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In vitro and ex vitro germination *of Phyllanthus niruri* L., an anti-plasmodial plant

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ABSTRACT

Three accessions of *Phyllanthus niruri* L., from three different localities were assessed for their fruit or seed germination *in vitro* and *ex vitro*. Dried fruits (undehisced seeds) of *P. niruri* accessions collected from Greater Accra (Kwabenya), Central (Kasoa) and Eastern (Aburi) regions of Ghana did not germinate when nursed both *in vitro* and *ex vitro*. However, seeds from 3, 5 or 7 days dehisced fruits germinated with 7 days dehisced seeds having the highest percentage (68.8%) germination when nursed (*ex vitro*) in the same soil substrate suggesting that there was fruit wall imposed dormancy. To improve percentage germination, dehisced seeds were cultured on Murashige and skoog (1962) (MS) medium supplemented with 0-1.2 mg/l BAP or kinetin. At these treatments, dehisced seeds cultured on MS medium supplemented with 1.2 mg/l BAP had the highest percentage (61.1%) of germination with poor germination of seeds occurring in MS medium supplemented with kinetin. Data were also taken on root and shoot proliferation as well.

Introduction

In recent times, the search for alternative treatment for malaria from medicinal plant species has been of major concern to World Health Organisation (WHO) and governments all over the world as a result of resistance to drugs by the *Plasmodium* parasite to the conventional drugs. *Phyllanthus niruri* L. (Euphorbiaceae) is an annual herbaceous plant widespread in temperate and tropical climates (Iizuka *et al*. 2006) and it has been found to be useful for treatment of malaria. It is native to the Americas (van Holthoon, 1999) and grows in West Coast of Africa (Burkill, 1985). *Phyllanthus niruri* can grow on marginal soils, roadsides, and rock crevices. The plant also does well in soils with high sand component on raised beds in areas with high water table and the application of organic fertilizer can improve the growth of the plant.

Phyllanthus niruri has several advantages. It has short seed to seed cycle of 2-4 weeks and can be harvested 8-10 weeks after planting (Http://www.hort.purdue.edu/newcrop/CropFactSheets/-*Phyllanthus*.html, 2010) indicating a very short propagation cycle compared to other members of the Euphorbiaceae family such as, *Manihot esculenta* and *Havea brasiliansis* which have a life cycle ranging from 9-18 months. Such a short propagation cycle augurs well for genetic improvement of the crop via either conventional or biotechnological techniques.

The collection of medicinal plants from the wild may lead to unsustainable utilisation of their genetic resources and possible genetic extinction. The problem may be aggravated by the high demand of the plant to combat the prevalence of malaria, particularly in Africa. Secondly, collection of these plants from the wild may also result in the destruction of the ecological habitat that serves as a niche for other organisms. Natesh (2000) reported that less than 10% of the medicinal plants utilised in India are collected from the wild in a destructive and unsustainable manner.

In vitro culture has proved useful for propagation of several plant species which are difficult to grow using conventional techniques (Fay, 1994). The technique is useful for rapid multiplication, of diseased-free planting materials, conservation and exchange of plant genetic resources including medicinal. For example, *in vitro* culture techniques have been employed for propagation of *Artemisia annua*, an anti-malarial plant for extraction of secondary metabolite artemisinin for the pharmaceutical industries (Graham *et al.* 2010); similar techniques can be applied to *P. niruri*. In spite of the usefulness of the plant, the traditional mode of propagation of the plant by seed is hindered by low seed germination (Unander *et al*. 1995) and subsequent low yield probably caused by dormancy. Thus, alternative mode of propagation via *in vitro* culture is highly recommended to meet the needs of pharmaceutical industries as well as traditional herbalists who usually collect the plants from the wild for preparation of concoction or poultice, making it labour intensive and hence the need for this work.

Materials and methods

Germination of fruits *ex vitro*

Fruits (undehisced seeds) of *Phyllanthus niruri* collected from plants growing in the three localities Kwabenya (Greater Accra Region), Aburi (Eastern Region) and Kasoa (Central Region) were harvested and put into petri dishes and covered. The fruits were dried at room temperature for 3, 5 and 7 days for adequate drying. After the period of drying, the fruits were nursed in the soil-cow dung-coconut husk mixture as described above. After 4 weeks, the number of fruits that germinated from each accession was recorded. The Randomised Complete Block Design (RCBD) was used with 4 replicates and fourteen fruits per replicate in each experimental unit.

