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Full Length Research Paper

In vitro regeneration and morphogenesis in *Phyllanthus niruri* L., an anti-plasmodial herb

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We investigated the use of *in vitro* techniques for regeneration of adventitious shoots in *Phyllanthus niruri*, an anti-malarial plant as an initial effort towards its domestication. Fruits cultured on Murashige and Skoog (1962) basal medium (MS) supplemented with BAP had low germination (61%) due to seed coat imposed dormancy. The culture of nodal cuttings explants on BAP, kinetin or 2-isopentyl adenine (2iP) amended medium to avoid seed dormancy resulted in shoot regeneration without roots in all accessions with BAP producing the highest number of shoots (9.0). Subsequent inclusion of either 1-naphthalene acetic acid (NAA) or indole-3-butyric acid (IBA) in the BAP, kinetin or 2iP amended MS medium also produced only shoots. Leaf lobe explants cultured on only 2,4-dichlorophenoxy acetic acid (2,4-D) amended medium led to a significant calli development with 1 mg/L 2,4-D producing 100, 88.9 and 95.8% callus, respectively from Kwabenya, Kasoa and Aburi accessions. Subsequent transfer of calli to MS medium supplemented with 0.1 mg/L BAP led to calli growth (increase in weight) and morphogenic response depending on the concentration of 2,4-D in the induction medium. Only 55 and 25% of these calli from Kwabenya and Kasoa, respectively produced shoots while roots development was significantly higher ranging from 48 to 88.9%. These shoots did not survive *ex-vitro* acclimatisation due to hyperhydricity while those regenerated from nodal cuttings or seeds had high percentage survival. The high morphogenetic response of *Phyllanthus niruri in vitro* can be used to propagate this anti malarial plant and enhance its utilization in the treatment of malaria.

Key words: Shoot regeneration, morphogenic response, *Phyllanthus niruri*, accessions.

INTRODUCTION

Malaria is reported to be one of the major fatal diseases endemic in tropical Africa (Asase et al., 2005) posing risk to over half a billion people in the tropics. It is transmitted by female *Anopheles* mosquito and its causative vector is *Plasmodium falciparum.* Several attempts by government agencies in most African countries to eradicate the disease has proved futile due to resistance developed by the plasmodium parasite against conventional therapeutic drugs. Also, the host female Anopheles mosquito has developed resistance to insecticides as a result of high rate of mutation (Anto et al., 2009). Coupled with these problems is the high cost of the classical drugs used for the treatment of the disease, thus, making them unaffordable by the indigenous people living in endemic areas.

The search for alternative medicine from medicinal plant species has therefore been a major concern to governments all over the world. *Phyllanthus niruri (amarus*) an annual euphorbia widely distributed in the tropics has traditionally been identified as an important

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Abbreviations: MS, Murashige and Skoog (1962); **BAP,** 6-benzylaminopurine; **2iP,** 2-isopentyl adenine; **NAA,** 1-naphthalene acetic acid; **IBA,** indole-3-butyric acid; **2,4-D**, 2,4-dichlorophenoxy acetic acid.

anti-plasmodial plant which can be used to treat the disease (Fasihuddin and Ghazally, 2003). Subeki et al. (2005) have shown that 1-O-galloyl-6-O-luteoyl-alpha-d-glucose obtained from boiled extracts of *P. niruri* had anti-plasmodial activities. Besides, its anti-plasmodial activity, extracts from the plant has been used for the treatment of jaundice, diabetes, and liver disorders (Banerjee and Chattopadhyay, 2010). It also has a strong activity against hepatitis B virus (HBV) (Shim et al., 2000) and C virus (HCV) (Bhattacharyya and Bhattacharya, 2003). The hepato protective effect of the plant has been attributed to the presence of the active ingredients phyllantin (PH) and hypophyllantin (HPH) (Banerjee and Chattopadhyay, 2010).

In spite of its medicinal importance, *P. niruri* is still harvested in the wild with no effort to domesticate or commercially grow it for sustainable exploitation and utilization. The continuous collection of the plant from the wild without its domestication may lead to genetic extinction as a result of over exploitation. Thus, *in vitro* mode of propagation should be employed to produce planting materials for commercial farming of the plant.

The use of *in vitro* regeneration techniques for the genus *Phyllanthus* is well documented (Liang and Keng, 2006; Ghanti et al., 2004). The technique offers several advantages not only for clonal multiplication of the plant but also for extraction of active secondary metabolites. *In vitro* multiple shoots induction in *Phyllanthus amarus* had been achieved on Murashige and Skoog (MS) 1962 basal medium (MS) modified with sucrose and low concentrations of benzyl amino purine (0.1 mg/L BAP) and naphthalene acetic acid (0.05 mg/L NAA (Ghanti et al., 2004). Also, calli induction in several species of *Phyllanthus* has been reported by several authors like Catapan et al. (2001), Cimanga et al. (2004) and Luyindula et al. (2004). Linyindula et al. (2004) successfully induced calli from apical shoots of *P. niruri* on a medium supplemented with 4 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D). Induction of calli in *P. niruri* can play a dual role in the commercial exploitation of the plant. Firstly, it can be used for plant regeneration and secondly, for extraction of secondary metabolites for the pharmaceutical industries. Cimanga et al. (2004) have reported that ethanol extracts from calli of *P. niruri* showed a higher anti-plasmodial activity than the same extract from intact plants. In this paper, we report on plantlet regeneration and callus induction from nodal cuttings and leaf lobes of *P. niruri*.

MATERIALS AND METHODS

Fruits of three accessions of *P. niruri* L. collected from Greater Accra Region, Eastern Region and Central Region were identified at the Department of Botany University of Ghana and labelled according to the location of collection (Figures 1A and B). Fruits were dried in Petri dishes for seven days and then nursed in trays filled with a mixture of soil-cow dung-coconut husk in a ratio of 3:1:1 to establish seedlings which were used for the various experiments in this study.

