ESTABLISHING A METHOD FOR THE MASS PROPAGATION OF *Piper guineense* (Schumach) VIA TISSUE CULTURE

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A FINAL YEAR PROJECT SUBMITTED TO THE DEPARTMENT OF PLANT SCIENCE AND BIOTECHNOLOGY, FACULTY OF SCIENCE IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (B.Sc.) DEGREE IN PLANT SCIENCE AND BIOTECHNOLOGY

FEDERAL UNIVERSITY, OYE EKITI, EKITI STATE, NIGERIA.

OCTOBER, 2015.

DECLARATION

I hereby declare that this project has been clarified by me, it is a record of my own research work and to the best of my knowledge and has not being published or presented in any form. All sources of information are duly acknowledged by means of references.

Okafor Ogechi

Sign.....

Date: October, 2015

CERTIFICATION

This is to certify that this final year project was carried out and compiled by OKAFOR OGECHI TOPE with matriculation number BTH/11/0256 in the Department of Plant Science and Biotechnology, Faculty of Science, Federal University, Oye Ekiti, Ekiti state under the supervision of PROF. SYLVIA UZOCHUKWU. All assistance and contributions received have also been acknowledged.

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DEDICATION

The work is dedicated to God my sufficiency who supplied all my needs according to his riches in glory throughout my stay in the University. It is also specially dedicated to my ever supportive parents Mr. and Mrs Okafor for their relentless efforts and compassion towards me during the pursuit of my degree

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ABSTRACT

Piper guineense (Schumach) has great potentials for economic exploration because of the proven use of its medicinal content in human health. The plant material is usually obtained from thick forests by women who have to walk long distances in the bush to reach it. A technique that improves its propagation and domestication, such as tissue culture, becomes necessary. This study was therefore initiated to develop a method for the mass propagation of *Piper guineense* seedlings using in vitro regeneration. Plants react differently to media concentrations and constituents for their in vitro regeneration. Comparative growth of *Piper guneensee* inoculated on Murashige and Skoog(MS) medium supplemented with some growth regulators were investigated. Mature nodes were collected from the medicinal garden of National Centre for Genetic Resources and Biotechnology (NACGRAB) Ibadan. Nodes of the species were inoculated onto MS media. The MS media was supplemented with the following concentrations of growth regulators 0.25, 0.50, 0.75, 1.0mg/l. BAP, the same concentration for KIN and 0.05mg/l of NAA for all replicate. After two weeks of inoculation it was observed that, the plantlet with BAP 0.50mg/l +NAA 0.05mg/l were found contamination-free and sprouted, while other treatments showed contamination including control. Furthermore, after 7 days the sprouted, plantlets with treatment of BAP 0.50mg/l +NAA 0.05mg/l were also contaminated, thereby yielding no positive result. Many repeats of the experiment also yielded contaminated products. It is therefore recommended that antibiotics should play an important role in the culture medium for *P. guineense* in order to eliminate deep tissue contaminants.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Piper guineense (Schumach) is a West African species of Piper. The spice derived from its dried fruit is known as West African pepper, Ashanti pepper, Benin pepper, false cubeb, Guinea cubeb, uziza pepper or (ambiguously) "Guinea pepper", and called locally kale, kukauabe, masoro, sasema and soro wisa. It belongs to the plant family called piperaceae (Rehm and Espig,1991;Amusan and Okorie,2002;Asawalam,2006).

The West African peppers are prolate spheroids, smaller and smoother in appearance and generally bear a reddish tinge (Katzer,2015). *Piper guineense* is a perennial woody climber that grows up to 10m or more in height(Hutchinson and Dalziel 1954; Irvine, 1961). Its' leaves are alternate and simple with a petiole 2-5cm long. These are native to tropical regions of Central and Western Africa and are semi-cultivated in countries such as Nigeria where the leaves (known as uziza) are used as a flavouring for stews (Facciola,1998). It is a plant of the wet tropics that requires heavy and well distributed rainfall and temperature. The ideal soil for production is a well drained alluvium with high humus content. It can also be grown in red laterite soil. The plant supports itself on other plants by means of adventitious roots which are produced along the stem. The plant is a popular spice in Africa, where it is often harvested from the wild, semi-cultivated and sometimes also cultivated for use both as a spice and also as a medicine. The use of derivatives of *Piper guineense* to protect stored grain legumes and cereals against several post harvest pests is a widespread age long practice in Africa (Olaiya *et al.*,1987).

West african pepper can be propagated by seeds, cuttings, layering, and grafting (Ravindran *et al.*, 2000). Seed propagation often results in genetic variation due to formation of recombinants while other methods of piper pepper propagation are slow and time consuming (Atal & Banga, 1962). So there is a need to introduce efficient methods for faster propagation of West African pepper. Also, the plant is not yet widely domesticated, and women have to journey far into thick tropical forests to harvest it for sale, at great personal risks from reptiles and other wild animals, apart from the great tedium involved. In view of the foregoing, plant tissue culture is the most efficient and reliable method for domestication and rapid and mass scale production of disease free, genetically stable and identical progeny (Hu &Wang,1983) of west African pepper throughout the year. Tissue culture techniques have taken significant part in clonal propagation, conservation of germplasm, and plant improvement in West African pepper (Bhat *et al.*, 1995; Sajc et al., 2000). Thus, *in-vitro* propagation is a superior option and alternative for traditional propagation (Abbasi *et al.*, 2007; George & Sherrington, 1984) especially when domestication is the primary aim.

