ISOLATION AND IDENTIFICATION OF FUNGI ASSOCIATED WITH *IRVINGIA* SPECIES KERNELS FROM SOME MARKETS WITHIN ZARIA METROPOLIS AND THEIR AFLATOXIN CONTENT

BY

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DECLARATION

The research work in this dissertationentitled “ISOLATION AND IDENTIFICATION OF FUNGI ASSOCIATED WITH *IRVINGIA* SPECIES KERNELS FROM SOME MARKETS WITHIN ZARIA METROPOLIS AND THEIR AFLATOXIN CONTENT”has been performed by me in the Department of Biological Sciences, Ahmadu Bello University, Zaria, under the supervision of Prof. S.O. Alonge and Prof. A. B. Zarafi.The information derived from the literature has been duly acknowledged in the text and the list of references provided. No part of this dissertation was previously presented for another degree or diploma at any university.

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CERTIFICATION

This dissertationentitled “ISOLATION AND IDENTIFICATION OF FUNGI ASSOCIATED WITH *IRVINGIA* SPECIES KERNELS FROM SOME MARKETS WITHIN ZARIA METROPOLIS AND THEIR AFLATOXIN CONTENT” by ONUOHA, GODDY CHIBUEZEmeets the regulations governing the award of the degree of Master of Science at Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

Prof. S. O. Alonge _____ _____
Chairman, Supervisory Committee Signature Date

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Member, Supervisory Committee Signature Date

Prof. A. K. Adamu _____ _____
Head,Department of Biological Sciences Signature Date

Prof. K. Bala _____ _____
Dean, School of Postgraduate Studies Signature Date

DEDICATION

This work is dedicated tothe blessed Holy Spirit, the ultimate teacher of all truths.

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ABSTRACT

This study was carried out, in order to isolate, identify the various fungi associated with *Irvingia* kernels and to ascertain whether or not there was significant aflatoxin content in *Irvingia*kernels sold in five markets in Zaria. The two species of *Irvingia*studied were *Irvingiagabonensis*and *Irvingiawombolu*. Commercially available Potato Dextrose Agar (PDA) served as the general purpose culture mediaandSabouraud Dextrose Agar (SDA) served as the selective media, which was used in the preparation of pure cultures. The cotyledons and thetesta were examined separately. *Aspergillusflavus, Aspergillusfumigatus, Aspergillusniger, Penicilliums*pecies and *Rhizopusstolonifer* were isolated from both *Irvingiagabonensis*and *Irvingiawombolu*testa and cotyledons. Four fungal species, namely, *Absidiacorymbifera, Aspergillusversicolor, Mucor* species and *Phomaherbarum* were only isolated from *Irvingiawombolu* cotyledons and testa.More *A.flavus, A .niger*and *Penicillium* sp. were isolated from the testa of both *I. gabonensis* and *I. wombolu*,than from the cotyledons. *Rhizopusstolonifer* had the highest percentage occurrence in the cotyledons of both *I.gabonensis* (69.44%) and*I.wombolu* (47.43%). *Aspergillusniger*had the highest percentage occurrence (37.59 %) of all the fungi isolated, and was closely followed by *R.stolonifer*(33.81 %).Similarly, *A. niger*had the highest percentage occurrence in the testa of both *I.gabonensis* (45.71%) and*I.wombolu* (42.94 %). Also, *I.gabonensis* samples from SabonGari market had the highest mean number of fungal species (12), while Dan Magaji/Wusasa market had the least (2). Similarly, *I.wombolu* samples from A.B.U Community, Dan Magaji/Wusasa and Tudun Wada markets all had the highest mean number of fungal colonies (24), while Samaru market had the least (22). Enzyme-Linked Immunosorbent Assay (ELISA) of the kernels showed that,*I.gabonensis* and *I.wombolu* kernels from SabonGari market had the highest aflatoxin concentrations (108.36 and 95.60µg/kg) respectively. Similarly, *I.wombolu* kernels from Dan Magaji/Wusasa market had the least

(70.28μg/kg) aflatoxin concentration, while *I.gabonensis* kernels from Samaru market had the least (75.02μg/kg). They were all beyond the World Health Organization (WHO) standard for food samples, which is 20μg/kg, hence not safe for human consumption. The study concludes that,the two *Irvingia* species kernels sold in some markets within Zaria metropolis were contaminated by several post-harvest fungi. One of these fungi isolated,*Aspergillusflavus*, had the capacity to produce aflatoxins, therefore the consumption of these kernels puts the consumers at risk.