Culture media and incubation conditions

The culture media consisted of MS basal medium amended with 30 g/L sucrose, 0.1 g/L myo-inositol, 1 ml of 2 µM $CuSO_4$ and vitamins (0.02 g/L thiamine, 0.1 g/L pyridoxine, 0.1g/L nicotinic acid and 0.04 g/L glycine) (Murashige and Skoog,1962). The media was supplemented with a range of growth regulators BAP, kinetin, 2,4-D or picloram at different concentration depending on the requirement of each experiment. The pH of the medium was adjusted to 5.8 ± 0.1 using 1 M NaOH or HCl prior to solidification with phytagel and autoclaved at 121°C for 15 min. All cultures were incubated at a temperature of 25°C under a 16 h photoperiod using white fluorescent lights (3000 lux).

Growth regulators and germination of dried fruits *in vitro*

Fruits or seeds of *P. niruri* were harvested and dried in Petri dishes at room temperature for seven days, washed under running tap water for 2 h and sterilized by immersion in 70% ethanol for 30 min and finally rinsed with three changes of sterile distilled water. Fruits were cultured on solid basal medium (MS) supplemented with 0.0, 0.3, 0.6, 0.9, or 1.2 mg/L BAP or kinetin in test tubes. All cultures were incubated under growth room conditions and the percentage germination was recorded. Also, the number of shoots or roots per seedling was also recorded four weeks after culture.

Plantlet regeneration from nodal cuttings

Eight weeks old seedlings were harvested from the nursery, trimmed of its leaves, washed with "Crystal Clear ™" detergent for 10 min and rinsed under running tap water for 1 h. They were then sterilized by immersion in 70% ethanol for 30 s and rinsed again in three changes of sterilized distilled water. To ensure effective decontamination, the shoots were again immersed in 10% commercial bleach (containing 3.5% sodium hypochlorite) with three drops of Tween-20 for 5 min and finally rinsed with sterile distilled water. The stem segments were cut into single nodal cuttings (1.5 to 3.0 cm) and cultured in test tubes containing 25 ml of MS basal medium amended with 0.0, 1.0, 2.0, 3.0, 4.0 or 5.0 mg/l BAP, kinetin or 2-isopentyl adenine (2iP). The cultures were incubated under growth room conditions as described earlier. The number of nodal cuttings that developed shoots, roots or shoots with roots was recorded eight weeks after culture.

Induction of roots on MS basal medium supplemented with NAA or indole-3-butyric acid (IBA)

Nodal cuttings were sterilized as described above and cultured on a MS basal medium supplemented with 0.0, 1.0, 2.0 or 4.0 mg/L BAP and 0.0, 0.5, 1.0, 1.5 or 2.0 mg/L IBA or NAA. The cultures were incubated under growth room conditions and the number of shoots or roots that developed per explants was recorded four weeks after culture.

