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Vegetative and micropropagation potential of *Piper guineense* (Schumach and Thonn)

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Summary: The continuous loss of forest plants due to deforestation, and the increasing demand for *Piper guineense* because of its medicinal and food value, has put a permanent pressure on its population in the wild where it is collected. A method for conservation and mass propagation is therefore required. This research was undertaken to determine the optimal concentration of auxin needed for vegetative propagation and to investigate the potential of *Piper guineense* for micropropagation. The auxin optimization study of vegetative propagation was based on the use of two-nodal stem cuttings treated with five different concentrations of indole-butyric acid (IBA). Growth parameters such as the number of sprouted, rooted and survived cuttings among others were determined. To investigate the potential of *Piper guineense* for micropropagation, nodal explants were subjected to different sterilizing treatments using ethanol, NaOCl, mancozeb, streptomycin and Plant Preservative Mixture (PPM). The effect of plant growth regulators (PGRs) was tested on sterilized nodal explants using full strength Murashige and Skoog (MS) hormone-free media alone as control and MS media supplemented with PGRs (BA, NAA and KIN) at different concentrations and combinations. Significant differences were observed across the treatments for all growth parameters measured. However, 2000 ppm IBA significantly ($p<0.05$) influenced sprouting and rooting of the stem cuttings. *Piper guineense* explants have deep tissue contaminants, which cannot be eradicated by surface sterilization alone except double sterilization using PPM. On control media, neither shoot nor root response was observed while the highest percentage of induced roots was obtained from explants cultured on MS +1 mg/L BA + 0.25 mg/L NAA. Shoot induction was only achieved when BA was used alone and when subcultured on media supplemented with NAA, which generated roots.

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Key words: African black pepper, conservation, deforestation, nodal cuttings, propagation, double sterilization, deep tissue contaminants

Introduction

Forest plants are essential for human survival and well-being because they provide us with food, oxygen, shelter and medicine. They are the source of many commercially traded products, ranging from pharmaceuticals to timber and clothing. Protection of biodiversity is essential in the fight to achieve sustainable development. However, the fight will not be holistic if only trees are considered since other plants also inhabit the forest.

Forests are home to many other species aside trees, plants like herbs, shrubs and vines are also found in the forests. Some of these plants have great economic potentials due to their medicinal or nutritional values but will go extinct due to deforestation, unless conscious efforts are made to propagate them outside the forest. One of them that have profitable economic possibilities is *Piper guineense* (Schumach and Thonn).

Piper guineense is a wild climbing shrub that originates from the tropical regions of Central and West Africa. It is a spice from West Africa and its fruits are called Ashanti pepper, African black pepper (Morufu et al., 2016), Benin pepper and false cubeb. In Nigeria, it is called Iyere in Yoruba and in Igbo, Uziza.

The leaves (***Figure 1***) are suitable as vegetable while the fruits (***Figure 2***) are used as spice in preparation of local dishes in the Igbo communities in Nigeria (Clair & Etukudo, 2000). Both the leaves and seeds are important ingredients in the preparation of special soups for post-partum women (Okoye and Ebeledike, 2013). It helps in stimulating uterine contractions, which brings about the return of uterine muscle to its original shape. It also has antimicrobial properties as reported by Okigbo & Igwe (2007). In some Yoruba communities in Nigeria, the dried fruits are utilized in preparing herbal drugs and the ashes are used to substitute salt in medicinal preparations. It has also been reported that *Piper guineense* has food preservatives and antioxidant properties among other values (Morufu et al., 2016).

The root, stem, fruits and leaves are used in Nigeria to treat different ailments, which include respiratory infections, rheumatism and syphilis (Morufu et al., 2016). According to Nwachukwu et al. (2010), the leaves are aseptic in nature and can be used for treating infertility in females and in males, the treatment of low sperm count. It can be used in the treatment of rheumatic pains as an adjuvant and it also has anti-asthmatic properties.

Though expensive, *Piper guineense* is highly sought-after in many communities in Nigeria. As Nigeria's population increases, so is the demand for this valuable forest species. However, increase in population and urbanization means increase in deforestation and the society depends on the forest for its supply. Okafor (1997) advocated that it is important to document, evaluate and monitor conservation efforts in order to assist farmers to achieve the objectives of conservation.

Unfortunately, this plant is hardly cultivated by the local farmers because its growth requirements are not yet fully understood (Udofia et al., 2016). Udosen & Sam (2015) reported that germination of the species is difficult while micropropagation attempts by Okafor & Uzochukwu (2015) failed due to contamination.