1.2 OBJECTIVES OF STUDY

The main aims and objectives of this study are :

i. To develop protocols for micro propagation of West African pepper in Nigeria in order to domesticate the plant.

ii. To remove the tedium involved in always having to go to the forest for supplies, for the women and men involved in the trade.

1.3 LITERATURE REVIEW

1.3.1 ORIGIN AND HISTORY

Pepper (*Piper species*) is native to South Asia and Southeast Asia and has been known to Indian cooking since at least 2 BCE. J. Innes Miller notes that while pepper was grown in southern Thailand and in Malaysia, its most important source was India, particularly the Malabar coast, in what is now the state of Kerala. Peppercorns were a much-prized trade good, often referred to as "black gold" and used as a form of commodity money. The legacy of this trade remains in some Western legal systems which recognize the term "peppercorn rent" as a form of a token payment made for something that is in fact being given. The ancient history of black pepper is often interlinked with (and confused with) that of long pepper, the dried fruit of closely related *Piper longum*. The Romans knew of both and often referred to either as just "piper". In fact, it was not until the discovery of the New World and of chili pepper that the popularity of long pepper entirely declined. Chili peppers, some of which when dried are similar in shape and taste to long pepper, were easier to grow in a variety of locations more convenient to Europe. Before the 16th century, pepper was being grown in Java, Sunda, Sumatra, Madagascar, Malaysia, and everywhere in Southeast Asia. These areas traded mainly with China, or used the pepper locally. Ports in the Malabar area also served as a stop-off point for much of the trade in other spices from farther east in the Indian Ocean. Following the British hegemony in India, virtually all of the black pepper found in Europe, the Middle East, and North Africa was traded from Malabar region.

Piper guineense, is also known as West African pepper, Ashanti pepper, Benin pepper, False cubebs, Guinea pepper, Uziza pepper . These are native to tropical and sub tropical regions of the world (Hutchinson and Dalziel, 1954;1963). They are also native to tropical regions of central and Western African and semi- cultivated in countries such as Nigeria where leaves are used as flavoring for stews. Though it was known in Europe during the middle ages, it was a common spice in Roven and Dieppen in 14th century, France. It is found almost in all countries in West Africa covering both the Savannah and forest region. The pepper is also sometimes one of the ingredients in the Berbere spice mix used in the cuisines of Ethopia and of Eritrea. However, West African Pepper is a highly esteemed spice in its region of origin and may be hard to get abroad; thus, long pepper is more often used in Berbere.

1.4 MORPHOLOGY AND BOTANICAL DESCRIPTION

Piper guineense is a climber up to 12m or 13m high on trees producing Ashanti pepper or West African pepper (Hutchinson and Dalziel,1954) and climbing by means of its adventitious rootlets. It is a perennial plant that is characterized by simple heart-shaped leaves and oval, petiolate, alternate,12 cm long. It has corky lower stem . It is a close relative of cubeb pepper, black pepper(*Piper nigrum*) and long pepper (*Piper longum*).Unlike cubeb pepper which is large and spherical in shape, West African pepper are prelate spheroids, smaller and smoother in appearance, they generally bear a reddish tinge and the stalk of *Piper guineense* are distinctively curved. The inflorescence is a pedicelled flower spike between 3 and 6cm, peduncle 5 millimeters long. The fruit is a drupe mesocarp or fleshy, oval, 5mm in diameter and are red-brown when ripe and black when dry. Flowers are greenish yellow and arranged in a spiral along the spine. The plant occurs in closed forests on trees in forests clearings.

1.5 DISTRIBUTION

Piper is a large genus of plant with about 700 species distributed throughout tropical and subtropical regions of the world (Hutchinson and Dalziel,1954;1963) but can also occur in clearings and in higher elevation life zones such as cloud forests. One species (Japanese Pepper, *P. kadsura*, from southern Japan and southernmost Korea) is subtropical and can tolerate light winter frost. Peppers are often dominant species where they are found. *Piper guinneense* is widely distributed in Africa from Guinea to Uganda. It is mostly found in almost all the countries in West Africa covering both the savannah and forest region.

1.6 SOIL AND CLIMATE

Piper vine thrives in a moist, hot climate at altitude from 450-600m. It tolerates temperature ranging from 24-30°c. An annual rainfall of over 250cm is ideal, but it also grows well in areas of lower rainfall provided the rain is evenly distributed throughout the year. It grows well in sandy loam soil rich in humus and well drained.

1.7 TAXONOMY OF PIPER SPECIES

The largest number of Piper species are found in the Americas (about 700 species), with about 300 species from Southern Asia. There are smaller groups of species from the South Pacific (about 40 species) and Africa (about 15 species). The American, Asian, and South Pacific groups each appear to be monophyletic; the affinity of the African species is unclear.