Morphogenesis via callus induction

Young leaves excised from eight weeks old plantlets were

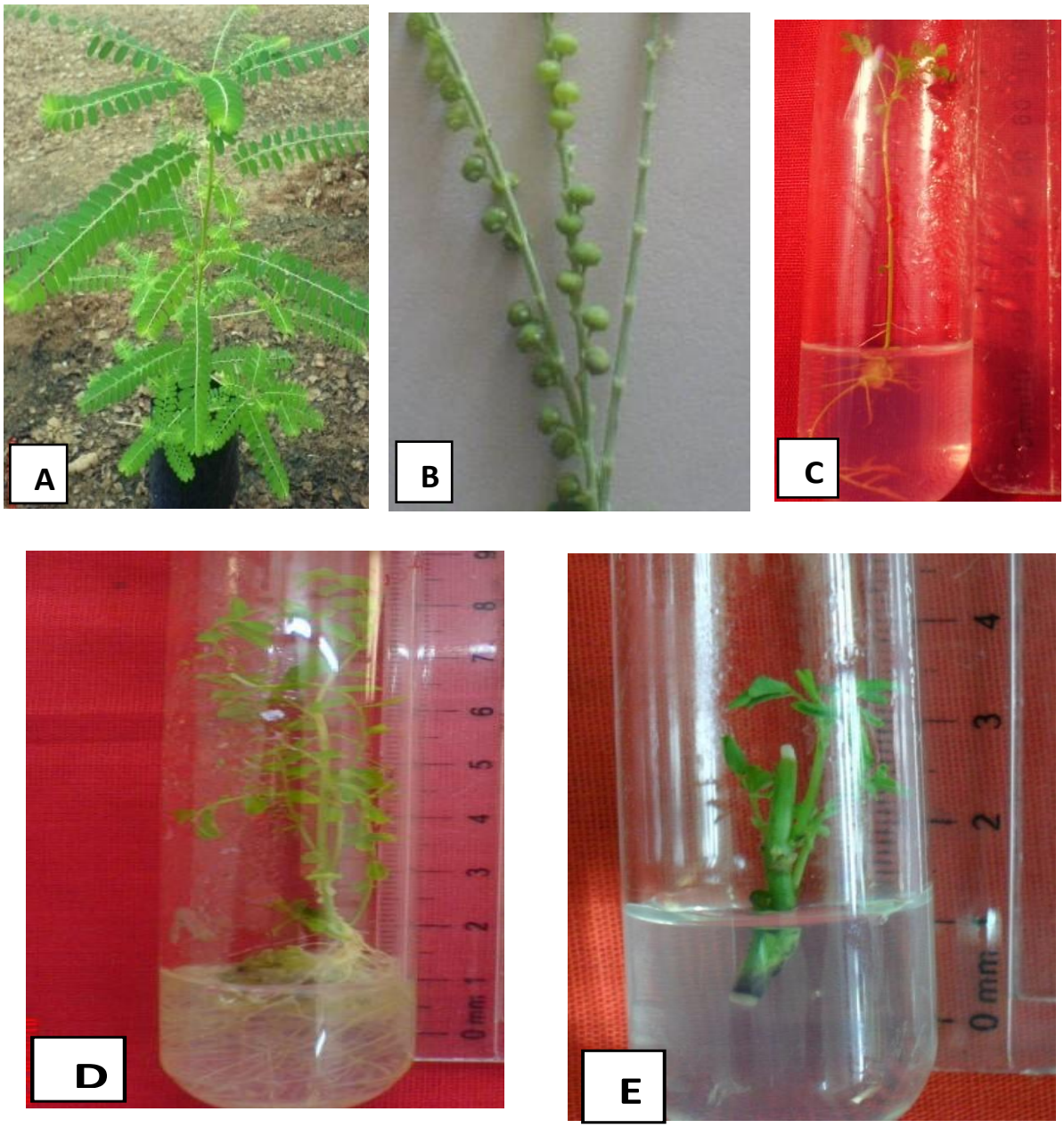

Figure 1. (A) *Phyllanthus niruri* plant growing in a pot. (B) Fruits of *Phyllanthus niruri* on a leaf of rachis. (C) *In vitro* plants regenerated from a fruit cultured in a pot without growth regulators (control). (D) 0.6 MG/l BAP. (E) Nodal cuttings cultured on 2.0 mg/L BAP and 1.0 mg/L IBA developing shoots *in vitro*.

wounded with scalpel blade and cultured on MS medium supplemented with 30 g/L sucrose, 1 ml of 2 µM $CuSO_4$ and vitamins (0.02 g/L thiamine, 0.1 g/L pyridoxine, 0.1 g/L nicotinic acid and 0.04 g/L glycine) and varying concentrations (0.0 to 1.0 mg/L) of 2,4-D or picloram with the adaxial surface in contact with the medium in Petri dishes. All cultures were incubated in darkness and the number of leaves that formed callus was recorded after 21 days. Also, the colour and morphology of the callus was recorded. The calli were transferred into a fresh MS basal medium supplemented with 0.1 mg/L BAP and incubated in the growth room at a temperature of 25°C and 16 h photoperiod (3000 lux). Each Petri dish with three leaf lobes constituted an experimental unit and was replicated nine (9) times. Calli were examined for the presence of shoots, roots or somatic embryos 42 days after culture.

Callus growth and weight

Proliferating calli without shoots or leaves were sub cultured

Table 1. Effect of different concentration of BAP and kinetin on seed germination of three *P. niruri* accessions after 4 weeks of culture *in vitro.*

Growth regulator	Concentration (mg/L)	Germination (%)		
		Kwabenya	Kasoa	Aburi
BAP	0.0	0.0[a]	33.3[b]	50.0[cd]
	0.3	33.3[b]	16.7[a]	16.0[a]
	0.6	50.0[cd]	0.0[a]	33.3[b]
	0.9	50.0[cd]	0.0[a]	50.0[cd]
	1.2	50.0[cd]	61.1[d]	50.0[cd]
Kinetin	0.0	77.8[d]	0.0[a]	0.0[a]
	0.3	22.2[ab]	0.0[a]	0.0[a]
	0.6	33.3[bc]	0.0[a]	0.0[a]
	0.9	44.4[c]	0.0[a]	0.0[a]
	1.2	11.1[a]	0.0[a]	22.2[a]

Each treatment was replicated three times with 30 seeds per treatment. Mean values in the same column followed by the same superscripts are not significantly different at (P≥0.05) according to Turkeys' test.

fortnightly onto fresh MS basal medium supplemented with 0.1 mg/L BAP for calli multiplication. The weight of each callus was measured using a weighing balance at each subculture stage. The diameter of the callus was also measured by placing a meter rule across the diameter of the callus below the glass jar.

Post-flask acclimatisation

In vitro plantlets regenerated from nodal cutting, seeds or calli with well developed roots were transferred to soil-cow dung-coconut husk mixture (3:1:1) in 12-cm diameter black polyethylene bags. Each plantlet was watered with a fungicide (Agri-fos 400) and then covered with plastic cups. The pots were kept under the plant barn for three days before the cups were removed. The survival rates of the plantlets were recorded after six weeks.

Experimental design, data collection and statistical analysis

The completely randomized design was used where appropriate. The number of replicates varied from three to five per treatment depending on the availability of explants and the type of experiment. All data were subjected to analysis of variance (ANOVA) and means were separated using Turkey's pair wise comparison. All statistical analysis was performed using Statistical Package for Social Sciences (SPSS) version 16.0 and Microsoft Excel Statistical Software.

RESULTS

Germination of *Phyllanthus* fruits *in vitro*

The presence of BAP and kinetin stimulated the germination of fruits in accessions of *P. niruri* collected from all the three localities (Figures 1C and D). The germination of the fruits was accompanied by callus development at the base of the seedling except seedlings cultured on the control (0.0 mg/L) medium. The number of shoots produced varied depending on the concentration of the growth regulator and the locality from where the accession was collected (Table 1). The optimal BAP concentration for higher germination of accession from Kwabenya was 0.6 mg/L while for Kasoa and Aburi the optimal was 1.2 and 0.9 mg/L, respectively. At these optimal concentrations, accessions from Kwabenya and Kasoa had significantly ($P \leq 0.05$) higher percentage of germination (50.0 and 61.2%, respectively) than the controls while accessions from Aburi showed no significant ($P \leq 0.05$) difference between the optimal concentration and the controls. In contrast, kinetin stimulated fruit germination in only accessions from Kwabenya. Even in these accessions, the percentage germination (11.1 to 44.4%) in the presence of the growth regulator was significantly ($P \leq 0.05$) lower than the controls (77.8%) and continuously declined as the concentration of kinetin increased to 1.2 mg/L.

