are valuable source of carbohydrates, fibers and low level of fats which make them a good dietary nutrient and also processed into various staple intermediate and end product forms (Jaleel, *et al.,* 2007).

Among the Dioscorea species, *Dioscorea bulbifera* has been widely utilized in the traditional medicine as the remedy of a vast range of ailments. Owing to its ethnobotanical and medicinal value, *D. bulbifera* has garnered

ample attention in the last few decades. *Dioscorea bulbifera* plant is being characterized by the emergence of its unique reproductive structures called bulbils or aerial tubers (Ekaette *et al.,* 2024). These bulbils are small, swollen structures that develop along the vine's stem, allowing the plant to propagate vegetatively. It is popularly known for its saline and sour taste. It had been traditionally used as the remedy for cough, epistaxis, goiter, hemoptysis, pharyngitis, skin infections, piles, throat infections and as an anti-dandruff (Guan *et al.,* 2017; Kumar *et al.,* 2017). The phytochemical analysis of *D. bulbifera*, revealed the presence of saponins, tannins, flavonoids, sterols, polyphenols, glycosides (Ghosh, 2015) and steroidal saponins (Tapondjou, *et al.,* 2013). The yam species is claimed to be rich in diosgenin, a steroid saponin as well as other therapeutic components. It also contains flavonol glycosides, namely, quercetin-3-*O*-galactopyranoside, myricetin-3-*O*-galactopyranoside, and myricetin-3-*O*-glucopyranoside (Kuroyanagi, *et al.,* 2002).

Several studies have been conducted to enhance our understanding of the in vitro propagation of *Dioscorea alata*, *Dioscorea rotundata*, and *Dioscorea bulbifera*. It has been performed by using different part of the plant's body as such as zygotic embryo's, shoot tips, bulbils, and roots as explants. The propagation of *Dioscorea* species through traditional methods often tend to be slow thereby, inadequate for proliferation. Tuber yield is drastically affected by viral and nematode infections. In some cases, the infected tubers alter the genes of the next generation which results in a compromised tuber quality. Moreover, lack of agronomic constraints, phytosanitary problems and unhealthy planting material, restrict production of tubers. In vitro propagation may help to overcome most of these constraints, especially those related with availability of high quality of planting material.

In a study conducted by Bhat, *et al.,* (2022), he established in vitro propagation of *Dioscorea bulbifera*, using mature nodal explants and of different concentration NAA and BAP, to optimize the culture conditions for shoot induction and multiplication. They observed significant shoot proliferation and subsequent plantlet regeneration. Another notable study by Birhan et al., 2021 investigated the in vitro propagation of *Dioscorea* alata, for shoot induction and multiplication, using cytokinins such as benzylaminopurine (BAP) and kinetin. Therefore, this study aimed to optimize the NAA and BAP of different concentration for the purpose of micropropagation of *Discorea bulbifera.*

MATERIALS AND METHODS

Plant materials and sterilization

Healthy plants of *Discorea bulbifera* were collected from the International Institute of Tropical Agriculture, Ibadan. The nodal segments were excised and washed on a running tap water and surface sterilized by 0.1% (V/V) aqueous solution of Tween-20 for about 10 min. Thereafter, washed with distilled water for 6 to 7 times and washed aseptically using sterile distilled water followed by sterilization with 70% ethyl alcohol for 60 sec and surface disinfected. It was rinsed again in 3 changes of sterile distilled water and then air-dried on sterile blotting paper.

Culture medium and growth conditions

About three-quarter sterilized distilled water was used for the preparation of culture medium. Murashige and Skoog medium 4.4 g conc were supplemented with 20 g sucrose, 8 g agar-agar and varying concentrations of Plant Growth Regulators (PGRs) BAP and NAA, separately and in combination and stirred on a magnetic stirring plate at 1500 rpm for 10 min. The pH of the solution was adjusted to 5.7–5.8 on a digital pH meter using either 0.1 N HCl or 1N NaOH before being topped to make a liter of the solution. For

solidification of the medium, agar powder (Tissue culture grade; agar-agar type) 0.7 % (w/v) was added to the luke-warm solution and then, melted on a heating and magnetic stirring plate (1500 rpms; 100 ◦C) for 15 min. The Plant Growth Regulators, BAP and NAA, each made to a stock solution of 5 mg/L (*w/v*) were separately added in the MS media at different concentrations. Each growth tube for incubation had 10 mls of MS media dispensed with and without plant growth regulators in respective treatments and sealed with a double aluminium foil. The growth tubes with media were autoclaved at 121 ◦C for 20 min (15 psi). For each treatment, 10 replicates were used and all the experiments were repeated three times. The cultures were incubated at 27 °C with a photoperiod of 16 h at an intensity of 10-20 µmol m-2s-1. After 3-6 weeks of culturing, cultures were subcultured in fresh basal medium depending on the experiments.