Several studies have been carried out on the pharmacological properties of *Piper guineense* as well as its phytochemical and nutritional status. However, only few of the researches has been on its' propagation. There is a need to determine and report more information regarding the propagation of this very important plant. This is one of the ways to ensure that the plant is conserved and its uses to mankind sustained. Therefore, this study investigated the optimal concentration of auxin needed for vegetative propagation and the potential of *Piper guineense* micropropagation.

Figure 1. *Piper guineense* plant.

Figure 2. Ripe *Piper guineense* fruits.

Materials and methods

Vegetative propagation

Origin of plant material, design and data collection

Herbaceous two-nodal cuttings were collected from healthy *Piper guineense* plants on the field in Obafemi Awolowo University, Ile –Ife, Osun state. The leaves on the cuttings were reduced to half their original sizes, because leaf retention has been reported as an important factor for the adventitious root formation in *Piper* species (Ferriani et al., 2018a). Five different concentrations (0 ppm, 1000 ppm, 1500 ppm, 2000 ppm and 2500 ppm) of indole-butyric acid (IBA) were prepared. The base of cuttings was soaked for 45 seconds in their respective auxin concentrations. Then the cuttings were planted in plastic bags containing moist river sand (preliminary studies showed that river sand was preferable to top soil). All the planted cuttings were placed in a humidified propagator. The cuttings were watered every other day for eight weeks. The experiment was replicated twice.

The number of days to the first sprout, the number of sprouted, rooted and survived cuttings, the number of leaves and roots per cutting were counted, and root length was measured using meter rule. Additionally, percentage of rooting and sprouting was estimated before acclimatization. After two months of monitoring the cuttings, rooted cuttings were transplanted into pots filled with top soil and observed daily for four weeks during acclimatization. After that, survival ratio was estimated.

Micropropagation

Murashige and Skoog (MS) basal medium with vitamins and sucrose, a product of Phyto Technology Laboratories (Lenexa, Kansas, USA) was used in this study. In the preparation of 1 L full strength MS medium, 34.43 g of MS powder was measured into a beaker and dissolved in 500 ml of sterile distilled water. The desired concentration(s) of plant growth regulators (PGRs) was added at this point and the mixture was made up to 1 L with sterile distilled water. The pH was adjusted to 5.7± 0.2 with either 0.1 N HCl or 0.1 N NaOH before the addition of 2 g of Gellan gum as gelling agent (Phyto Technology Laboratories, Lenexa, Kansas, USA). The control was basal medium without PGR. All the media were autoclaved for 15 minutes at 121℃ and 108 KPa to allow the Gellan gum dissolve completely, and dispensed into McCartney bottles (10 ml each). The top of the bottles were wrapped in aluminum foil before sterilizing them by autoclaving for 15 minutes at 121℃ and 108 KPa again. The culture was maintained in the incubator at 25 ± 2℃.

Sterilization and in vitro seed germination of Piper guineense

40 fresh *Piper guineense* seeds were first treated with 70% ethanol for 5 minutes and then divided into 4 groups. Each of them was subjected to a sterilization treatment using varying concentrations of sodium hypochlorite (NaOCl) at different exposure time, having a total of four treatments (10% NaOCl for 5 minutes; 10% NaOCl for 10 minutes; 10% NaOCl for 15 minutes; 15% NaOCl for 15 minutes). A drop of Tween-20 (surfactant) was added to each solution.

At the expiration of the duration, the solutions were decanted with seeds being rinsed 3 times with sterile distilled water and then cultured on basal MS medium. There were 10 seeds per treatment. The cultures were maintained in the incubator at 25 ± 2℃ and observed periodically. The experiment was replicated twice.

Sterilization of Piper guineense nodal explants using sodium hypochlorite and mancozeb

Nodal explants were collected from 3-6 months old *Piper guineense* seedlings raised in a screenhouse. The explants were first rinsed several times with tap water to get rid of dust, pre sterilized with 70% ethanol for 5 minutes and then soaked for 5, 10 or 15 minutes in different concentrations of sodium hypochlorite solutions. Some explants were not treated with

ethanol at all but soaked in mancozeb [Z-Force Fungicide (Mancozeb 80% WP) Sabero Organic Gujarat Ltd, Mumbai, India] solutions for 30 minutes, 1 hour and 2 hours before the sodium hypochlorite treatment.