Majority of the fruits germinated into shoots with or without roots (Figures 1C and D). The mean number of shoots produced in each accession did not follow any particular trend although; germination seems to be influenced by the concentration of the growth regulator (Table 2). With accessions from Kwabenya and Aburi, more shoots (6.4 and 3.3, respectively) were obtained in the presence of BAP than the controls while in Kasoa the controls developed significantly more shoots (4.0) than the treatments. However, on a medium with the highest concentration (1.2 mg/L) of the growth regulator, there was no significant difference between the controls and the treatment. The highest number of shoots (6.4) was produced by accessions from Kwabenya on a medium

Table 2. Mean number of shoots and roots developed per fruit of three *P. niruri* accessions after 4 weeks of culture *in vitro.*

Growth regulator	Concentration (mg/l)	Mean number of shoots and roots ± SE					
		Kwabenya		Kasoa		Aburi	
		Shoot	Root	Shoot	Root	Shoot	Root
BAP	0.0	0.0±0.0^{a}	0.0±0.0^{a}	4.0±0.8^{c}	6.9±3.9^{d}	2.4±0.6^{b}	1.7±1.0^{b}
	0.3	6.4±1.5^{e}	8.2±4.2^{e}	2.5±0.5^{b}	1.7±1.6^{b}	3.3±0.3^{b}	1.9±1.5^{b}
	0.6	4.4±0.9^{c}	3.9±2.9^{c}	0.0±0.0^{a}	0.0±0.0^{a}	3.1±0.3^{b}	1.5±2.9^{b}
	0.9	2.5±0.4^{b}	1.3±0.5^{b}	0.0±0.0^{a}	0.0±0.0^{a}	2.7±0.4^{b}	1.3±0.2^{b}
	1.2	3.7±0.6^{c}	1.3±0.3^{b}	3.6±0.6^{c}	1.2±0.2^{b}	5.6±0.5^{de}	1.2±0.1^{b}
Kin	0.0	3.3±0.3^{b}	11.2±5.6^{d}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	0.3	6.1±0.9^{d}	5.0±4.5^{c}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	0.6	4.4±0.5^{bc}	2.8±2.3^{b}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	0.9	3.6±0.7^{b}	2.0±0.6^{b}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
	1.2	5.0±0.8^{c}	0.7±0.3^{a}	0.0±0.0^{a}	0.0±0.0^{a}	7.4±0.7^{e}	0.5±0.3^{a}

Each treatment was replicated three times with 30 seeds per treatment. Mean values in the same column followed by the same superscripts are not significantly different at (P≥0.05) according to Turkeys test.

supplemented with 0.3 mg/L BAP. Fruits cultured on BAP amended medium developed more roots than kinetin cultured ones (Table 1). In both BAP and kinetin amended medium, the mean number of roots developed decreased with increasing concentration of the growth regulators. However, fruits of accessions from Kasoa and Aburi failed to developed roots independent of the growth regulator.

Induction of adventitious shoots from nodal cuttings

Nodal cuttings cultured on MS medium supplemented with BAP, kinetin or 2iP developed either single or multiple adventitious shoots with no roots (Figure 1E). Only those cultured on MS medium without growth regulators developed roots. Independent of the growth regulator used, the number of shoots produced decreased as the concentration of the growth regulators increased (Table 3). With the exception of accession from Kwabenya cultured on 2iP, the addition of 1.0 mg/L of the BAP or kinetin significantly ($P \leq 0.05$) produced more shoots than the controls in all the accessions from the three localities. Accessions from Kwabenya and Aburi produced the highest mean number of shoots (9.0 and 8.3 shoots, respectively) per nodal cutting on a medium supplemented with 1.0 mg/L BAP. On a medium supplemented with 5.0 mg/L BAP, most of the shoots developed showed stunted growth indicating phytotoxic effect of the growth regulator. Of the three cytokinins, BAP amended medium produced more shoots followed by kinetin and 2iP in that order. Pearsons' correlation analysis showed a low non significant positive relation (r = 0.5) between the concentration of the cytokinins and shoot production.

NAA and IBA on shoot and root development

A combination of BAP with NAA or IBA in the culture medium resulted in shoot development in all the accessions but again these shoots failed to develop roots while the controls did (Table 4). Similarly, the number of shoots produced decreased with increasing concentration of the growth regulators in the culture medium. The optimal concentration of BAP and the auxins (NAA or IBA) were 1.0 and 0.5 mg/L, respectively; accessions from Kasoa developed the highest mean number of shoots, 6.0 and 4.7, respectively in NAA and IBA amended medium. Nodal cuttings from Aburi accessions cultured on BAP with NAA amended medium showed the poorest shoot development with the mean number of shoots ranging from 0.2 to 1.3 at the optimal concentration of the growth regulator. Generally, the presence of IBA in the culture medium stimulated more shoot development from the nodal cuttings than NAA.

Callus induction and morphogenetic response

Callus from young leaf lobes were readily obtained within three weeks of culture on an MS medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) (Figure 2A). The percentage callus developed increased with increasing concentration of the auxin in the culture medium (Table 5). On a medium amended with 2,4-D, all accessions (100%) from Kwabenya developed calli while in Kasoa and Aburi, 88.9 and 95.8% of the leaf lobes, respectively developed calli (Table 5). However, calli development from leaf lobes cultured on picloram amended medium was erratic and occurred only at concentration of 0.6 mg/l, thus the data was not

Table 3. Effect of different concentrations of BAP, Kinetin or 2iP on multiple shoot induction and root development from nodal cuttings of three *P. niruri* accessions after 4 weeks of culture.