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LIST OF ABBREVIATIONS

A.corymbifera	=	*Absidiacorymbifera*
A .B. U	=	Ahmadu Bello University
A C S	=	American Chemical Society
A. flavus	=	*Aspergillusflavus*
A .fumigatus	=	*Aspergillusfumigatus*
A. niger	=	*Aspergillusniger*
A. versicolor	=	*Aspergillusversicolor*
ELISA	=	Enzyme Linked Immunosorbent Assay
F. A. O.	=	Food and Agricultural Organization
HCl	=	Hydrochloric acid
HR P	=	Horse Radish Peroxidase
H K M	=	Huankai Microbial company
I B A	=	Indole – 3 –Butyric Acid
I A R	=	Institute for Agricultural Research
I R A D	=	Institute de la Recherche´Agricole pour le Development,Cameroon
I C R A F	=	International Centre for Research in Agroforestry, Ibadan
I. gabonensis	=	*Irvingiagabonensis*
I. wombolu	=	*Irvingiawombolu*

$KHCO_3$	=	Potassium Hydrogen Carbonate
NaCl	=	Sodium Chloride
N A E R L S	=	National Agricultural Extension Research and LiaisonServices
N P K	=	Nitrogen Phosphorus Potassium
N T F P	=	Non –Timber Forest Product
N W F P	=	Non –Wood Forest Product
P R C	=	Peeople´s Republic of China
P. herbarum	=	*Phomaherbarum*
P D A	=	Potato Dextrose Agar
P S I	=	Pounds per Square Inch
R. stolonifer	=	*Rhizopusstolonifer*
S D A	=	Sabouraud Dextrose Agar
Sp. ; Spp.	=	Species
W H O	=	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 General Introduction/Background Information on *Irvingia* species

The natural forests of West and Central Africa are rich in resources, and have tremendous biodiversity (FAO, 1983), particularly in trees that provide food, fuel, fibre, medicines and various other products, including construction and building materials (Ladipo*et al.,* 1996).

The tropical rainforests of West and Central Africa is the home to many native economically important trees such as the *Irvingia* species, namely *Irvingia gabonensis*, and *Irvingiawombolu*(Harris, 1996; Lowe *et al.,* 2000).

The *Irvingia* plant is dicotyledonous, and belongs to the *Irvingia*ceae family, and order Malpighiales. It is known by a variety of local names, both within and outside Nigeria; among which are bush mango or African mango for English speakers. In Yoruba language, the tree is known as "Oro", while the kernel is known as "Apon" (Adebayo-Tayo*et al.,* 2006). Ndigbo call it Adu, Ugiri or Ogbono depending on the species (Okafor, 1978). In Nupe, it is called "Pekpeara", "Ogwi" in Bini (i.e. Edo), "Uyo" in Efik (Okafor, 1984), 'Oro-Akpele' in Igala. The Eton people of Lekie division in Cameroon call it 'Azang', 'Andok' or 'Beti'. The Mpong Mpong people of Haut Nyong division in Cameroon call it "Ogno'k", while the Bulu people of Mvila division in Cameroon call it 'Andoh'. In Gabon, it is known as the "Dika" nut (Ayuk *et al.,* 1999). In french, it is known as 'Manguier sauvage'. The paste produced from the kernels is termed "dika bread" in Gabon, while in Cameroon, it is known as "etima" (Ndoye *et al.,* 1997), it is also called 'Borborou' by the Abbey (Cote D'ivoire) and 'Ewewe' by the Bolon tribe (Gabon) (Vivien and Faure, 1988; 1996; Ake-Assi,1991; Ndoye and Tchamou, 1994; Tabuna, 1997).

All these names cut across South East, South South, South Western and North Central Nigeria, as well as some other Western and Central African countries where the *Irvingia* plants are grown, or where the kernels are eaten in one form or the other. The generally accepted common name in Nigeria is "Agbono" or "Ogbono" (Festus and Nwala, 2012).

The *Irvingia* tree is large, attaining a height of about 50m when it grows naturally in the wild, but under improved agricultural practices, especially when budded seedlings are planted, it could attain a height of about 22.5m, with a straight bole, cylindrical, slightly buttressed (Festus and Nwala, 2012). The buttresses reach a height of 3m (9.8ft) (Orwa*et al.*, 2009). The cylindrical trunk exceptionally exceeds one metre in diameter, with a well developed and maintained support (Festus and Nwala, 2012). It has a hard, heavy and durable wood, with a fine grain, easy to polish, as well as being resistant to termite.