Growth estimation of *D. bulbifera*

Growth of the Shoot and root of *D. bulbifera* was assessed at 4-6 weeks. Measurements include shoot number, root number, number of nodal segments (buds) on shoots and root length.

Experimental design

Each experiment was arranged in a complete randomized design with ten replications that were repeated three times. The BAP and NAA each had four treatments each that were 0.5, 1.0, 2 0, 3.0, and 1.0, 2.0, 3.0, 4.0 mg/L respectively including the control 0 mg/ L. In all the treatments, each replication had two explant per growth tubes for experimentation.

Data collection and analysis

Initiated explants for each treatment were determined after 14 days after culturing. Daily records were made on the number of days and the total number of shoots and roots to assess the effect of BAP and NAA on the shooting and rooting potential of the plant. In the experiment, the length of shoots was evaluated among ten randomly selected explants in each replication of the treatments. explants were carefully cleaned off the media with gently running water and the length and number of all shoots and roots were determined at day 45 of the end of the experiment. The results were analyzed using ANOVA tests with Turkey's multiple comparison tests and expressed as mean ± standard error at $p < 0.05$ level of significance.

Results

Effect of different concentrations of NAA on the number and lengths of shoots and roots

In the present study, it was observed that MS medium + NAA (3.0 mg/L) produced the highest number of shoots and roots of (25 and 21) respectively as shown in Table 1.

The treatment with PG-free MS medium showed a very low number of shoots and roots having 3 and 7 respectively. The number of the shoots increased as the concentration of NAA increased from 1.0 mg/l to 3.0 mg/l (10 and 15) but drastically reduced to 7 shoots at a concentration of 4.0 mg/l NAA. This observation was also seen in the number of roots but the number of roots produced by 4.0 mg/l NAA (14) was more than those produced by 1.0 mg/l and 2.0 mg/l (10 and 8) roots. The shoot and root germination having percentage of 40.98% and 35.00 % respectively was observed at 3.0 mg/l of NAA and was statistically different among other treated groups in table 1.

Table 1: Different concentration of Plant Growth Regulator of NAA on number of shoot and root

Treatment (mg/l)	Control	1NAA	2NAA	3NAA	4NAA	Sum	Mean
No of Shoots	3	11	15	25	7	61	12.20
No of Roots	7	10	8	21	14	60	12.5
Initiation percentage of Shoot	4.92^{d}	18.03^{c}	24.59^{b}	40.98^{a}	11.48^{cd}		
Initiation percentage of Root	11.67^{c}	16.67^{cd}	13.33^{c}	35.00^{a}	23.33^{b}		

Means with the same letter within a column are not significantly different at P≤0.05.

Table 2 shows the effect of different concentrations of NAA on the mean root and shoot length. MS medium + NAA (2.0 mg/L) produced the highest mean shoot length of (4.01± 0.49) cm with single root of mean length (2.26 ± 0.64) cm (Figure 1). It was also observed that the highest mean root length was produced by the PG-free MS medium of (3.01± 0.74) cm whereas the medium produced the lowest mean shoot length of (1.93± 0.22) cm.

Table 2: Different concentration of Plant Growth Regulator of NAA on shoot and root lengths

Treatment (cm)	Control	1NAA	2NAA	3NAA	4NAA	Mean	LSD (P≤0.05)
Shoot length (cm).	1.93 ± 0.22^{a}	2.73 ± 0.37^{a}	4.01 ± 0.33^{a}	2.92 ± 0.16^{a}	3.01 ± 0.33^{a}	2.92	0.60
Root length	3.01 ± 0.74^{a}	0.8 ± 0.16^{bc}	2.28 ± 0.64^{a}	0.55 ± 0.06^{b}	0.69 ± 0.08^{c}	1.47	0.66

Results are expressed as mean ± SE. Means with the same letter within a column are not significantly different at P≤0.05.