Growth regulator	Concentration (mg/L)	Mean number of shoots and root ± SE					
		Kwabenya		Kasoa		Aburi	
		Shoot	Root	Shoot	Root	Shoot	Root
BAP	0	6.8±0.5^{c}	3.0±0.6^{c}	3.2±1.6^{b}	1.4±0.3^{b}	7.1±1.1^{c}	0.9±0.1^{b}
	1	9.0±2.1^{e}	0.0±0.0^{a}	1.2±1.0^{a}	0.0±0.0^{a}	8.3±3.2^{de}	0.0±0.0^{a}
	2	6.5±1.3^{c}	0.0±0.0^{a}	1.0±0.8^{a}	0.0±0.0^{a}	7.8±1.3^{cd}	0.0±0.0^{a}
	3	4.7±1.3^{b}	0.0±0.0^{a}	0.8±0.6^{a}	0.0±0.0^{a}	6.2±0.8^{c}	0.0±0.0^{a}
	4	4.3±1.2^{b}	0.0±0.0^{a}	0.7±0.6^{a}	0.0±0.0^{a}	4.0±2.1^{b}	0.0±0.0^{a}
	5	3.7±0.9^{b}	0.0±0.0^{a}	0.6±0.4^{a}	0.0±0.0^{a}	3.4±1.8^{b}	0.0±0.0^{a}
Kin	0	2.0±0.4^{b}	3.4±0.4^{b}	1.8±1.0^{ab}	3.4±0.4^{b}	6.2±0.1^{e}	5.4±1.1^{c}
	1	2.7±0.9^{bc}	0.0±0.0^{a}	0.8±0.5^{a}	0.0±0.0^{a}	4.3±0.0^{d}	0.0±0.0^{a}
	2	1.7±0.9^{ab}	0.0±0.0^{a}	0.7±0.4^{a}	0.0±0.0^{a}	3.7±0.6^{cd}	0.0±0.0^{a}
	3	1.2±0.5^{a}	0.0±0.0^{a}	0.5±0.3^{a}	0.0±0.0^{a}	3.3±0.4^{c}	0.0±0.0^{a}
	4	1.0±0.6^{a}	0.0±0.0^{a}	0.3±0.1^{a}	0.0±0.0^{a}	2.5±0.7^{bc}	0.0±0.0^{a}
	5	0.3±0.1^{a}	0.0±0.0^{a}	0.2±0.1^{a}	0.0±0.0^{a}	2.3±0.2^{bc}	0.0±0.0^{a}
2iP	0	1.8±0.9^{a}	2.6±0.5^{b}	0.7±0.6^{a}	4.2±0.6^{d}	0.9±0.5^{a}	3.6±0.6^{cd}
	1	0.9±0.4^{a}	0.0±0.0^{a}	1.3±0.7^{a}	0.0±0.0^{a}	2.2±0.5^{a}	0.0±0.0^{a}
	2	0.8±0.4^{a}	0.0±0.0^{a}	1.0±0.5^{a}	0.0±0.0^{a}	1.6±0.8^{a}	0.0±0.0^{a}
	3	0.7±0.4^{a}	0.0±0.0^{a}	0.8±0.7^{a}	0.0±0.0^{a}	1.1±0.5^{a}	0.0±0.0^{a}
	4	0.6±0.5^{a}	0.0±0.0^{a}	0.7±0.6^{a}	0.0±0.0^{a}	1.0±0.5^{a}	0.0±0.0^{a}
	5	0.3±0.2^{a}	0.0±0.0^{a}	0.4±0.2^{a}	0.0±0.0^{a}	0.4±0.3^{a}	0.0±0.0^{a}

Fifteen nodal cuttings were cultured per treatment. Mean values in the same column followed by the same superscripts are not significantly different at (P≥0.05) according to Turkeys' test.

Table 4. Effects of BAP and IBA or NAA on shoot and root development in nodal cutting explants *P. niruri* accessions after 4 weeks of culture.

Concentration of combined growth regulator (mg/L)		Mean number of shoots and roots ± SE					
BAP	IBA	Kwabenya		Kasoa		Aburi	
		Shoot	Root	Shoot	Root	Shoot	Root
0	0.0	4.8±1.2^{ab}	4.7±1.5^{b}	7.8±0.3^{b}	7.8±1.9^{b}	6.5±0.4^{ab}	4.2±0.4^{b}
1	0.5	5.2±2.9^{ab}	0.0±00^{a}	6.0±0.8^{ab}	0.0±00^{a}	3.3±0.2^{ab}	0.0±00^{a}
2	1.0	2.7±0.0^{ab}	0.0±00^{a}	5.5±0.9^{ab}	0.0±00^{a}	2.0±0.2^{a}	0.0±00^{a}
3	1.5	2.7±1.5^{ab}	0.0±00^{a}	2.7±0.2^{ab}	0.0±00^{a}	1.7±0.4^{a}	0.0±00^{a}
4	2.0	1.3±0.7^{a}	0.0±00^{a}	2.3±1.4^{ab}	0.0±00^{a}	1.0±0.6^{a}	0.0±00^{a}
BAP	**NAA**						
0	0.0	3.1±1.7^{a}	6.0±1.0^{c}	7.2±4.1^{a}	2.7±0.9^{b}	1.3±0.7^{a}	3.2±0.4^{b}
1	0.5	3.2±1.7^{a}	0.0±00^{a}	4.7±2.4^{a}	0.0±00^{a}	1.3±0.6^{a}	0.0±00^{a}
2	1.0	2.7±1.4^{a}	0.0±00^{a}	3.8±2.0^{a}	0.0±00^{a}	0.7±0.3^{a}	0.0±00^{a}
3	1.5	2.0±1.2^{a}	0.0±00^{a}	3.6±1.8^{a}	0.0±00^{a}	0.4±0.2^{a}	0.0±00^{a}
4	2.0	0.8±0.7^{a}	0.0±00^{a}	2.6±1.3^{a}	0.0±00^{a}	0.2±0.1^{a}	0.0±00^{a}

Fifteen (15) nodal cuttings were cultured per treatment. Mean values in the same column followed by the same superscripts are not significantly different at (P≥0.05) according to Turkeys' test.