The leaves are about 1.5 – 1.7cm long, spirally arranged and grouped towards the tips. It is alternately arranged, simple, elliptic and shiny on both surfaces (Festus and Nwala, 2012).Under natural conditions, the *Irvingia* tree matures (reproductive maturity) between the ages of 8 – 10 years in some cases, while in some others maturity is delayed till between 15 – 20 years (Festus and Nwala, 2012). However, with modern improved cultural management practices, which include budding and topping, especially with the use of budded seedlings, the vegetative phase is considerably reduced, and flowering and fruiting can start from 4 – 5 years, while economic yield can be attained after 7 – 8 years from planting (Ladipo *et al.*, 1996).

Maturity of the *Irvingia* tree refers to the stage when the plant begins to produce flowers, in readiness for fruitification. The first stage in the flowering process is floral induction or evocation (Sedgley and Griffin, 1989). It is not known what triggers this process in *Irvingia* species, but a substantial variation was observed in the number of floral flushes within the

population of 182 trees planted in 1990, with most trees not flowering at this age, or flowering only once a year, with a few flowering 2 – 4 times per year (Ladipo *et al.,* 1996).The flowers produced during the flowering period are arranged in axillary racemes, fragrant, small, greenish and hermaphrodite. They bear disks that become bright and yellow during ripening stage (Alston, 1992).*Irvingia gabonensis* flowers in February – March, and fruits in the rainy season (July – September), while *Irvingia wombolu* flowers in October, and fruits in the dry season (January – March), (Franzel *et al.,* 1996).Fruiting on the other hand, sets in when the flowers produced at the stage of flowering are pollinated by insects such as Coleopterans, Dipterans, Hymenopterans and Lepidopterans (Orwa *et al.,* 2009), as well as mammals and birds.The maturedfruit is greenish when unripe, but turns yellow when ripe, with a fleshy mesocarp. The fruits are broadly ellipsoid, about 4 – 7 cm long, and 3 – 5 cm in diameter. The fruits of the two *Irvingia* species resemble those of the common mango (*Mangifera indica*), hence the name African or bush mango (Okafor, 1975; Simons and Leakey, 2004; Matos*et al.,* 2009).The fruit of *Irvingia gabonensis* is usually bigger than that of *Irvingia wombolu.*When ripe, the fruit of *Irvingia gabonensis* has a sweet edible mesocarp, hence it is usually consumed by man and animals fresh, whereas *Irvingiawombolu* has a bitter or sour taste, and very slimy mesocarp, hence rarely eaten by man and animals (Etukudo, 2000; Fajimi *et al.,* 2007).

1.2 Uses of *Irvingia*

Every part of the *Irvingia gabonensis* and *Irvingia wombolu*tree is useful, ranging from the stem, fruits and kernels, to the leaves. The stem supplies durable timber for construction purposes. The branches supply firewood, as well as chewing stick. The fruit mesocarp of *Irvingia gabonensis* in particular, is consumed by both man and livestock (Ayuk *et al.,* 1999).

It serves as dessert fruit or snack throughout Western and Central Africa. The fruit mesocarp is equally used for the preparation of juices, jams, jellies, wine, as well as in soap making (Okafor, 1985; Shiembo *et al.,* 1996; Leakey *et al.,* 2003). The fruit juice obtained from the pulp is rich in vitamin C (Ejiofor, 1994; Leakey and Newton, 1994).The leaves and stem bark are employed as purgative, for gastro-intestinal and liver conditions, for hernias and urethral discharge, or for sores and wounds (Ayuk *et al.,* 1999).In some traditional/cultural practices, the split seed shell is used in prediction or fortune telling (Abbiw, 1990). The mature fruits serve as ripening agent for bananas and plantains. Environmentally, the *Irvingia* tree serves as wind break (Ladipo, 2000).

1.3 Fungal Attack of *Irvingia* kernels

Irvingia gabonensis(Plate I) and *Irvingia wombolu*(Plate II) kernels are economically valuable due to the fact that they have both health and medicinal benefits (Duguma *et al.,* 1990; Ndoye *et al.,* 1997; Van 2010). However, the sales and consumption of *Irvingia* kernels has a major set back, since the kernels are susceptible to post-harvest spoilage fungi, with their attendant health risks (Etebu and Bawo, 2012). Fungal contaminated *Irvingia* kernels are potentially harmful to those who consume them (Adebayo-Tayo *et al.,* 2006), as it has been observed that these contaminated kernels could possess aflatoxin.