Two drops of Tween-20 were added to the NaOCl solutions. At the expiration of the disinfection period, the sterilant was decanted under the flow chamber and the nodal explants were rinsed with sterilized distilled water thrice. The explants were cut into 1 cm length with sterile forceps and scalpels, and then inoculated vertically on MS media. For each treatment, five explants were utilized and the experiment was repeated.

Sterilization using streptomycin antibiotics

Some nodal explants were washed under running water and thereafter treated with streptomycin antibiotics solutions. Three methods were tested; quick-dip in 30 mg/L streptomycin, soaking the explants for five minutes in 30 mg/L Streptomycin solution before culturing and by adding 20 or 30 mg/L Streptomycin to the media and disinfecting the explants with 10% NaOCl solution. At the end of the disinfection time, the explants were rinsed thrice with sterile distilled water, sectioned with sterilized forceps and scalpel to 1 cm length and cultured on full strength MS media.

Sterilization using Plant Preservative Mixture (PPM)

Plant Preservative Mixture (PPM) (Plant Cell Technology Inc., Washington, DC, 20009 USA) was added to the media during preparation at the concentration of 0.2 ml/L or 0.4 ml/L. Some nodal explants were treated with 10% sodium hypochlorite solution before inoculating into the media containing PPM, while some were treated by soaking in 2% or 5% PPM for 1 or 2 hours before inoculating onto the media with PPM. At the expiration of the exposure time, only the explants treated with NaOCl solutions were rinsed thrice with sterile distilled water before inoculation, those treated with PPM solutions were not rinsed. The sterilant was simply decanted and the explants sectioned to 1 cm length with the sterilized tools. Observation was then made for any contamination and percentage of clear cultures determined.

Effect of Plant Growth Regulators (PGR) on shoot and root induction of Piper guineense nodal explant

The effect of plant growth regulators was tested on sterilized nodal explants using full strength Murashige and Skoog (MS) media alone as control and MS media supplemented with varying concentrations and combinations of plant growth regulators, benzylaminopurine (BA), naphthalene acetic acid (NAA) and kinetin (KIN). The cultures were maintained in an incubator at a temperature of 25 ± 2 ℃ and 16/8-hour photoperiod. Data collected included the number of sterile and contaminated cultures, percentage of shoot and root induction.

Statistical analysis

Data were evaluated with one-way analysis of variance. Means were separated using the Duncan's Multiple Range Test (DMRT) at 5% probability level.

Statistical analysis was carried out with the Statistical Analytical Software (SAS) 2002 package.

Results

Vegetative propagation

Two-nodal stem cuttings of *Piper guineense* planted in river sand without any rooting hormone sprouted after 31 days. Shoot induction (***Figure 3a***) was observed in all the treatments with the control taking the longest time to sprout (***Table 1***). The highest mean number of sprouted cuttings, mean shoot height and shooting percentage was recorded in stem cuttings treated with 2000 ppm and they were significantly higher than that of all the other treatments. No significant difference was observed in the mean number of sprouted cuttings treated with 0 ppm, 1000 ppm, 1500 ppm and 2500 ppm (***Table 2***), although 0 ppm had the lowest value (1.33±0.33).

Significant differences were also detected across the treatments with regards to root induction, as cuttings treated with 2000 ppm had the highest mean number of rooted cuttings as shown in ***Table 2***. The number of roots per cutting was also affected by IBA (***Figure 3b***).

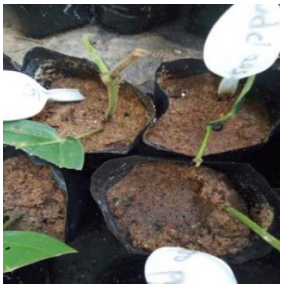

Figure 3a. Sprouted *Piper guineense* cuttings.

Figure 3b. Rooted *Piper guineense* cutting.

Table 1. Effect of different concentrations of IBA on the number of days from planting to sprouting of *Piper guineense* cuttings (abbreviations: ppm – parts per million, IBA: indole-butyric acid).

IBA (ppm)	Days to sprouting
0	31
1000	19
1500	19
2000	19
2500	19

Sterilization and in vitro seed germination of Piper guineense

Germination was first observed in treatment with 10% NaOCl for 10 minutes after seven days (***Figure 4a***), but different ratios of germination and sterility were recorded in all the treatments after 14 days. The highest percentage sterile culture was achieved in treatment with 15% NaOCl for 15 minutes, although only 20% of the seeds germinated as shown in ***Table 3***. One of the seedlings (14 days after germination) is shown on ***Figure 4b***.