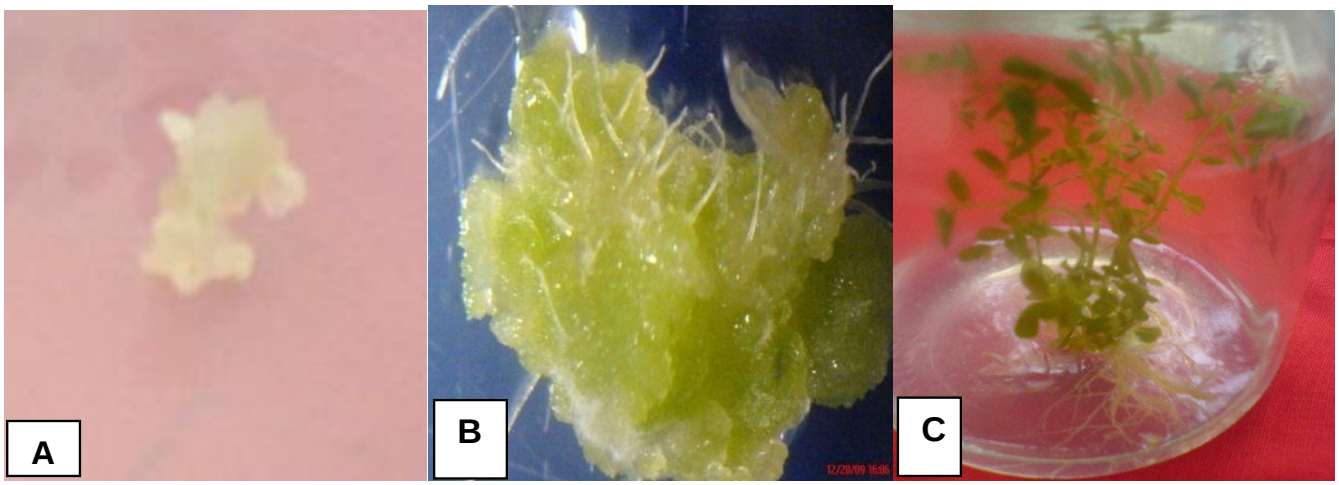

Figure 2. Morphogenesis in *Phyllanthus niruri.* (A) Calli induced from young leaf lobes on 0.2 mg/l 2,4-D (B) callus growth after six weeks of culture and (C) plantlet development after transfer to 0.1 mg/l 2,4-D.

Table 5. Effect of 2,4-D on percentage of leaf lobes of *P. Niruri* that developed callus on MS basal medium.

Growth regulator	Concentration (mg/L)	Callus formation (%)		
		Kwabenya	Kasoa	Aburi
2,4-D	0.0	0.0^{a}	0.0^{a}	0.0^{a}
	0.2	81.5^{b}	70.4^{b}	77.8^{b}
	0.4	81.5^{b}	74.1^{bc}	81.5^{b}
	0.6	81.2^{b}	74.1^{bc}	92.6^{bc}
	0.8	92.6^{bc}	81.5^{cd}	86.7^{b}
	1.0	100^{c}	88.9^{cd}	95.8^{bc}

Mean values in the same column followed by the same superscripts are not significantly different at (P≥0.05).

Table 6. Percentage of shoots or roots regenerated from low (0.2-1.0 mg/l) concentrations of 2,4-D-induced callus of three *P. niruri* accessions on MS medium supplemented with 0.1 mg/l BAP.

2,4-D conc (mg/L)	Percentage calli with shoots or roots					
	Kwabenya		Kasoa		Aburi	
	Shoot	Root	Shoot	Root	Shoot	Root
0.2	0.0^{a}	48.1^{a}	0.0^{a}	70.4^{c}	0.0^{a}	48.1^{a}
0.4	33.3^{b}	66.7bc	33.3^{b}	74.1^{cd}	22.2^{b}	51.9^{ab}
0.6	55.6^{c}	88.9d	25.9^{b}	88.8^{d}	0.0^{a}	85.2^{d}
0.8	33.3^{b}	77.8cd	37.1^{b}	72.1^{c}	0.0^{a}	48.1^{a}
1.0	22.2^{b}	48.1^{a}	0.0^{a}	63.0^{bc}	0.0^{a}	51.9^{ab}

Each treatment was replicated nine times with 27 explants per treatment. Mean values in the same column followed by the same superscripts are not significantly different at (P≥0.05) according to Turkeys' test.

presented.

The transfer of 2,4-D induced calli to fresh MS basal medium supplemented with 0.1 mg/L BAP led to morphogenic response indicated by continuous growth (Figure 2B), increased weight as well as shoot and root development (Tables 6 and 7) The optimal concentration for shoot induction varied. For accessions from Kwabenya and Kasoa the optimal concentration was 0.6 and 0.8 mg/l respectively while accessions from Aburi had erratic shoot development. The weight and diameter

Table 7. Weight and diametre of 2,4-D -induced callus on MS medium supplemented with 0.1 mg/l BAP of three *P. niruri* accessions for three subculturing cycles

Days for subculturing	Conc (mg/l)	Kwabenya		Kasoa		Aburi	
		Weight (g)	Diametre (cm)	Weight (g)	Diametre (cm)	Weight (g)	Diametre (cm)
14	0.2	0.037±0.030^{a}	0.248±0.123^{a}	0.023±0.002^{a}	0.211±0.0118^{a}	0.117±0.078^{a}	0.265±0.115^{ab}
	0.4	0.041±0.018^{a}	0.570±0.151^{abc}	0.052±0.016^{a}	0.57±0.752^{abc}	0.084±0.017^{a}	0.404±0.135^{abc}
	0.6	0.082±0.003^{a}	0.611±0.091^{bc}	0.150±0.110^{a}	0.670±0.097^{abc}	0.098±0.020^{a}	0.685±0.143^{abc}
	0.8	0.093±0.003^{a}	0.704±0.096^{bc}	0.167±0.034^{a}	0.852±0.109^{abc}	0.092±0.035^{a}	0.752±0.153^{abc}
	1.0	0.093±0.044^{a}	0.803±0.115^{bc}	0.169±0.043^{a}	0.963±0.131^{c}	0.150±0.036^{a}	0.922±0.084^{c}
28	0.2	0.041±0.012^{a}	0.267±0.131^{a}	0.028±0.052^{a}	0.244±0.168^{a}	0.096±0.03^{a}	0.282±0.139^{a}
	0.4	0.043±0.012^{a}	0.664±0.149^{bc}	0.0741±0.027^{a}	0.598±0.101^{abc}	0.123±0.049^{a}	0.496±0.140^{ab}
	0.6	0.093±0.026^{a}	0.674±0.129^{bc}	0.156±0.044^{a}	0.692±0.152^{bc}	0.303±0.213^{a}	0.748±0.197^{bcd}
	0.8	0.114±0.023^{a}	0.735±0.066^{bc}	0.169±0.031^{a}	0.906±0.094^{cd}	0.354±0.163^{ab}	0.800±0.091^{cd}
	1.0	0.120±0.110^{a}	0.819±0.159^{cd}	0.170±0.047^{a}	1.028±0.080^{d}	0.778±0.207^{b}	1.207±0.086^{d}
42	0.2	0.064±0.036^{a}	0.356±0.152^{a}	0.076±0.045^{a}	0.285±0.196^{a}	0.074±0.041^{a}	0.367±0.161^{a}
	0.4	0.068±0.031^{a}	0.670±0.097^{ab}	0.094±0.049^{a}	0.614±0.137^{ab}	0.139±0.050^{a}	0.544±0.195^{ab}
	0.6	0.118±0.056^{a}	0.640±0.146^{ab}	0.134±0.060^{a}	0.717±0.174^{bc}	0.390±0.100^{a}	0.785±0.252^{b}
	0.8	0.159±0.047^{a}	0.756±0.166^{bc}	0.188±0.068^{a}	0.937±0.094^{bc}	0.409±0.101^{a}	0.818±0.125^{bc}
	1.0	0.236±0.090^{a}	0.870±0.182^{bc}	0.281±0.127^{a}	1.041±0.067^{c}	0.792±0.094^{b}	1.037±0.120^{c}