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Days to dehiscing and seed germination under *ex vitro* conditions

Fruits of *Phyllanthus niruri* collected from plants growing in the three localities Kwabenya (Greater Accra Region), Aburi (Eastern Region) and Kasoa (Central Region) were harvested and put into separate labelled Petri dishes and covered. The fruits were dried at room temperature for 3, 5 and 7 days to allow for dehiscing. After the period of dehiscing, the seeds were collected and nursed in the soil-cow dung-coconut husk mixture as described. After 4 weeks of seedling establishment, the percentage germination of each accession was recorded. Each experimental treatment was replicated 4 times using the randomised Complete Block Design (RCBD). There were fourteen seeds per replicate for each experimental unit.

Growth regulators and germination of dried fruits *in vitro*

Fruits (undehisced seeds) of *Phyllanthus niruri* collected from plants growing in the three localities Kwabenya (Greater Accra Region), Aburi (Eastern Region) and Kasoa (Central Region) were harvested and put into Petri dishes and covered. The fruits were dried at room temperature for 7 days to allow for adequate drying. After the period of drying, the fruits (undehisced seeds) were washed under running tap water for 2 hours and then sterilised by immersing in 70% ethanol for 30 minutes under the laminar flow hood and thereafter rinsed with three changes of sterile distilled water. The sterilised fruits were inoculated in test tubes containing 25 ml of Murashige and Skoog (1962) (MS) basal medium supplemented with 30 g/l sucrose, 0.1 g/l myoinositol, 1 ml of 2µM $CuSO_4$, vitamins (0.02 g/l thiamine, 0.1 g/l pyridoxine, 0.1g/l nicotinic acid and 0.04 g/l glycine) and varying concentrations (0.0-1.2 mg/l) of 6-benzyl aminopurine (BAP) or kinetin. The pH of the medium was adjusted to 5.8 ± 0.1 using 1M NaOH or HCl prior to addition of the phytagel and autoclaving at 121ºC for 15 minutes at 15 psi. The cultures were kept in a growth room at a temperature of 25ºC under a 16/8-h (light/dark) photoperiod with light provided by white fluorescent tubes (T 5 fluorescent fitting, UK) at an intensity of 3000 lux. After 4 weeks of culture the number of shoots, roots and percentage germination were recorded. Each experimental treatment was replicated three times using completely randomised design (CRD). There were ten fruits (undehisced seeds) per replicate for each experimental unit.

***In vitro* germination of *Phyllanthus niruri* seeds**

Seeds (dehisced seeds) of *Phyllanthus niruri* collected from Kwabenya, Aburi and Kasoa were washed under running tap water for 1 hour. The seeds were then sterilised by immersing in 70% ethanol for 20 minutes under the laminar flow hood, rinsed with three changes of sterile distilled water (SDW) and inoculated in Murashige and Skoog (1962) (MS) medium supplemented with varying concentrations (0.0-1.2 mg/l) of 6-benzyl aminopurine (BAP) or kinetin. The pH of the medium was adjusted to 5.8 ± 0.1 and autoclaved at 121ºC for 15 minutes at 15 psi. The cultures were kept in a growth room at a temperature of 25ºC under a 16/8-h (light/dark) photoperiod with light provided by white fluorescent tubes (T 5 fluorescent fitting, UK) at an intensity of 3000 lux. After 4 weeks of culture, the number of seeds (dehisced seeds) that germinated was recorded. Also, the number of multiple shoots or roots per germinated seed was recorded. Each experimental treatment was replicated three times using Completely Randomised Design (CRD). There were ten seeds per replicate.

Results

Effects of days to dehiscing on seed germination

Dried undehisced fruits (seeds with intact fruit coat) of *Phyllanthus niruri* nursed in trays for seven days did not germinate in spite of the repeated experiments conducted. In contrast, dehisced seeds (seeds without fruit coat) nursed under similar conditions germinated (Fig.1). The percentage germination, however, varied depending on days to dehiscing and the locality from which the fruits were collected (Table 1).

With the exception of seeds obtained after 3 days to dehiscing from Kwabenya, all the other accessions had more than 50% germination. The highest percentage germination (68.8%) was obtained from seeds that dehisced after 7 days from Kwabenya. Of the three localities, accessions from Aburi had the lowest percentage germination after 7 days to dehiscing. A factorial analysis between days to dehiscing and accessions did not show any significant interaction. Comparatively, 7- day - dehisced seeds produced the highest percentage germination independent of the locality from which the accession was collected after 28 days of sowing.

The number of shoots developed from the seeds also followed the same trend. The number of shoots that developed per seed increased as the days to dehiscing increased (Table 2). There was significant interaction between days to dehiscing for the mean number of shoots developed.