Disinfection of nodal explants

Brownish discoloration was observed from cut ends of explants while inoculating nodal explants collected from screenhouse grown seedlings subjected to different concentrations of 3.5% NaOCl (Jik - Reckitt Benckiser, Agbara, Ogun State, Nigeria) at different exposure time after 70% ethanol treatment for 5minutes. After one week, 100% contamination was recorded in all treatments (***Table 4***).

Table 2. Effect of varying concentrations of IBA treatments via soaking for 45 seconds on root and shoot induction on 2-nodal stem cuttings of *Piper guineense* (abbreviations: MNSC - mean number of sprouted cuttings, MNLPC - mean number of leaves per cutting, MNRC - mean number of rooted cuttings, MNRPC - mean number of roots per cutting, MRL – mean root length, SH – Shoot height, SP – Shooting percentage, ppm – parts per million).

IBA	MNSC	MNLPC	MNRC	MNRPC	MRL (cm)	SH (cm)	SP (%)
0 ppm	1.33 ± 0.33^{b}	0.40 ± 0.20^{b}	1.00 ± 0.00^{c}	5.25	1.70 ± 0.91^{b}	2.52 ± 1.14^{b}	26.6
1000 ppm	2.00 ± 0.57^{b}	0.80 ± 0.28^{b}	2.00 ± 0.58^{bc}	8.33	4.50 ± 1.50^{b}	4.28 ± 1.42^{b}	40.0
1500 ppm	2.33 ± 0.33^{b}	1.27 ± 0.46^{b}	3.00 ± 0.00^{ab}	6.78	5.23 ± 1.39^{b}	6.05 ± 1.75^{a}	46.6
2000 ppm	4.00 ± 0.57^{a}	3.87 ± 0.56^{a}	4.00 ± 0.58^{a}	11.17	10.52 ± 1.50^{a}	11.91 ± 1.60^{a}	80.0
2500 ppm	2.00 ± 0.57^{b}	1.07 ± 0.37^{b}	2.33 ± 0.33^{b}	7.00	4.47 ± 1.40^{b}	5.02 ± 1.66^{b}	40.0

Means with the same letter along the column are not significantly different at $p<0.05$

Table 3. Effect of 70% ethanol for 5 minutes and different concentrations of sodium hypochlorite on the sterilization of *Piper guineens*e seeds (abbreviations: NaOCl – sodium hypochlorite).

Treatment (3.5% NaOCl)	Duration	Sterile cultures (%)	Germinated seeds (%)
10% v/v	5 minutes	40	40
10% v/v	10 minutes	70	80
10% v/v	15 minutes	85	35
15% v/v	15 minutes	95	20

Figure 4a. Germinating Piper guineense seeds cultured on MS media.

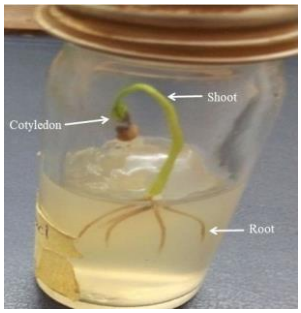

Figure 4b. Young Piper guineense seedling.

When the above treatment failed to give any sterile culture, the experiment was repeated, but this time Clorox (6.05% NaOCl) (The Clorox Company, Oakland, CA 94612, USA) was used instead of Jik (3.5% NaOCl). Although explant discoloration was still observed in all the treatments, there was delay in contamination, but by the 12th day, 100% contamination was recorded on explants treated with 5% Clorox for 5 minutes. By the 28th day of inoculation, all cultures were infected (***Table 4***).

The use of ethanol was dropped and 2g/100 ml mancozeb was applied to disinfect the explants by soaking for 30 minutes, 1 or 2 hours before using 10% Clorox. Although there was no explant discoloration, all the samples were totally contaminated (***Table 4***) within 28 days.

Thereafter, streptomycin was used to sterilize the explants. Although both treatments with Streptomycin delayed contamination, every culture was infected again (***Table 5***).

Sterile culture in ratio 40 % was recorded when 0.4 ml/L PPM was added to the media and the explants treated with 5% PPM solution for 2 hours. For the remaining cultures, the contamination was minimal, root and shoot were still induced on the explants (***Table 6***).