Each treatment was replicated nine times. Mean values in the same column followed by the same superscripts are not significantly different at (P≥0.05) according to Tukeys test.

of the calli increased proportionally as the concentration of the 2,4-D in the culture medium increased in all the accessions. At each subculture cycle, 1.0 mg/L 2,4-D-induced calli significantly increased the weight of the calli two- or three-fold higher than 0.2 mg/L in all the accessions. For instance, with accessions from Kwabenya, 1.0 mg/L 2,4-D-induced calli increased calli weight to 0.093 g, which was three times (0.037 g) that produced by 0.2 mg/L 2,4-D (Table 7). Similar observation was seen in all the accessions cultured on 2,4-D amended medium.

For morphogenic response, the creamy calli initially turned green indicating shoot or root primordial which later developed into either shoot or roots (Figures 2B and C). Although, the percentage leaf lobes that formed calli was high, most of them did not develop shoots. Only 22.2 to 55.6% of calli of accessions of Kwabenya developed shoots while 25 to 37.1% from Kasoa had shoots; with accessions from Aburi, only 22.2% of calli induced on 0.4 mg/L 2,4-D developed shoots (Table 6). Contrary to shoot development, the percentage calli with roots was comparatively higher than shoot formation. 0.6 mg/L 2,4-D induced calli developed the highest percentage of roots; 88.8% of calli from Kwabenya and Kasoa accessions developed roots while 85.2% of Aburi accessions developed roots at this concentration. Morphogenically, shoots developed by calli were hyperhydric and were more brittle in texture when touched.

Post flask plant development

There was successful plantlet establishment four weeks after transfer of seed or nodal cutting regenerated plantlets to sandy soil, soil-cow dung-coconut husk mixture while none of the plantlets from calli survived post flask weaning and hardening (Figure 3). Plantlets acclimatized in soil or cow dung-coconut husk mixture had higher survival rates than those hardened in sandy soil. The survival rates ranged from 93 to 99% for plantlets regenerated from nodal cuttings and 80 to 91% for those from seed. There was significant effect of both the accession and substrate on post flask survival of plantlets generated from seeds. In contrast, only the substrate had significant effect on survival of plantlets generated from the nodal cuttings.

DISCUSSION

The continuous exploitation of *P. niruri,* an anti-malarial herbaceous plant from the wild limits its utilization in herbal medicine. It may also lead to genetic extinction and/or erosion due to over exploitation. Thus, we aimed at developing *ex vitro* and *in vitro* culture procedures for alternative propagation of *P. niruri* as an initial effort towards its domestication. Most cytokinins especially, BAP and kinetin have been reported to promote seed