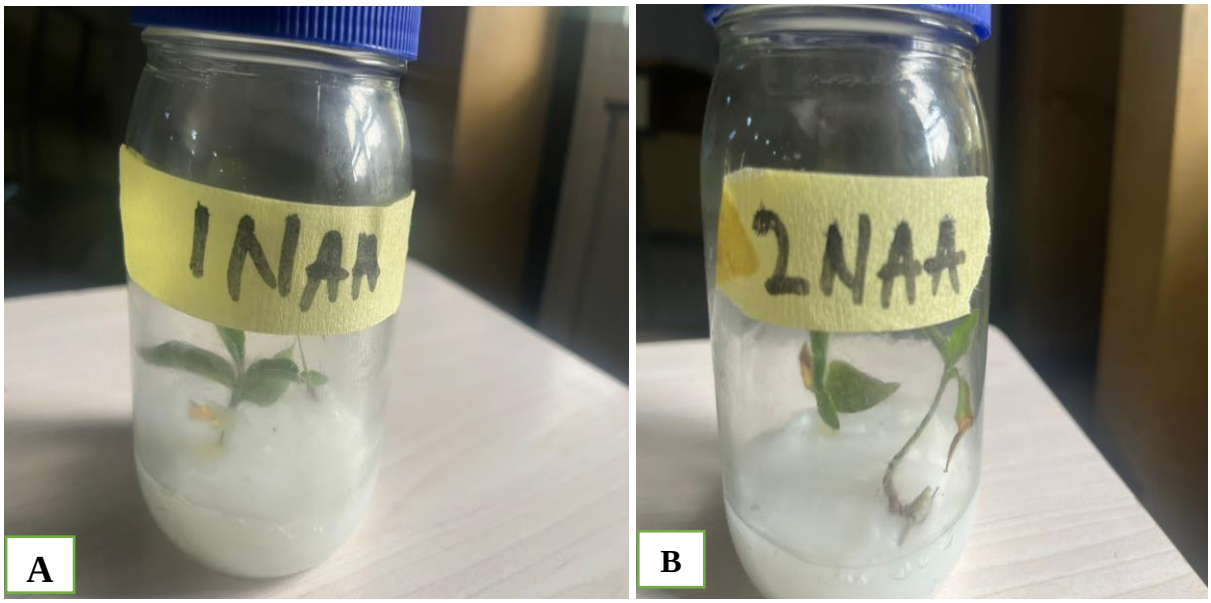

Fig. 1: *Dioscorea bulbifera* after 6 weeks of culture in single MS medium with 1 NAA (A) and 2 NAA (B).

Fig. 2: *Dioscorea bulbifera* after 6 weeks of culture in single MS medium with 1 BAP (A) and 2 BAP (B).

Table 3: Different concentration of Plant Growth Regulator of BAP on number of shoot and root

Treatment (mg/l)	Control	0.5 BAP	1 BAP	2 BAP	3 BAP	Sum	Mean
No of Shoots	7	9	7	6	4	33	6.6
No of Roots	6	7	5	4	3	25	5.0
Initiation percentage of Shoot	21.21^{ab}	27.27^{a}	21.21^{ab}	18.18^{b}	12.12^{c}		
Initiation percentage of Root	2.4^{a}	2.8^{a}	2.0^{b}	1.6^{bc}	1.2^{c}		

Means with the same letter within a column are not significantly different at P≤0.05.

Table 3 depicts a steady decline in the number of shoots produced with increase in BAP concentration The BAP concentration which gave rise to the greatest number of shoots is the 0.5 BAP treatment (28%), While the BAP treatment with the lowest shoot number is the 3.0 BAP treatment (12%), there is a significant difference in number of shoots among BAP treatments from 0.5 mg/l BAP to 3.0 mg/l BAP ($p \leq 0.05$) which inferred that an increase in BAP concentration results in a decrease in the number of shoots produced. The BAP treatment which produced the highest number of roots is the 0.5 mg/l (2.80%), while the BAP treatment with the lowest number of shoots is the 3.0mg/l (1.2%). This result suggests that an increase in BAP concentration results in a decrease in number of roots produced.

Table 4 shows a steady increase in the shoot length with an increase in BAP concentration from 0.5 to 2.0mg/l ($p= 0.03$), and there is a decline after 2.0 mg/l BAP treatment. there is a significant difference at 2.0 mg/l BAP treatment (2.92±1.16). This could suggest that an increase in BAP concentration would most likely lead to an increased shoot length of *D. bulbifera*.

Table 4: Different concentration of BAP on shoot and root lengths

Treatment (cm)	Control	0.5 BAP	1 BAP	2 BAP	3 BAP	Mean	LSD (P≤0.05)
Shoot length (cm).	1.93±0.22[a]	1.73±0.37[a]	1.96±0.33[a]	2.92±1.16[a]	0.78±0.07[b]	0.08	0.12
Root length	3.01±0.74[a]	0.8±0.16[a]	0.71±0.06[a]	0.75±0.06[a]	0.69±0.08[a]	1.47	0.66

Results are expressed as mean ± SE. Means with the same letter within a column are not significantly different at P≤0.05.