1. *Piper guineense*

- Scientific classification of *Piper guineense* according to Schumach and Thonn(2005)

  Kingdom: Plantae

  Division: Magnoliopyta

  Class: Mangnoliopsida

  Order: Piperales

  Family: Piperaceae

  Genus: *Piper*

  Species: *guineense*

2. - Black pepper (*Piper nigrum*) is a flowering vine in the family Piperaceae, cultivated for its fruit, which is usually dried and used as a spice and seasoning. The fruit, known as a peppercorn when dried, is approximately 5 millimetres (0.20 in) in diameter, dark red when fully mature, and, like all drupes, contains a single seed. Black pepper is native to south India, and is extensively cultivated there and elsewhere in tropical regions. (Mathews and Rao, 1984).

- Scientific classification of Piper nigrum.L

  Kingdom: Plantae

  Unranked: Fruits

  Unranked: Magnolids

  Order: Piperales

  Family: Piperaceae

  Genus: *Piper*

  Species: *p.nigrum*

3. Cubeb or tailed pepper (Piper cubeba): It's a plant whose fruits are quite similar to black pepper, although they are pedicellate ("with a twig or tail"). It was formerly a widely used spice, although, due to its bitter taste, it possibly fell into medicinal properties.

Scientific classification of *Piper cubeba*

Kingdom: Plantae

Unranked: Angiosperm

Unranked: Mangolids

Order: Piperales

Family: Piperaceae

Genus: *Piper*

Species: *P.Cubeba*

4. - Long pepper (*Piper longum*): Originally from India, it is characterized by its welding fruits in fruity compact spikes. Dried and crushed are suitable for cooking. It is added to curry mixes or used as a substitute for black pepper

Scientific classification of *Piper longum*

Kingdom:	Plantae
(unranked):	Angiosperm
(unranked):	Mangolids
Order:	Piperales
Family:	Piperaceae
Genus:	*Piper*
Species:	*P. longum*

5. - Acuyo, Cordoncillo, Yerba Santa, Mexican pipperleaf (*Piper auritum*). Although it is popularly referred as pepper, the leaves of this plant are used as spice, in the kitchen of Mesoamerica, providing a nice spicy flavor.

Scientific classification

Kingdom:	Plantae
(unranked):	Angiosperm
(unranked):	Mangolids
Order:	Piperales
Family:	Piperaceae
Genus:	*Piper*
Species:	*P. auritum*

-6. Kava or kava-kava (Piper methysticum) (Piper: Latin for "pepper", methysticum: Latinized greek for "intoxicating") is a crop of the western Pacific. The name kava(-kava) is from Tongan and Marquesan (kava,2008) other names for kava include 'awa(Hawai'i) , ava (Samoa), yaqona (Fiji), and sakau (Pohnpei). Its aromatic compounds are highly valued as an anxiolytic for aromatherapy. It has medicinal properties , but may have toxic effects.

Scientific classification

Kingdom:	Plantae
(unranked):	Angiosperm
(unranked):	Mangolids
Order:	Piperales
Family:	Piperacea
Genus:	*Piper*
Species:	*P.methysticum*

7. *Piper marginatum* is also known as the cake bush, Anesi wiwiri, marigold pepper, Ti Bombé in Creole or Hinojo and is a plant species in the genus *Piper* found in moist, shady spots in the Amazon rainforest in Surinam, French Guiana and Brazil(Paris RR ,1978).

Kingdom:	Plantae
(unranked):	Angiosperm
(unranked):	Magnolids
Order:	Piperales
Family:	Piperacea
Genus:	*Piper*
Species:	*P.marginatum*

8. The betel (*Piper betle*) is the leaf of a vine belonging to the Piperace family, which includes pepper and kava. It is valued both as a mild stimulant and for its medicinal properties.

Scientific classification

Kingdom: Plantae

Unranked:Angiospermae

Unranked: Magnolidae

Order: Piperales

Family: Piperaceae

Genus: *Piper*

Species: *P.betle*

1.8 MEDICINAL AND CONVENTIONAL USES

The leaves of *Piper guineense* are used as a leafy vegetable pepper in many African soups. The leaves and fruits are also used as flavour in many dishes. Like other members of the pepper family, some organic compounds present in this specie include alkaloids, terpenoids, phenols, alcohols and phenolic esters but piperine is most abundant(Webbe and Lambart, 1983). According to Dada *et., al.* (2013), *Piper guineense* 'Uziza' is a local spice that comprises of dillapiol, 5-8% of piperine, elemicine, 10% of myristicine and safrole and these chemicals exhibit bactericidal and antimicrobial effects on certain microorganisms.. They contain large amounts of beta-caryophyllene, which is being investigated as an anti-inflammatory agent. Traditionally, people believed that they are medicinal. It is added to food meant for pregnant and nursing mothers as a medicinal spice and among the post partum women, it is claimed that it assists in the contraction of the uterus (Achimewhu *et al.*,1995). It is generally known to possess antimicrobial and antioxidant properties (Iwu, 1993). The leaves and fruits of *Piper guineense* are used in preparing food for the pregnant and lactating mothers, because people believed that they promote good health. The leaves are used as spice to flavor meat preparations and fresh pepper soup. They have played important role in the history of civilization, exploration and commerce. *Piper guineense* is used for the treatment of boils, bronchitis, catarrh, chest pains, coughs, dyspepsia, impotence, insect repellant, lumbago and rheumatism. It is also used for treating uterine fibroids and wounds. (Busia, 2007).