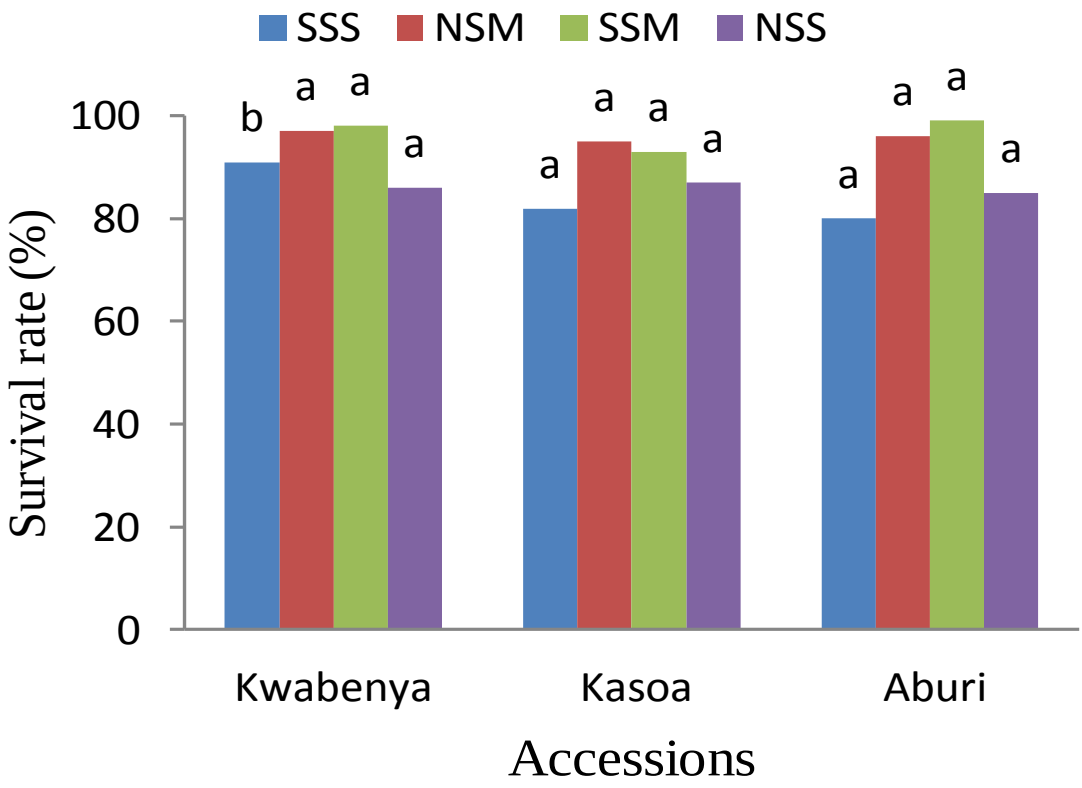

Figure 3. Effect of substratum on post flask hardening and weaning of *in vitro* regenerated plantlets of *Phyllanthus niruri*.

germination in numerous plant species (Ketring and Morgan, 1971). However, in the present study, while the presence of BAP led to comparatively higher fruit germination, kinetin significantly reduced germination as compared to the controls. The low germination response of the fruits may be attributed to fruit wall imposed or other endogenously dormancy inducing factors in the fruits. Fruits of *P. niruri* like other members of Euphorbiaceae have thick and hard seed coat which hindered the germination of the embryos *in vitro.* Unlike chemical induced-dormancy, fruit wall-induced dormancy is often released by scarification or stratification treatments and not growth regulators, hence, the low percentage germination.

To avoid the limitation of the fruit wall imposed dormancy, we cultured nodal cuttings on an MS medium amended with BAP, kinetin or 2iP to induce shoot regeneration. Lower concentrations of the cytokinins resulted in multiple shoot production while higher concentrations (5.0 mg/L) adversely decreased shoot development. Also, none of the shoots produced developed roots independent of the concentration of the cytokinin used, an observation similar to that reported on other medicinal plant species by Kalidass and Mohan (2009) and Nasib et al. (2008). de Klerk et al. (1995) also reported similar antagonistic effects of higher concentrations of cytokinin on root initiation. According to Thimann (2008), higher concentrations of cytokinin have adverse effect on morphogenesis in plants.

Interaction between cytokinin and auxin play significant regulatory roles in shoot and root regeneration *in vitro* (George et al., 2008). However, the culture of nodal cuttings on MS medium amended with a combination of BAP with either IBA or NAA at low concentrations also failed to induce shoots with roots. Sen et al. (2009) were also unable to initiate root in nodal cuttings of *P. amarus* using a combination of NAA and IAA at 0.5 and 0.8 mg/L, respectively. The present results coupled with observations by Sen et al. (2009) suggest that other factors other than auxins may be influencing root induction in *P. niruri* which we were not able to investigate in this study.

Indirect plant regeneration via callus induction occurred by initially culturing leaf lobe explants on MS medium amended with 2,4-D or picloram followed by the transfer to 0.1 mg/L BAP. While callus development on 2,4-D amended medium was readily achieved, callus development on Picloram amended medium was erratic. However, none of the calli developed into somatic embryos when transferred to the 0.1 mg/L BAP amended

medium indicating that they were embryogenically incompetent. The non- embryogenic calli grew in size with green spots indicating shoot or root primordium which eventually developed into shoots or roots after a series of subculture. *In vitro* calli and subsequent shoot development on an MS medium with 2,4-D and NAA had been reported in *P. amarus* and other members of the Euphorbiaceae family (Chitra et al., 2009). Several factors may account for this morphogenetic response leading to shoot and root development. It is likely that exogenous addition of 2,4-D or picloram in the induction medium signaled the genes in the somatic cell cycle to move from G_0 to G_1 phase, thereby making it competent for calli induction or shoot regeneration (George et al., 2008). Secondly, the transfer of the calli from 2,4-D (auxin) amended medium to a BAP (cytokinin) based medium provided the needed cytokinin-auxin ratio requirement leading to both shoot and root regeneration.

The morphogenetic potential, however, favoured roots development than shoots which may be explained by the initial long exposure of the calli to the synthetic auxin 2,4-D. 2,4-Dichlorophenoxy acetic acid, a synthetic auxin is known to inhibit shoot production through ethylene biosynthesis (Kantharajah and Golegaonkar, 2004). In spite of the low shoots production, the technique may provide an alternative pathway for plant regeneration in *P. niruri*. However, the plants were hyperhydric and therefore could not survive post flask weaning and hardening.

Besides shoot regeneration, calli formation may play a significant role in the extraction of secondary metabolites. The two or three fold increase in calli weight at each subculture cycle by all the accessions may be beneficial in the pharmaceutical industries as it provides more raw materials for extraction of anti-plasmodial compounds from the plant. Cimanga et al. (2004) reported that ethanol extracts from *P. niruri* calli cultured on 2,4-D amended medium had comparatively more anti-plasmodial activity than whole plant extracts.

The transition from *in vitro* to variable *ex vitro* environment determines the success of micro-propagation technique. Plantlets regenerated from nodal cuttings and seeds had higher survival rates after six weeks of acclimatisation on soil-cow dung-coconut husk mixture than sandy soil. The higher survival rate may be attributed to provision of growth nutrients to the plantlets as well as high porosity of the substrate which enhanced root penetration for absorption of minerals and water. The sandy soil lacked these conditions, thus, the lower percentage survival *ex vitro*. Although, plantlets regenerated from calli had well developed roots, they did not survive the transitory acclimatization phase and therefore died due to hyperhydricity of the shoots. We have shown in this study that BAP in the culture medium enhanced comparatively higher fruit germination. Additionally, nodal culture and callusgenesis was successfully used to regenerate shoots of *P. niruri in vitro* shoots. These *in vitro* techniques can be used to complement effort to domesticate the plant to provide alternative option for treatment of malaria in tropical Africa as both the *Plasmodium* parasite and its female *Anopheles* mosquito vector that has developed resistance to the conventional medicines.

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