Shoot and root regeneration from nodal explants

Varying responses resulted from the culture of nodal explants on MS without or with different concentration and combinations of plant growth regulators (***Table 7***). On control, neither shoot nor root development was observed. Roots were induced in cultures on MS supplemented with BA in combination with NAA (***Figure 5a***) and on MS supplemented with BA in combination with NAA and KIN (***Figure 5b***). The highest percentage of explants that induced roots was recorded from explants cultured on MS containing 1 mg/L BA + 0.25 mg/L NAA.

Shoot induction was only achieved when BA was used alone (***Figure 5c***), however, the inclusion of 0.25 mg/L of NAA inhibits shoot formation and roots were induced instead. Higher concentration of BA (without NAA) resulted in more shooting (***Figure 6a***), as shown in ***Table 7***.

On the other hand, no combination of BA + NAA or (and) kinetin induced both shoot and root formation on an explant in one culture. Therefore, the explants which produced shoots were sub cultured on MS supplemented with 0.5 mg/L NAA in order to induce roots. In this way, multiple roots were generated (***Figure 6b***).

Discussion

The best rooting rates are observed (Ferriani et al., 2018b) to be obtained with sand substrate. Alaje et al. (2022) also reported that river sand performed better in terms of the root component which they attributed to the aeration potential and drainage properties of river sand which enhance development and spreading of roots.

In vegetative propagation, auxins especially IBA, are known to support root induction activity (Abshahi et al., 2022). They reported that this is because the compound induces adventitious roots and shortens rooting time. *Piper guineense* cuttings treated with water only (0 ppm) had the least mean number of rooted cuttings. This could be as a result of endogenous hormones (auxin) which may be at low levels in the cuttings. Pessarakli (2002) reported that endogenous hormones are usually at suboptimal level in plant tissues and the applying appropriate exogenous hormones can significantly affect the rooting of cuttings of different species (Zhao et al., 2022).

IBA had significant effects on root induction of the stem cuttings, as 2000 ppm had the highest mean number of rooted cuttings, the highest number of roots per cutting and the

Table 4: Effect of 70% ethanol for 5 minutes and different concentration of sodium hypochlorite and mancozeb at different exposure time on disinfection of *Piper guineense* nodal explants [abbreviations: NaOCl – sodium hypochlorite, Clorox – 6.05% NaOCl, Jik – 3.5% NaOCl, mancozeb – Z-Force Fungicide (Mancozeb 80% WP)].

Treatment	Duration	Observation	Sterile culture (%)
70% ethanol for 5 min + 10% v/v Jik	5 minutes	Brownish while inoculating	0
70% ethanol for 5 min + 5% v/v Jik	10 minutes	Brownish while inoculating	0
70% ethanol for 5 min + 5% v/v Jik	15 minutes	Brownish while inoculating	0
70% ethanol for 5 min + 10% v/v Jik	10 minutes	Brownish while inoculating	0
70% ethanol for 5 min + 15% v/v Jik	5 minutes	Brownish while inoculating	0
70% ethanol for 5 min + 15% v/v Jik	10 minutes	Brownish while inoculating	0
70% ethanol for 5 min + 5% Clorox	5 minutes	Brownish discoloration	0
70% ethanol for 5 min + 5% Clorox	10 minutes	Brownish discoloration	0
70% ethanol for 5 min +10% Clorox	5 minutes	Brownish discoloration	0
70% ethanol for 5 min + 10% Clorox	10 minutes	Brownish discoloration	0
2 g/100 ml mancozeb for 30 min + 10% Clorox	5 minutes	No discolored explant	0
2 g/100 ml mancozeb for 1 hr + 10% Clorox	5 minutes	No discolored explant	0
2 g/100 ml mancozeb for 1 hr + 10% Clorox	10 minutes	No discolored explant	0
2 g/100 ml mancozeb for 2 hrs + 10% Clorox	10 minutes	No discolored explant	0

Table 5. Effect of streptomycin on disinfection of *Piper guineense* nodal explants (abbreviations strept. – streptomycin, Clorox – 6.05% NaOCl).