Effect of different concentration of NAA and BAP on the number and lengths of shoots and roots

The effect of different concentrations and combinations of BAP and NAA on the numbers of shoots and roots as shown in table 5. Treatment with only the MS- medium produced 13 shoots and 7 roots. Treatment with 2 mg/l BAP and 3 mg/l NAA produced a significantly higher number of shoots (31) with 37% of germination, followed by those treated with 0.5 mg/l BAP and 1 mg/l NAA produced a significant high number of roots (40%).

Table 5: Different concentration of Plant Growth Regulator combination (BAP and NAA) on number of shoot and root

Treatment (mg/l)	Control	0.5BAP, 1NAA	1 BAP, 2NAA	2BAP, 3NAA	3BAP, 4NAA	Sum	Mean
No of Shoots	13	24	4	31	12	84	16.80
No of Roots	7	19	2	15	5	48	9.6
Initiation percentage of Shoot	15.5[c]	28.6[a]	4.8[d]	36.9[ab]	14.3[c]		
Initiation percentage of Root	14.6[b]	39.6[a]	4.2[c]	31.3[a]	10.4[b]		

Means with the same letter within a column are not significantly different at P≤0.05.

Table 6: Different concentration of Plant Growth Regulator combination (BAP and NAA) on shoot and root lengths

Treatment (cm)	Control	0.5BAP, 1NAA	1 BAP, 2NAA	2BAP, 3NAA	3BAP, 4NAA	Mean	LSD (P≤0.05)
Shoot length	2.2±0.41[a]	2.2±0.25[a]	3.1±0.46[a]	2.4±0.25[a]	2.2±0.27[a]	2.4	0.68
Root length	0.81±0.18[a]	0.6±0.05[a]	0.80±0.25[a]	0.5±0.06[ab]	0.5±0.12[a]	0.65	1.9

Results are expressed as mean ± SE. Means with the same letter within a column are not significantly different at P≤0.05.

Figure 3: the different concentrations of BAP and NAA

Combination of 2 BAP and 3 NAA on root length was found to be highly significant, while there was no significant difference among all the treated groups for the shoot length having the combination of BAP and NAA. However, it is noteworthy that the highest mean shoot length 3.1±0.46 cm was obtained on MS medium supplemented with 1.0 mg/l of BAP and 2.0 mg/l of NAA, while, mean shoot length in the MS-medium (PGR-free medium) was observed to be (2.2±0.41). From all treatments the minimum rate of shoot length was observed in MS containing 3.0 mg/l BAP and 4.0 mg/l NAA (2.2±0.27). It was generally observed that an addition of BAP to NAA showed reduced mean length of roots.

Discussion

Micropropagation is regarded as a fast method of multiplying plants and has great potential to develop high-quality and disease-free plants. Advancements in this field have led to the development of several techniques for the rapid multiplication and improvement of a wide range of horticultural and tuberous crops and their production systems (Butt, *et al*., 2015). *Dioscorea bulbifera* is a seasonal medicinal plant, which appears with the beginning of rainy season. Nodal segments are the preferred explant type used in numerous investigations for culture initiation of medicinal plants (Farooq, *et al*., 2021; Nandagopal, *et al.*, 2015; Kher, *et al*., 2014;). These explants are found to be superior for in vitro regeneration with retained genetic stability (Shekhawat, *et al*., 2014). Tissue culture (micropropagation) plays an important role in producing controlled nutritional and environmental conditions to produce the clones of plants irrespective of the season and weather on a year-round basis. Recent studies have shown that the single use of NAA for the in vitro propagation of plants has not been broadly reported as the in vitro propagation has been done via the combinations of different promoting regulator hormones such as BAP, IAA, Kinetin, etc.

Exogenous application of synthetic auxins, such as NAA, is crucial for root initiation and development (Matsuo, *et al*., 2018). They are important factors involved in rooting because they promote adventitious root formation in the vast majority of species (De Klerk, 2002). Auxins trigger complex growth and developmental processes. They facilitate fast switching between gene activation and transcriptional repression via the auxin-dependent degradation of transcriptional repressors. The nuclear auxin signaling pathway consists of a small number of core components, but each component is represented by a large gene family (Lavy and Estelle, 2016). Previous studies had shown that MS media fortified with NAA alone

showed good root formation in many *Dioscorea* spp. (Zhang et al., 2025; Nazir, *et al.* 2020a, b; Maheswari, *et al*. 2012;). Cultures maintained on NAA supplemented media produced profuse callus, which retarded shoot formation and subsequent shoot growth (Dwumawa, *et al*., 2016).