1.9 TRADITIONAL PROPAGATION OF *Piper guinennse*

Different propagation strategies have been used to increase the healthy biomass of this important specie. Conventionally, this species are propagated through seeds and stem cuttings (Ravindran *et al.*, 2000) . *P. guineense* is a perennial climbing vine which needs support (standards) for fruit production (Susan *et al.*, 2008). All established plants of *P. guineense* produce seeds but due to short viability and high sterility the production is cumbersome. Therefore, this plant is propagated through cutting with 2-6 nodes and 4 leaves for nursery production and field plantation (Sivaraman, 1988). Micro propagated shoot tips of mature vine and seedling of other piper species are also used for plantlets production (Mathews and Rao, 1984; Philip et al., 1992). Vegetative propagation is also limited by the low number of individuals obtained from a single plant, requires enough stocks of stem cuttings and high labor inputs. For the last few years substantial degree of decline has been noticed in production of piper species due to plant aging and multi pathogenic attacks like mycoplasma, bacteria, virus, fungus and pests (Abbasi *et al.* 2010; Phillip *et al.*, 1992). In this regard, in vitro tissue culture techniques appear to be a very promising alternative

1.10 TISSUE CULTURE OF MEDICINAL PLANTS

Since ancient times, mankind has been dependent on plants for food, flavours, medicinal and many other uses. Ancient written records of many civilizations (i.e. Egyptian, Roman, Chinese) give strong evidence regarding use of medicinal plant(Cowan, M.M. 1999) , for example ayurveda documents record the use of medicinal plants to cure many ailments . At present there are many well established herbal and plant medicine practices (Ayurvedic medicine in India) which are popular in many parts of the world. The World Health Organization (WHO) reported that 80% of people in the developing world use medicinal plants for their primary health care (Vines. G. 2004).

The use of herbal medicines is growing in developed countries. At present, 25% of the UK population use herbal medicine and about 40% of compounds used in pharmaceutical industry are directly or indirectly derived from plants (Rout G.R *et al,*2000) because the chemical synthesis of such compounds is either not possible and/or economically not viable . Therefore a large number plant species (especially medicinal) are under threat of extinction because of their over exploitation. In the last two decades there has been a great increase in research on medicinal plant. A number of new medicines have been discovered and advancements in production technology to harvest pharmaceutical important metabolites. During this period there has also been an increase in research publication on medicinal plants .Plant tissue culture plays relevant role in *ex situ* conservation and multiplication of endangered and commercially exploited medicinal plants.

1.11 IMPORTANCE OF *IN VITRO* TECHNIQUES FOR MASS PROPAGATION OF PIPER SPECIES

In vitro techniques bypass conventional methods and rapidly produce identical progenies of many medicinal and economical plant species (Hu and Wang, 1983). *In vitro* culture and regeneration is an alternative method to the traditional propagation of plants that are less responsive to be cloned by conventional means (Sajc *et al.*, 2000). These techniques play an important role in propagation, conservation and improvement of *Piper* and other plant species (Bhat *et al.*, 1995). Micro propagation provides an efficient method for *ex situ* conservation of plant biodiversity and multiplication of the endangered species from minimum plant material available. Micro propagation can be used as a routine method of multiplication in species where conventional modes of propagation are inadequate to meet the demands of the planting stock, either due to poor seed set or low germination. Also, micro propagation is a tool for cloning of elite plants. Even in species where vegetative multiplication is possible, tissue culture has been important for multiplication of disease certified material and multiplication of elite clones. The latter requires culture initiation from mature explants. There are several reports of shoot regeneration from explants having pre-existing meristems such as nodal segments (Selvakumar *et al.* 2001, Arya *et al.*, 2002, Benniamin *et al,*.2004), shoot tips (Bhattacharya and Bhattacharya 2001, Thiem 2003) and vegetative buds (Borthakur *et al.*, 1998, Narula *et al.*, 2003).

1.12 EXPLANT SELECTION OF PIPER SPECIES

The technique of plant tissue culture starts with selection of suitable explant from a desirable donor plant(mother plant) and its successful establishment in aseptic and suitable culture conditions. The choice of appropriate explant for culture establishment varies with plant species and plays a key role in determining the efficiency of propagation (Abbasi *et al.*, 2007). In early experiments, leaf, nodal, internodal, shoot tips, roots and seed derived plantlets have been used for direct and indirect regeneration of relevant *Piper* species (Bhat *et al.*, 1995; Nazeem *et al.*, 1992; Philip *et al.*, 1992; Babu *et al.*, 1993; Joseph *et al.*, 1996; Sujatha *et al.*, 2003; Bhat *et al.*, 1992; Nair and Gupta, 2005; Rout *et al.*, 2000). The *in vitro* propagation of *Piper guineense* has not been carried out but some other piper species have been micropropagated over time.