Mycotoxins are toxic secondary metabolites naturally produced by molds (fungi), (Pittet, 2005). Mycotoxin is a term derived from the Greek word “Mykes” meaning fungus, and the Latin word “toxicum” meaning by poison (Adjou *et al.,* 2012).Aflatoxin is just one out of the many mycotoxins produced by harmful fungi. Aflatoxin is a group 1 carcinogen, proven to cause liver cancer, and also suppresses the immune system and ultimately death when consumed in high doses (Lewis *et al.,* 2005; Strosnider *et al.,* 2006).

Plate I: *Irvingiagabonensis* kernels

Plate II: *Irvingiawombolu* kernels

1.4 Statement of Research Problem

The fungal attack on post-harvest crops is quite alarming (Harris, 1996). These fungi of post-harvest crops, naturally produce mycotoxins which could constitute health hazard to the unsuspecting consumers (Cotty *et al.*, 1994). These mycotoxins may remain in food long after the fungal source has died, and are hardly destroyed by cooking or freezing as mycotoxins can remain stable for up to 268 – 269^0C (Frazier and Westhoff, 1988). Mycotoxins resist decomposition or being broken down in digestion, so it remains in the food chain (Mahendra *et al.*, 2012).

Mycotoxins are quite numerous, among which are aflatoxins, Ochratoxins, Fumonisins, Zearalenone. Out of these, aflatoxins have assumed economic importance, because of their influence on the health of human beings and livestock, as well as the marketability of agricultural products. In most developing countries, limited or no facilities exist for monitoring these toxins in foods and feeds. Since aflatoxins are ubiquitous in the food and feeds substances consumed locally, there is therefore the need to monitor their levels so that they do not exceed the World Health Organization (WHO) standard for food samples, which is 20 μg/kg (Oladejoh and Adebayo-Tayo, 2011). To the best of my knowledge, the aflatoxin content in *Irvingia gabonensis* and *Irvingia wombolu* kernels sold in some markets in Zaria, is not known.

1.5 Justification

The *Irvingia gabonensis* and *Irvingia wombolu* kernels are widely consumed by man globally (Matos *et al.,* 2009), either as sauce thickening agent and oil for the preparation of the famous, viscous "Ogbono soup", or in the making of "Dika cake" especially in Gabon. The two species of *Irvingia* kernels are also valued for their health and medicinal benefits (Duguma *et al.,* 1990; Ndoye *et al.,* 1997; Van, 2010). Studies have shown that seed extract of the kernels caused a significant reduction in body weight among obese people in Cameroon (Ngondi *et al.,* 2005). These benefits therefore, make the market for *Irvingia* kernels very robust and economically valuable. The trade of kernels of the two species from Nigeria, Gabon, Equitorial Guinea, Central African Republic, and Cameroon, has been valued at US$260,000 per annum (Ayuk *et al.,* 1999).

Unfortunately, fungi that produce harzardous mycotoxins attack these kernels, thereby making them potentially harmful to both man and livestock that consume them (Adebayo-Tayo *et al.,* 2006; Iyayi *et al.,* 2010). One major mycotoxin that fungal contaminated kernels possess is aflatoxin (Wu and Khlangwiset, 2010). *Aspergillus flavus* and *Aspergillus parasiticus* have been implicated in the possession of aflatoxins (Rizzo *et al.,* 2004), and in this work, *Aspergillus flavus*was identified from the samples, including other fungal species that are potentially harmful.

For these reasons, the need to quantify the level of aflatoxin present in the *Irvingia* species kernels sold in some markets in Zaria is therefore imperative, as this would provide information on the safety of the kernels for human and animal consumption.

1.6 Aim

The aim of this study was to isolate and identify the fungal species that infest the kernels of *Irvingia gabonensis* and *Irvingia wombolu* kernels sold in some markets within Zaria metropolis, as well as to quantify their aflatoxin content.