In this study, it was observed the PGR free MS medium produced limited number of roots. The lowest concentration of NAA employed in this study was 1 mg/L. It was observed that this low NAA concentration produced a remarkable number of root and even shoots. This result is not in agreement with the study conducted by Jane, *et al*., (2016) who reported the lowest number mean root. He observed a significant decrease in root formation with the increase in the concentration of NAA. Unlike Behera *et al.*, (2009) that described that lower concentration of auxin, NAA produced very few or no root.The results of the current study point to the need for more research to compare the effects of other auxin-promoting growth regulators in order to achieve optimal development and micropropagation of *D. bulbifera*.

The concentration of cytokinin, BAP used in this study is in line with several other reports. A previous study by Birhan et al., (2021) explained the various nature and amount of cytokinin which can significantly determine the growth of various genotypes of Dioscorea spp. And these corroborates other findings according to (Mahesh et al. 2010; Poornima and Ravishankar, 2007). Also, Gopitha et al. (2010), reported low concentrations of cytokinin can induce shoot multiplication and elongation. Manoharan et al. (2016), on the other hand obtained MS media with 0.4 mg l-1 BAP as the optimum concentration for shoot proliferation.

This study was carried out to evaluate the combination effect of different concentrations of 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA) of *D. bulbifera.* Synergistic effect of BAP when combined with an NAA has been confirmed in many medicinal plant species from the genus *Dioscorea*, viz. *Dioscorea alata*, *D. hispida*, and *D. fordii* (Behera, *et al*., 2008; Das, *et al*., 2013; Yan, *et al*., 2011), *Dioscorea floribunda* (Borthakur and Sing, 2002) and *D. zingiberensis* (Yan, *et al*., 2002).

Concentrations of (0.5, 1.0, 2.0 and 3.0 mg/l) and (1.0, 2.0, 3.0 and 4.0 gm/l) was employed for BAP and NAA respectively. All media combinations produced a high number of shoots and roots as compared to the PGR free MS medium (control group). A lower number of shoot production in comparison to the control was observed in the combination having 3 BAP and 4NAA. This study is in agreement with Fotso, *et al.,* (2013) who used a combination of BAP and NAA and recorded a higher number of microtubers in the shoots of *D. alata*. Yuan, *et al.* (2005) also reported a higher effect of BAP and NAA on the callogenesis of *D. zingiberensis*. From this study, it was observed that 1 BAP and 2 NAA provided an optimum number of shoots and roots.

After 6 weeks, the interaction effect of the explant with different concentrations of BAP and NAA was also observed on the root and shoot length. Observations reported the combinations of 1 BAP and 2 NAA produced a shoot mean length of (3.10±0.46) cm which was higher than the control and other PGR combinations in this study. This result corroborated Mubo and Adedapo, (2014), which observed the highest root proliferation obtained from Murashige Skoog medium of 6-benzylamino purine (0.05 mg/L) and α-naphthaleneacetic acid (0.01 mg/L) with mean root length of (27.0±01.25) mm and elongated single shoot of mean length (38.00±11.09) mm. This comparison shows that the micropropagation of *D. bulbifera* requires a small concentration of BAP and NAA to

produce an optimum number of roots and shoots along with a better mean length. The combination of BAP and IAA on the micropropagation of *Dioscorea bulbifera* has been reported to be more effective than NAA (Musadiq, *et al*., 2022).

The technique of micropropagation can be utilized as an alternative strategy to grow plantlets quickly and in great quantities, as well as to improve the production of primary and secondary metabolites in medicinal plants. The synergistic and advantageous effects of BAP and NAA on *D. bulbifera* cultures boosted shoot induction and proliferation, according to current studies. The development of an enhanced, effective, and cost-effective methodology for the micropropagation of this plant was made possible by the use of plant growth regulators and in vitro conditions that were tuned for shooting and roots in *D. bulbifera*. The study may help to designate, identify, and standardize active chemicals for the manufacture of new therapeutic formulations for future research.

Conflict of Interest

The author state that there is no conflict of interest in this study.

Acknowledgements

The author wishes to acknowledge the support of Professor Omobolanle E. Ade-Ademilua for creating a bench space in her tissue culture laboratory at the University of Lagos, Nigeria.

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