In the literature cited the most appropriate explant for efficient and successful regeneration of *Piper nigrum* are seed derived plantlets and shoot tips. However, seed derived explant leads to genetic variation from the mother plant. The type and source of explant is very important in determining the morphogenetic potential which are significantly influenced by the phytosanitary and physiological conditions of the donor plant (Debergh and Maene, 1981; Read, 1988). Maintenance of the donor plants in hygienic and controlled conditions provides fresh, healthy and sterile explants (Sagare *et al.*, 2001). The type, size and physiological age of explant are other factors which play an important role in regeneration of complete plantlets *via* callus cultures or through direct organ formation (Rout *et al.*, 2000). In conclusion, leaf and shoot tip explants are useful for regeneration of *Piper* species.

1.13 *IN VITRO* SEED GERMINATION

The viable seeds of *Piper species* are commonly infected by different pathogens including bacteria, fungi, mycoplasma and virus (Philip *et al.*, 1992). It is very difficult to control viral and mycoplasmal contamination because these internal pathogens are nearly always transferred by vegetative propagation (Philip *et al.*, 1992). Several workers reported that these endogenous contaminations induced delay in response of *in vitro* cultures of *P. nigrum* and some other species (Mathews and Rao, 1984; Fitchet, 1990; Philip *et al.*, 1992). Joseph *et al.* (1996) reported that these internal pathogens can be minimized, if excised embryos from the seeds are used for *in vitro* cultures establishment. Another alternative for minimization of these pathogens is the use of sterilization techniques. Different surface sterilizing agents include ethanol, mercuric chloride and sodium hypochlorite are used for *in vitro* establishment of Piper. Mujib (2005) reported that seeds sterilization with 0.1 % HgCl2 for 4 min is very effective in eliminating of several contaminations. Similarly Azad *et al.* (2003) also reported similar concentration of HgCl2 for removal of contamination for 3-20 min. But Fitchet (1990) and Philip *et al.* (1992) reported that repeated surface sterilization of seeds and other explants inhibit the growth of bacteria but does not eliminate them completely.

1.14 REGENERATION

In vitro regeneration of *P. nigrum* can occur both by organogenesis and somatic embryogenesis (Philip *et al.*, 1992; Joseph *et al.*, 1996; Nair and Gupta, 2006). Both solid MS (Murashige and Skooge, 1962) and liquid SH media (Shchenk and Hildebrandt, 1972) are widely used for micropropagation of this species (Bhat *et al.*, 1995; Nazeem *et al.*, 1992; Babu *et al.*, 1993; Sujatha *et al.*, 2003; Bhat *et al.*, 1992; Nair and Gupta, 2005).

However, the use of other media such as Woody Plant Medium (WPM) has only been reported for the regeneration of *P. barberi* (Babu *et al.*, 1996.).

1.15 SHOOT ORGANOGENESIS OF PIPER

Shoot organogenesis means the regeneration of shoots directly from differentiated tissues or indirectly from undifferentiated cells under *in vitro* conditions (Nalawade and Tsay, 2004). Shoot organogenesis needs several factors and biochemical processes for differentiation and complete organ formation (Chawla, 2000). Generally the type of plant growth regulators, explant types and its inoculation direction play an important role in regulating the differentiation process (Jones *et al.*, 2007). Additional media supplements, alteration and modification also play a key role in the morphogenesis (Narayanaswamy, 1977). Culturing of leaf explants on MS medium containing Indole Acetic Acid (1 μM) in combination with BAP (benzyl amino- purine acid) produced whitish or green friable calli. The addition of silver nitrate (AgNO3; 05-15ppm) significantly influenced shoot regeneration (Sujatha *et al.*, 2003). The addition of BA alone or in combination with adenosine sulphate (AdSO4) and IBA are effective for initial shoot proliferation but higher concentrations of BA (5.0 μM) reduced shoot proliferation and further growth of shoots. But higher concentrations of BA (5.0-10 μM) produced 40% shoots from nodal explants. However, the combination of BA (1.5 μM) and IBA (3.0 μM) promote shoot organogenesis in this specie (Philip *et al.*, 1992). Supplementing of Kinetin (Kn) to the medium influenced the axillary bud proliferation but failed to induce shoot buds in *P. nigrum* (Bhat *et al.*, 1995). However, Anand and Rao (2000) reported that the addition of BA and Kn to the medium produced 88% response. The BA in combination with activated charcoal had a significant effect on the number of shoot production. Shoot tips and nodal explants failed to induce shoots and bud

break on cytokinin-free medium. But the higher concentration of BA (6.6 μM) promotes the response in both types of explants. The combination of Kn and IAA are also effective in shoot organogenesis (Rubluo and Barroso, 1992). Furthermore, direct and indirect shoot regeneration from leaf and stem explants are widely reported in other piper species such as *P. attenuatum, P. betle, P. chaba, P. colubrinum and P. longum* (Babu *et al.*, 1996; Bhat *et al.*, 1992, 1995; Madhusudhanan and Rahiman, 2000).