Fig.1. Seedlings of *P. niruri* collected from (A) Kasoa (B) Kwabenya and (C) Aburi growing in soil- cow dung- coconut husk mixture 14 days after sowing.

Effects of BAP or kinetin on seed germination and seedling development *in vitro*

The presence of BAP stimulated germination of seeds in most of the accessions of *P. niruri* collected from the three localities (Table 3). However, effect of the growth regulator was observed when the BAP concentration was 1.2 mg/l. At this optimal concentration, germination in all the accessions was comparatively higher than the controls but the difference was not significant ($P \geq 0.05$). However, in accession from Kwabenya where there was no germination at the controls, there was significant ($P \geq 0.05$) difference between controls and treatments. The accession from Kasoa had the highest percentage (61.1%) germination when cultured on medium supplemented with 1.2 mg/l BAP.

In contrast, germination in kinetin supplemented medium did not follow any particular trend. With the exception of seeds collected from Kwabenya, shoot development was poor in the remaining accessions. Even with the accession from Kwabenya, the presence of kinetin did not enhance germination as the controls had the highest (77.8%) germination, suggesting that the concentration of kinetin used was either below the threshold needed or may have had adverse effect on shoot development.

The germinated seeds developed into plantlets (Fig.2). However, the mean number of shoots and roots developed per seedling varied (Table 4). With the exception of accessions from Kasoa, the mean number of shoots developed decreased as the concentration of BAP increased. The decrease in mean number of shoots did not follow any particular trend, thereby making it difficult to determine the influence of the growth regulator on shoot development. The effect of kinetin on seed germination is similar to that of BAP except that accessions from Kasoa did not germinate for any of the kinetin treatments whereas Aburi germinated at concentration (1.2 mg/l) of kinetin.

Generally, the seeds cultured on an MS medium without growth regulator (BAP or kinetin) did not develop callus at the basal portion but only roots. In contrast, seeds cultured in either BAP or kinetin supplemented medium developed both callus and roots (Fig.2.B and C). The mean number of roots developed followed the same trend as the shoots, decreasing as the concentration of the cytokinins (BAP or kinetin) in the culture medium increased. Root development was also poor in medium supplemented with kinetin, an observation similar to shoot development.

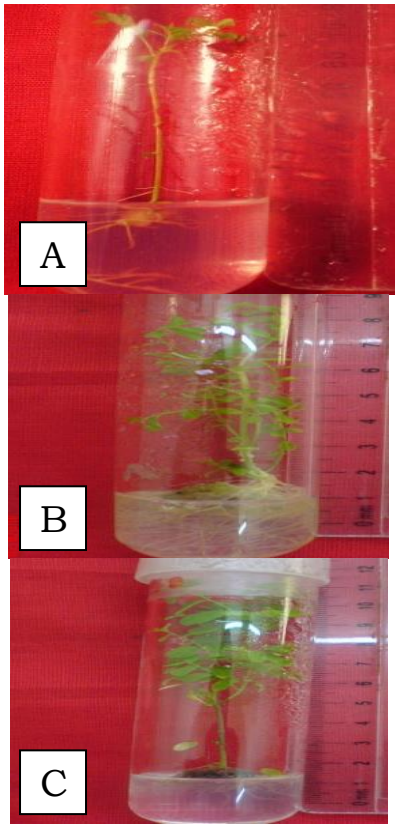

Fig.2. Seedlings of *Phyllanthus niruri* collected from Kasoa on MS without growth regulators (control) (A) 0.3 mg/l BAP (B) and 0.6 mg/l (C)

Discussion

Germination of undehisced fruit and influence of days to dehiscing on seed germination *ex vitro*

Dried undehisced fruits of *Phyllanthus niruri* nursed under *ex vitro* as well as *in vitro* conditions did not germinate. However, seeds obtained from naturally dehisced fruits germinated under *ex vitro* condition. The failure of the fruits to germinate may be attributed to fruit wall imposed dormancy. Most members of the family Euphorbiaceae have hard fruit wall or seed coat which needs to be removed before they can germinate (Nassar, 1979). Thus successful germination of seeds from dehisced fruit may be due to the rapture of the fruit wall.

The percentage germination of seeds dehisced seven days was high compared to three or five days. According to Hoyle *et al* (2008), seeds are considered to be matured when they naturally dehisce. Thus, seven days allowed the fruits or seeds enough time to reach proper maturation stage for germination. However, the percentage germination of seeds *ex vitro* for all the accessions was below 70%. Unander *et al* (1995) have reported poor seed germination in some accessions of *Phyllanthus niruri*. The exact reason for this observation could not be elucidated in this study because the seeds had enough time to dry to maturity. This clearly indicates that if the *P. niruri* is to be exploited for malaria treatment a method that will increase germination and growth significantly should be searched for.