Streptomycin (mg/L)	Duration	Observation	Sterile cultures (%)
30 mg/L quick dip	-	Contaminated in one week	0
30 mg/L soak explant	5 minutes	Contaminated in two weeks	0
20 mg/L strept. in media+ 10% Clorox	10 minutes	Contaminated in two weeks	0
30 mg/L strept. in media+ 10%Clorox	10 minutes	Contaminated in two weeks	0

Table 6. Effect of Plant Preservative Mixture (PPM) alone and with 10 % Clorox on sterilization of nodal explant of *Piper guineense* (abbreviations: PPM - Plant Preservative Mixture, Clorox – 6.05% NaOCl, +++ - Heavily contaminated, ++ - Moderately contaminated, + - Slightly contaminated).

Treatment	Duration	Degree of cont.	Contamination (%)
0.2 ml/L PPM in media + 10% Clorox	5 minutes	+++	100
0.2 ml/L PPM in media + 10% Clorox	10 minutes	+++	100
0.4 ml/L PPM in media + 10% Clorox	10 minutes	+++	100
0.4 ml/L PPM in media + 2% PPM explants soaking	1 hour	++	100
0.4 ml/L PPM in media + 5% PPM explants soaking	1 hour	+	90
0.4 ml/L PPM in media + 5% PPM explants soaking	2 hours	+	60

Table 7. Effect of various concentrations of plant growth regulators on shoot and root induction from nodal explants of *Piper guineense* (abbreviations: BA – benzylaminopurine, NAA – naphthalene acetic acid, KIN – kinetin).

BA (mg/L)	NAA (mg/L)	KIN (mg/L)	Shoot (%)	Root (%)
0	0	0	0	0
0.25	0.05	0	0	20
0.25	0.05	0.05	0	30
0.5	0	0.25	0	40
0.5	0.25	0	0	40
1.0	0.25	0	0	60
1.0	0	0	60	0
1.5	0	0	80	0

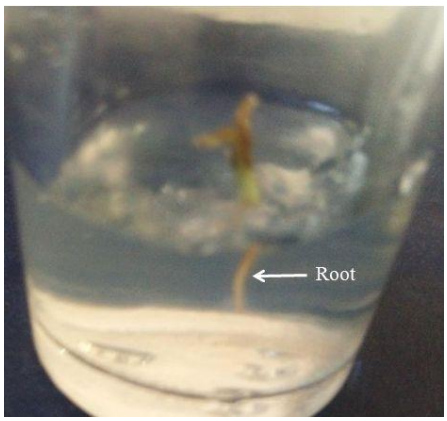

Figure 5a. Nodal explant of *P. guineense* cultured on MS + 0.25 mg/L NAA + 0.5 mg/L BA.

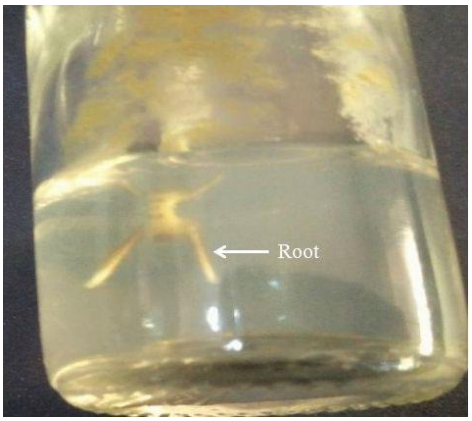

Figure 5b. Nodal explant of *P. guineense* cultured on MS + 0.25 mg/L BA + 0.05 mg/L NAA + 0.05 mg/L KIN.

Figure 5c. Nodal explant of *P. guineense* cultured on MS + 1 mg/L BA.

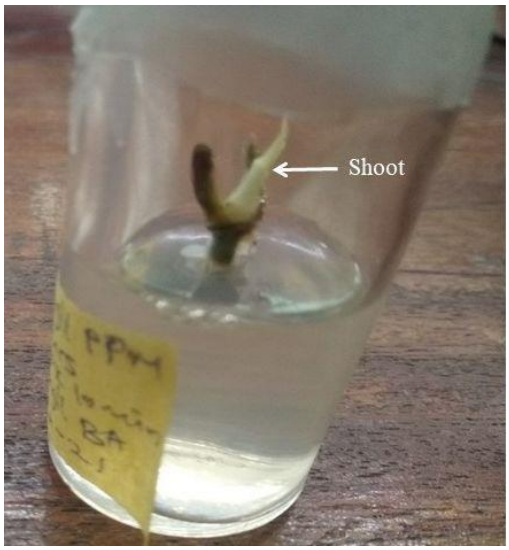

Figure 6a. Nodal explant of *P. guineense* cultured on MS + 1.5 mg/L BA.