1.16 ROOT ORGANOGENESIS OF PIPER

Once the objective of mass multiplication is achieved, the regenerated shoots are rooted on suitable culture media. The process of root initiation and complete development normally needs different auxin concentrations in the medium (Azad *et al.*, 2003). In general, root development in *Piper* on different media was best induced by the addition of Naphthalene Acetic Acid (NAA), IAA(Indole Acetic Acid) and Indole Butryric Acid(IBA) (Bhat *et al.*, 1992; Joseph *et al.*, 1996; Philip *et al.*, 1992). Bhat *et al.* (1992) reported that *P. nigrum* shoots can be easily rooted on MS-medium containing α-naphthalene acetic acid (NAA; 1 mg l-1) only. Bhat *et al.* (1995) reported best root initiation and development on GAMBORGs (B5) medium containing 6-benzyladenine (BA; 0.5 μM) in combination with Indole acetic acid (IAA; 1.0 μM). However, Joseph *et al.* (1996) reported root development from somatic embryos in liquid SH medium with 1.5% sucrose without the incorporation of any plant growth regulator.

1.17 ACCLIMATIZATION OF PLANTLETS

The acclimatization and establishment of *in vitro* regenerated plantlets in green house and field conditions are a critical step for commercial applications and also because it finally determines the success of micropropagation protocol (Preece and Sutter, 1991). During acclimatization the tissue cultured plantlets need relatively high humidity for establishment in soil conditions (Preece and Sutter, 1991). Generally, 4-6 weeks old well rooted plantlets are transferred to different pots in a growth chamber and completely covered with polyethylene bags to maintain maximum humidity. These polyethylene bags are gradually removed for acclimatization. The prevailing conditions (humidity and temperature) of the transplanting season greatly influence the initial survival of potted plantlets (Azad *et al.*, 2003). Bhat *et al.* (1995) reported the successful acclimatization of micropropagated plantlets in sterilized soil in green house after two weeks of transferring. These plantlets are then successfully established in field conditions after 4 weeks. Philip *et al.* (1992) reported the acclimatization of plantlets in compost (peat: sand: soil, 2: 1: 1). Nalawade and Tsay (2004) and Anand and Rao (2000) also reported similar conditions for acclimatization of micropropagated plantlets of *P. nigrum*. Different reports suggested that micropropagated plantlets of piper species are superior to traditional propagules in field performance. Additionally these plantlets showed better establishment and early flowering compared to conventionally grown plants (Nazeem *et al.*, 1992). Micropropagated plantlets are analogous clones of parental plants with good plant height, number of spikes per unit area, number of laterals per unit area, internodal length, mean yield, better fruiting, fresh weight, dry weight and higher oil contents as compared to seed-derived plants (Nazeem *et al.*, 2004)

CHAPTER TWO

2 .0 MATERIALS AND METHODOLOGY

2.1 STERILIZATION OF GLASSWARES AND EQUIPMENTS

Glassware such as culture vessels, petridishes, beakers, test tubes and dissecting tools (forceps, blade holder) were soaked for two hours inside a basin containing water, sodium hypochlorite and liquid soap. After two hours, glasswares was scrubbed with brush and rinsed with water containing sodium hypochlorite and again rinsed two to three times with running tap water so as to remove components of the liquid soap. The washed glassware were placed in the oven along with the forceps and scalpel for 15 minutes at 250^0c. After oven drying the glassware, the tools(forcep and scalpel) were wrapped with aluminium foil and placed in the autoclave for 30 minutes at 121^0c temperature and pressure (15 psi)

2.2 MEDIA PREPARATION

Half a litre of Murashige and Skoog Media was prepared (500ml) according to the manufacturer's instructions..

- 300ml of sterile distilled deionized water was measured into 500ml beaker and the beaker was placed on a hotplate. A stir bar was dropped into the beaker which stirred the deionized water.
- While the water was stirring, 25mls of macro-nutrient solution (stock 1) was added to the water.
- 2.5ml of micro nutrient solution (stock 2) was added to the deionized water.
- 2.5ml of vitamins and 15g of sucrose(stock 3) was added.

- Other supplements were added, which included,

  0.0187 of EDTA,0.05g of insitol,0.013g of iron (Fe_2so_4).

  After the Ms media preparation, 10ml each was pipetted into 10 smaller vessels. The vessels were labeled from A to J and covered with aluminium foil.

- BAP (0.1mg/l), 0.1mg/l of NAA and 0.1mg/l of kinetin was prepared. BAP (0.1mg/l) was prepared by pipetting 10ml of BAP solution into a measuring cylinder and then adding 90ml of distilled water to make it up to 100ml.

- Same method of preparation was used for 0.1mg/l of NAA and 0.1mg/l of kinetin.