Effects of cytokinins on undehisced fruit and seed germination *in vitro*

Cytokinins may promote seed germination in numerous plant species (Bewley and Black, 1994). Thus, to improve germination in *Phyllanthus niruri*, fruits and seeds were cultured on MS medium supplemented with BAP or kinetin at varying concentrations under *in vitro* conditions. In spite of these treatments, the percentage germination achieved was not more than those grown under *in vivo* conditions, suggesting that hormonal concentrations did not influence germination and that other factors might have triggered germination in the seeds grown under *ex vitro* conditions. It has also been suggested that the degree of dormancy may also vary with climate (Tilki and Guner, 2007) and this may account for variation in germination response of *Phyllanthus niruri* fruits and seeds under both *in vitro* and *ex vitro* conditions from the various localities.

Besides the fruit wall imposed dormancy, many factors including immature embryos and presence of chemical inhibitors (Bewley and Black, 1994; Arditti and Pray, 1969) may cause dormancy. The consistent watering regime under nursery conditions coupled with variation in temperature might have caused the *ex vitro* seeds to soften hence leading to high germination percentage than *in vitro* cultured seeds which were subjected to uniform temperature, humidity and light in the growth room. The poor percentage germination achieved in kinetin treated cultures attests to the fact that other factors probably ecological may be causing poor germination or low viability in *P. niruri.*

The reduction in the number of roots per seedlings that germinated on a medium supplemented with growth regulators could be due to endogenous hormones in the seedlings that had an antagonistic effect since root initiation is reported to be inhibited by exogenous cytokinins (de Klerk *et al.* 1995). The presence of cytokinins resulted in the formation of callus at the base of the germinated seeds. Consequently, the number of roots produced by the seedlings that germinated in the medium supplemented with growth regulators was low compared to seedlings in hormone-free medium (control). The exogenous cytokinin added to the medium might have increased the endogenous auxin higher than required for root initiation.

Conclusion

Nursery establishment using undehisced fruits was difficult and may be due to fruit coat which acted as a mechanical barrier or coat imposed dormancy. However, dehisced seeds germinated under both *in vitro* and *ex vitro* conditions. The highest germination percentage was recorded in 7 day-dehisced seeds of *Phyllanthus niruri* sown in soil, cow dung and coconut husk in a ratio of 3:1:1. However, percentage germination recorded in seeds cultured in MS medium supplemented with 30g/l of

sucrose, 0.1 g/l myoinositol, 1 ml of 2µm $CuSO_4$, vitamins (0.02 g/l thiamine, 0.1 g/l pyridoxine, 0.1 g/l nicotinic acid and 0.04 g/l glycine), 3.5 g/l phytagel and 1.2 mg/l BAP was not as high as that of *ex vitro*. In contrast, none of the fruits germinated in either BAP or kinetin for all accessions.

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Table.1. Effect of days to dehiscing on percentage germination of three accessions of *P. niruri* seeds after 4 weeks of sowing.

Days to dehiscing	Germination (%)		
	Kwabenya	Kasoa	Aburi
3	45.5	55.3	52.8
5	54.3	67.9	50.9
7	68.8	64.3	53.6
Means	56.2	62.5	52.4

Each treatment was replicated four times (A total of 56 seeds were sown)
There was no significant difference at (P≥0.05) according to Tukeys' Test.

Table.2. Effect of days to dehiscing on number of shoots developed by three accessions of *P. niruri* seeds after 4 weeks of sowing

Days to dehiscing	Mean number of shoots ± SE		
	Kwabenya	Kasoa	Aburi
3	4.1 ± 0.5	4.8 ± 0.5	4.5 ± 0.5
5	4.8 ± 0.5	4.5 ± 0.5	4.5 ± 0.5
7	7.0 ± 0.5	6.5 ± 0.5	7.8 ± 0.5
Means	5.3 ± 0.3	5.3 ± 0.3	5.6 ± 0.3

Each treatment was replicated four times (A total of 56 seeds were sown)
There was no significant difference at (P≥0.05) according to Tukeys' Test.

Table.3. Effect of BAP or kinetin at different concentrations on seed germination of three *P. niruri* accessions after 4 weeks of culture *in vitro*

Growth regulators	Conc (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 Tukeys' Test.

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

Growth regulator	Conc (mg/l)	Mean number of shoots and roots ± SE					
		Kwabenya		Kasoa		Aburi	
		Shoot(s)	Root(s)	Shoot(s)	Root(s)	Shoot(s)	Root(s)
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 ($P \geq 0.05$) according to Tukeys test.