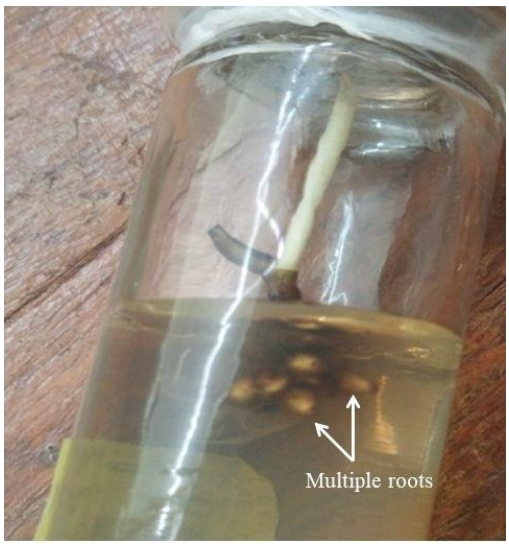

Figure 6b. Nodal explant of *P. guineense* cultured on MS + 1.5 mg/L and subcultured on MS + 0.5 mg/L NAA.

longest root length. This study agrees with Sundharai et al. (2002), who reported that the metabolite activity at the cut ends is improved by IBA which also assists the translocation of carbohydrates and other root inducing substances to them. Cuttings treated with 2500 ppm of IBA performed generally lower than those treated with 2000 ppm indicating that higher concentration of IBA is inhibitory to root and shoot induction in *Piper guineense.* Zhao et al. (2022) suggested a dose-response relationship between exogenous hormones and adventitious roots formation, referencing that adequate concentrations of plant growth hormones are important because auxin concentrations that are too high may inhibit the formation of adventitious roots". Therefore, they emphasized the importance of the optimal concentration of IBA application during clonal propagation of different species.

The length of cuttings is similar to that which was adequate for *Piper samentosum* (Waman et al., 2019). They reported that 2-nodal cuttings were the best for vegetative propagation of this species. According to Waman et al. (2019), varying number of nodes on nodal cuttings was reported in different *Piper* species with responses that were species dependent. They intimated that the use of 1000 mg/L IBA promoted better rooting of *Piper sarmentosum*. However, higher concentration (2000 mg/L IBA) was required for maximum rooting and shooting of *Piper guineense* cuttings as opposed to that of *P. sarmentosum*. Ferriani et al. (2018a) reported 3.0 mg/L for the rooting of *Piper cernuum* and *P. arboreum* cuttings and 1.5 mg/L for *P. diospyrifolium* all with varying degrees of rooting. They showed from the results, the need of evaluating rooting production for each *Piper* species because they present different responses in root induction and development. Abshahi et al. (2022) also determined that the capacity of cuttings to root can vary among species. Moreover, according to Zhao et al. (2022), there is a complex modulation, balance, and signaling interaction between auxin and other phytohormones apart from the fact that, apart from auxins, other hormonal signaling pathways have been reported to affect rooting. Therefore, they asserted that maintaining the endogenous hormone balance during rooting in the vegetative propagation process is necessary.

Surface sterilization is the most important step in the preparation of explants that are not *in vitro* grown for micropropagation, because the surface of plants carries a wide range of microbial contaminants. Contamination is regarded as the single reason for losses during *in vitro* culture of plants. The microbial contaminants can be, bacteria, viruses, yeast or fungi (Omamor et al., 2007). These microbes compete with cultured tissue for available nutrients adversely and often lead to increased culture mortality, tissue necrosis, limited shoot proliferation and inhibited rooting (Kane, 2003).

The common sterilizing agents that have been used to disinfect many explant types are ethanol and sodium hypochlorite (NaOCl). However, in this study ethanol and

NaOCl achieved effective sterilization only with seeds. Rinsing the seeds in 70% ethanol for 5 minutes + 10% Jik (3.5% NaOCl) for 10 minutes resulted in 80% clean, germinated culture. Nodal explants collected from *Piper guineense* plants raised in the screenhouse required double sterilization treatment with Plant Preservative Mixture (PPM) to reduce the contamination to minimal level.

For some reasons *Piper guineense* nodal explants start turning brown at both ends when ethanol is included in the sterilization protocol even at reduced concentration and exposure time. The browning effect could be caused by phenolic compound secretion. Anny (2011) noted that the genus *Piper* contains high concentration of phenolic compounds, and some of these compounds are leached from the explants of the species causing browning and might ultimately kill the explants. It could be that ethanol increases the secretion of phenolic compounds in *Piper guineense* explants. Sodium hypochlorite proved to be ineffective in reducing the contamination rate and so was soaking of the explants in mancozeb (a fungicide) before culturing, indicating that the contaminants are not only present on the surface of the explants.