  Concentration of BAP, NAA and kinetin to be measured.

TABLE 1 : `Concentration of hormones in the labeled vessels

Labeled vessels	Concentration/treatment
A	0.25mg/l of BAP + 0.05mg/l of NAA
B	0.50mg/l of BAP + 0.05mg/l of NAA
C	0.75mg/l of BAP + 0.05mg/l of NAA
D	1.0mg/l of BAP + 0.05mg/l of NAA
E	Control Ms only
F	0.25mg/l of kinetin + 0.05mg/l of NAA
G	0.50mg/l of kinetin + 0.05mg/l of NAA
H	0.75mg/l of kinetin + 0.05mg/l of NAA
I	1.0mg/l of kinetin + 0.05mg/l of NAA
J	Control Ms only.

Note: BAP (6-benzyl amino purine) and kinetin (6-furfuryl amino purine) vary while NAA (α - Napthalene acetic acid) remain constant for all treatments.

- Different volumes of BAP and NAA were pipetted into vessel A to D while different volume of kinetin and NAA was pipetted into vessels F to I.

Note: Vessel E and J served as the control, it is a hormone-free medium containing only the MS medium.

- The PH of each solution in the vessels was adjusted to 5.8 using NaoH and HCl.
- Deionized water was added to the initial 10ml/g of Ms medium in the vessels so as to make it up to 25m/l which is the final volume.

- After this 0.2g of agar was added to each solution in the vessel, the vessels was taken to the oven so as to heat them until the solution becomes clear.
- The media was dispensed into 50 culture tubes i.e 5mls each was dispensed into each culture tube. The test tubes (culture tubes) containing the medium was placed in a validated autoclave for sterilization for 30minutes at 121^0C.

2.3 COLLECTION OF MOTHER PLANTS

The mother plant of *Piper guineense* was collected from the field at NACGRAB's compound, Moor plantation, Ibadan Oyo State. The plant tissue culture was carried out in the tissue culture laboratory at National Centre For Genetic Resources and Biotechnology (NACGRAB) Ibadan Oyo state.

2.3.1 EXPLANT SELECTION

The nodes of the plant was used as the explant. The explants were excised from the mother plant using surgical blade and the nodes were placed inside a vessel containing distilled water.

2.4 SURFACE STERILIZATION OF EXPLANTS

The nodes were thoroughly washed under running water with a drop of liquid soap to eliminate the surface dirt. It was rinsed several times with running tap water until there was no trace of liquid soap on the explant. The final rinsing was done by rinsing the explant with distilled water. The nodes were placed in different vessels, 70% of ethanol was added to each of the vessels for 5 minutes. After 5 minutes, the ethanol was decanted and the explant was rinsed with distilled water, 25% and 30% concentration of sodium hypochlorite was prepared for

surface sterilization of explant. The laminar flow hood was switched on and swabbed with 70% ethanol using cotton wool. Prepared concentration of Sodium hypochlorite was placed inside the laminar flow hood. The explant was sterilized using double disinfection method in which the explants was submerged in 30 % of the hypochlorite for 10 minutes and rinsed with sterile distilled water 4 times. After this , 25% of the hypochlorite was added to the explants for 5 minutes and rinsed with sterile distilled water 4 times.

2.5 INOCULATION/ CULTURE PROCEDURE

After the laminar air flow hood had been switched on and surface sterilized, the spirit lamp was filled with ethanol and the culture tools were dipped inside ethanol. The culture materials was sprayed with 70% ethanol and neatly arranged inside the flowhood. The spirit lamp was lit , the forceps and scalpel were flamed and allowed to cool and dipped back inside the ethanol. The forceps were used to hold the explant while the scalpel was used to cut the explants into smaller sizes and inoculated on to the media in the culture tubes. The culture tubes were sealed with parafilm and labeled with name of the plant and the date in which it was cultured.