1.7 Objectives

1. To determine whether the *Irvingia gabonensis* and *Irvingiawombolu* kernels sold in some markets within Zaria metropolis are attacked by fungi.
2. To isolate and identify the species of fungi attacking the *Irvingia* kernels from the markets
3. To determine whether the fungal species identified were potentially aflatoxic
4. To determine the aflatoxin concentration or level of the kernels of the two species

1.8 Hypotheses

1. The *Irvingia gabonensis* and *Irvingiawombolu* kernels sold in some markets within Zaria metropolis are not infested with fungi
2. The species of fungi infesting the *Irvingia* kernels from the five markets cannot be isolated and identified.
3. The fungal species identified were not potentially aflatoxic
4. The aflatoxin concentration or level cannot be determined.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Ecophysiology of *Irvingia* plant species

Irvingia gabonensis and *Irvingia wombolu*, belong to the group of plants classified as "non timber forest products" (NTFP), or "non wood forest products" (NWFP) (Ladipo, 1999; Ayuk *et al.,* 1999). They have as their natural habitat, Angola, Cameroon, Congo, Cote d'ivore, Siera Leone, Sudan and Uganda (Orwa *et al.,* 2009). It is now planted in parts of Benin, Cote d'ivore, Ghana, Southern Cameroon, South-Western Nigeria and Togo (Ladipo*et al.,* 1996; Orwa *et al*., 2009).

The *Irvingia*species trees are present in the tropical wet and dry climate zone, occurring naturally in the canopied jungle, gallery forests and semi deciduous forests, growing at altitudes 200 – 500 m. their growth is favoured by temperature ranges from 20 – 38^{o} C under slightly shaded to very bright clear skies.

The *Irvingia* species preferred habitat is undisturbed lowland tropical forest (Van Dijk, 1997). They are better adapted to utisol soils (sandy clay loams and loamy clay soil) in high rainfall areas, than to less acidic soils (Kang *et al.,* 1994), but the two species still differ within these areas. According to Okafor (1975), *Irvingia gabonensis* prefers well-drained sites. Deep soils with more than 150 cm (59in) are needed, with a moderate fertility, with pH ranging from 4.5 to 7.5 (Ayuk *et al.,* 1999). The annual rainfall requirement for *Irvingia gabonensis* is 1000 – 1500 mm. In Nigeria, the tree is found growing between latitudes 4.15 and 8.99^{0}N of the equator, especially along streams and the banks of riversand in village homesteads (Okafor, 1983).

Irvingia wombolu on the other hand, thrives in wetter conditions, with mean annual rainfall ranging between 1500 – 2500 mm, preferring dry or seasonally flooded soils (Orwa *et al.,* 2009).

In some localities, *Irvingia wombolu* is common in swamps and seasonally flooded forest than in adjacent dry land forest.

Irvingia gabonensis and *Irvingia wombolu* are very similar, and are often difficult to differentiate from herbarium specimens alone (Harris, 1996). There are howver, some characteristics that differentiate the two, such as the edibility of the fruit mesocarp, where *Irvingia gabonensis* fruit mesocarp is sweet, while *Irvingia wombolu* mesocarp is very bitter and quite slimy (Orwa *et al.*, 2009). Other diagnostic characters that differentiate them include the density of the fibres in the mesocarp, the amount of mucilage in the cotyledons, the size of the endosperm, the height of first branching, the disc shape in unfertilized flowers, and the shape of the tree (Harris, 1996).

Irvingiagabonensis is a tree 10 – 40 m tall, buttress to 3m high, straight and unbranched (in wild trees), crown is approximately spherical, or fatter than wide; foliage dense and dark green leaves are elliptic, about 4.5 – 8 cm in length, and 2 – 4 cm in diameter. Inflorescence is axillary, laxly branched panicle to 9 cm long flowers in fascicles, pedicels up to 5 mm long; sepals 1 – 1.5mm; petals yellowish white; 3 – 4 mm long; filaments 4 – 5 mm long. Fruit is ellipsoid to cylindrical, occasionally almost spherical, only slightly laterally compressed; length 4.6 – 5 cm, width 4.2 – 6.4 cm green when ripe, mesocarp bright orange, soft and juicy when ripe with few weak fibres and sweet seeds 2.5 – 3.8 cm long, 1.7 – 2.7 cm wide and seedling have purple to red cotyledons,hypocotyl and stipules; first pair of leaves opposite and stem pale green to purple (Harris, 1996).