Piper guineense explants have endogenous or deep tissue contaminants which cannot be eradicated by surface sterilization alone. In this study, streptomycin (an antibiotic) did not yield any positive result in reducing the contamination, probably because antibiotics are heat sensitive, therefore useless when added to the media before autoclaving. Soaking the explants in streptomycin solutions only delayed contamination for a week. Although Okafor and Uzochukwu (2015) suggested the use of antibiotics to eliminate endogenous bacterial contamination in *Piper guineense*, and these agents were also recommended by Kulkarni and Krishnamurthy (2002), this study agrees with Philip et al. (1992), who reported that antibiotics do not eliminate the systemic bacteria in cultured explants, but delayed their onset.

Plant Preservative Mixture (PPM) is a broad-spectrum biocide/fungicide for plant tissue culture and it is not heat sensitive. Addition of 4 ml/L PPM to the media and soaking of the explants in 5% PPM for two hours before culturing (double sterilization) gave 40% sterile and 60% contaminated cultures. Soaking the explant in PPM solution for a longer duration would have resulted higher percentage of sterility. Sameer and Nabeel (2016) reported 100% sterile culture when nodal explant of *Cestrum nocturnum* L was sterilized by soaking in 4.6% PPM solution for 4 hours.

Nodal *Piper guineense* explants inoculated on MS media without PGRs did not induce any root or shoot. The differentiation process is modulated by PGRs (Chawla, 2000), therefore, the absence of shoot or root induction in nodal explants cultured on MS without PGRs may be due to low level of endogenous PGRs in the explant.

20% rooting response was observed when 0.25 mg/L BA + 0.05mg/L NAA was introduced into the media, as against the 0% obtained with the basal media. But increasing the BA concentration in the combination in order to induce shoot production as well only increased the percentage of rooting as observed when 60% roots was developed on explants cultured on MS +1 mg/L BA + 0.25 mg/L NAA, but no shooting was induced. When only 1 mg/L BA was added to MS media, 60% shoot induction was recorded, but Khan et al. (2019) reported no shooting response of *Piper betle* when MS was supplemented with BA alone and 60% shoot induction in the addition of 1.5 mg/L BA + 0.2 mg/L NAA. Also, Khan et al. (2017) experienced 80% shooting (without rooting) of *Piper nigrum* on medium with 1.0 mg/l BA + 1.0 mg/l IAA. These results shows that different *Piper* species require different PGR(s) combinations and concentrations for successful *in vitro* shoot and/or root induction.

In this study, NAA in the media inhibited shoot formation and promoted root induction in *Piper guineense*. The level of endogenous auxin in the explant is probably higher than that of cytokinin and so the balance of auxin to cytokinin may not have been adequate to induce shoot formation. Gostin (2008) reported that the optimum level of BA needed for shoot induction depends on the concentration of endogenous cytokinin present in the plant species. The higher the concentration of BA used alone the more shoots were induced with 80% of the cultured explants inducing shoots on MS media supplemented with 1.5mg/L BA alone. Afroz (2020) also reported that maximum shoot induction in *Piper nigrum* was obtained with 2.0 mg/L BA.

However, no combination of BA + NAA induced both shoot and root on *Piper guineense* nodal explant in one culture. This agrees with the reports of Khan et al. (2017 and 2019); Da Silva et al. (2012). Explants with induced shoots were sub cultured on MS media supplemented with 0.5 mg/L NAA and multiple roots were generated.

Conclusions

This study has revealed that herbaceous, 2-nodal stem cuttings treated with 2000 ppm IBA can be used to raise *Piper guineense* seedlings vegetatively. For *in vitro* propagation, sterile *in vitro* seedlings can be obtained by treating fresh seeds with 70% ethanol for 5 mins and 10% v/v 3.5 % NaOCl for 10 mins before culturing. The endogenous contamination observed in nodal explants collected from field grown plants can be reduced through double sterilization with 0.4 ml/L PPM in media plus soaking the explants in 5% PPM for more than 2 hours. The use of BA alone (1.5 mg/L) promotes shoot induction from nodal explants. This can then be sub cultured to obtain roots on MS media supplemented with 0.5 mg/L NAA.

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