2.6 INCUBATION:

The culture vessels were placed in the growth room at 26±2°C temperature

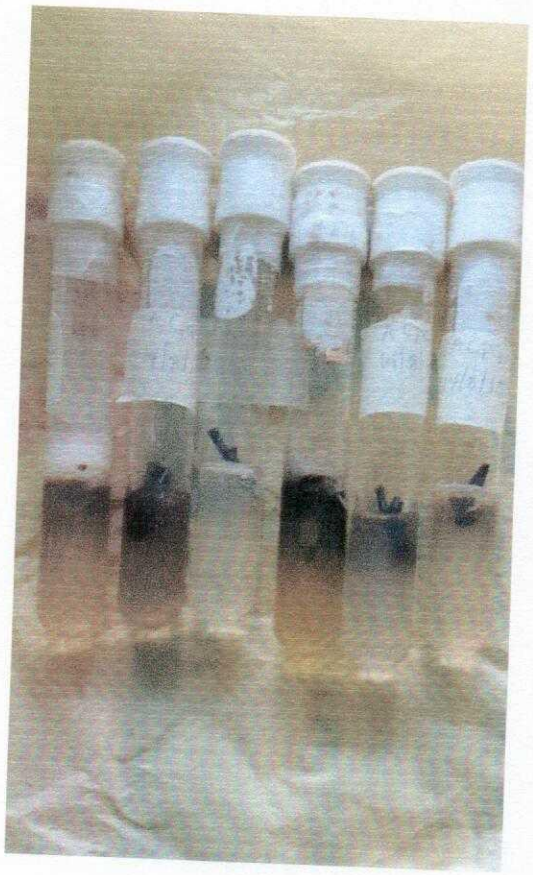

Plate 3: plantlet inoculated after 3 weeks with contamination

Plate 4: plantlet inoculated after 5 weeks with contamination

TABLE 2 : Concentration of growth regulators and their replicates

TREATMENT	REPLICATE
BAP 0.25mg/l + NAA 0.05mg/l	5
BAP 0.50mg/l + NAA 0.05mg/l	5
BAP 0.75mg/l + NAA 0.05mg/l	5
BAP 1.0mg/l + NAA 0.05mg/l	5
CONTROL	5
KIN 0.25mg/l + NAA 0.05mg/l	5
KIN 0.50mg/l + NAA 0.05mg/l	5
KIN 0.75mg/l + NAA 0.05mg/l	5
KIN 1.0mg/l + NAA 0.05mg/l	5
CONTROL	5

CHAPTER FOUR

4.0 DISCUSSION

As shown in the experiment, *Piper guinnense* might have high innoculum of micro organism within its tissue. Tissue culture provides a best tool for large scale production of propagules especially in the case of endangered medicinal plants. *Piper guineense* is reputed for its various medicinal and pharmaceutical properties. Due to its high demand in local, national and international drug manufacture, illegal, unscientific and indiscriminate extraction of *Piper guineense* from its wild habitat has increased. Micro propagation provides a best tool for large scale production of propagules and its conservation especially in case of endangered medicinal plant, where explant material is available in a very small quantity. Viability of seeds, age of explant and the tissue source from which the explant is excised are very important for high frequency of regeneration. The most important treatment prior to culture initiation is perhaps surface sterilization of plants, because repeated surface sterilization was not successful in establishing healthy cultures of *Piper guinnesse*. Since *in-vitro* propagation provides suitable environments for growth of fungi and bacteria, unsuccessful sterilization hindered the progress of micro propagation in this study. Many of the organisms that are resident on mammalian skin can survive in *in-vitro* cultures and therefore faulty aseptic techniques can also result in contamination.

Explants from field–grown plants, diseased specimens or from plants parts which are located close to or below the soil may be difficult or impossible to disinfect due to both endophytic and epiphytic microbes (Leifert *et al* ., 1994). Epiphytic bacteria may lodge in plant structures where disinfectants cannot reach (Gunson and Spencer- Philip,1994 ; Leifert *et al* ,1994).

Contaminants found at explant initiation, present in explants from several collection dates and resistant to surface disinfection are likely to be endophytic (Reed, *et al,* 1995). Therefore, reduction of contamination requires efficient aseptic techniques in tandem with effective sterilization methods (Falkiner, 1990). Sterilization of a material (explants/ seeds) before subjecting them for *in-vitro* propagation is essential for the production of 'clean' *in-vitro* plantlets that ensures the reduction of the contaminants as well as high survival rate of explants. Chang *et al*. (2001) found that only few of this species with explants from mature trees/climber have been propagated by tissue culture methods. The longer life span of trees/climbers may add to the problem of contamination *in vitro* by the symbiotic association of microorganisms. It is evident that there are few micropropagation protocols for mature tree explants. Also, many woody plant species and particularly tree legumes are known for their *recalcitrant* nature for regeneration (Jha *et al*., 2004; Anis *et al*., 2005; McCown, 2000). In many woody plants, it is much easier to establish juvenile than adult explants *in vitro*.

Generally, preconditioning of explants with media supplements such as growth regulators, culture condition and media composition are necessary conditions for multiplication in new candidates when establishing tissue culture protocol. Thus more work on preconditioning of *Piper guineense* for tissue culture needs to be done if this very useful forest plant is to be domesticated

4.1 CONCLUSIONS

Preventing or avoiding microbial contaminat in plant tissue culture generally is critical to successful micropropagation. Epiphytic and endophytic organisms can cause severe losses to micro propagated plants at each stage of growth and also bacterial and fungi contaminats are often difficult to detect because they remain mostly within the plant tissue. These problems with decontamination of explants have delayed the development of *Piper guineense* micro propagation system. Establishing natural regeneration trend of threatened species such as *P. guineense* is important as it may provide bench mark for utilization trend and conservation strategies. Further, for effective propagation there is a need for developing alternative *in vitro* methods for the regeneration of the climber species in the red list – i.e., species threatened with extinction.

4.2 RECOMMENDATIONS

There are several other aspects that have to be evaluated before *Piper guineense* micro propagation system could be recommended. The use of antibiotics in culture media may help in the successful culture of *Piper guineense* explants. This is the area of further work in the establishment of culture methods for micro propagation of *Piper guineense*.

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