Irvingia wombolu is a tree up to 25m tall; buttresss to 2m high; bole often slightly leaning; first branch usually at 7 – 10m, foliage regular, not as dense as *Irvingia gabonensis*. Leaves are elliptic to obovate at least some leaves distinctly obovate; apex rounded often with a barely distinct blunt acumen. Inflorescence a laxly branched panicle, to 9 cm long, axillary and on older

twigs. Flowers crowded together at the end of the inflorescence branches or in fascicles; pedicels to 6mm long; sepals 1mm; petals whitish 3 – 4mm long; filaments 5mm long; disc bright yellow, diameter 2 – 3mm. fruit ellipsoid, only slightly compressed laterally; length 4.5 – 5.8cm, width 4.5 – 5 cm; green on falling often turning bright yellow and then black as the mesocarp starts to rot; mesocarp soft and juicy when ripe, fibres more obvious than in *Irvingia gabonensis* mesocarp very bitter and completely inedible. Seeds 3.5 cm – 5 cm long, 1.7 – 2.6 cm in diameter, 8 – 10 mm thick (Harris, 1996).

2.2 Cultivation practices of *Irvingia* plant species

2.2.1 Propagation of *Irvingia* plant

At present, most farmers maintain mature bush mango trees that are already growing on their land, and will also transplant wild seedlings onto their own farms, or grow up new seedlings (Ayuk *et al.*,1999).

Naturally, that is to say, in the wild, *Irvingia gabonensis* and *Irvingia wombolu* are commonly propagated by seeds. These seeds are usually from the ripe fruits that fall to the ground, leading to the rottening of the mesocarp, leaving the stony endocarp to germinate epigealy with time. The ripe fruits are sometimes eaten by forest elephants (*Loxodonta Africana cyclotis*) (Tchamba and Seme, 1993). Howes (1948) also reported that gorillas (*Gorilla gorilla*) eat the fruits of the *Irvingia.* These wild animals including man, eat the mesocarp, and discard the stony endocarp, either in the dung of the forest elephant, or gorilla, and man that throw away the endocarp after consuming the mesocarp. These discarded endocarp later germinate into the *Irvingia* plant wherever they are released.

*Irvingia gabonensis*and *Irvingia wombolu* are now planted and maintained on farms, throughout their range in Central and Western Africa. Planting is common in Nigeria (Ejiofor *et al.*,1987),

more predominantly on outlaying farms than on compound farms or homestead (Okafor, 1983; Orwa *et al.,* 2009). Farmers select seeds from trees that are known to produce high yields of good fruit, either on their land, or from a neighbour. Germination of *Irvingia gabonensis* seeds takes upwards of 14 days, and first requires that the seeds are extracted from the fruits and dried for at least 2 days. Okafor (1997) reported 80% germination if seeds are treated this way. *Irvingia* trees grown from seeds may take over 10 years to start producing fruit and although growing seeds may be the easiest way to improve cultivation, farmers would like to see a financial return much sooner (Moss, 1995). Faster propagation techniques are hence needed to produce trees for the selection of desirable phenotypes needed for further breeding (Ainge and Brown, 2001). Efforts are on to obtain early flowering (and fruiting) through cultural management practices which include budding and "topping" (Orwa *et al.,* 2009), and many workers are involved.

Shiembo *et al.* (1996) experimented with different methods for growing cuttings taken from mature trees. They found that sawdust or a similar organic substrate is the best medium for rooting, and that the cuttings must have a leaf area of at least 80 $cm^2$to survive and grow well. The Auxin, indole-3-butyric acid (IBA) makes no significant difference to growth if applied, hence not necessary. The technique is ideal for farmers in tropical Africa because it does not require electricity or piped water. Tchoundjeu *et al.* (1997), however, were of the opinion that propagation by stem cuttings is notoriously difficult because the cuttings do not root. Tchoundjeu *et al.*(1997) investigated a technique that he claims to be preferable, known as marcotting (or air layering) where the branch of a mature tree is bark girdled, and the girdled ring is wrapped in a damp medium, inducing rooting. Despite a low rooting success rate (30%), and an even lower survival rate (10%), he states that this is still a useful technique, which can be improved. Rooting

hormones, more careful handling of the rooted propagules, and initial growth under a non-mist propagator could all significantly increase the survival rate of the propagules.

Okafor (1997), carried out bud grafting, where a bud was grafted from a mature tree, onto a seedling. This technique, he reported, can reduce the fruiting age of the tree from 2-4 years, and also lower the height of the fruit set from 8 m or more, to 1 – 3 m. However, Leakey *et al.* (2000), are of the view that this process or technique may not be as successful as propagation by stem cutting, because of problems with grafting incompatibilities. In Nigeria, workers have detailed standard methods for growing *Irvingia gabonensis* in nurseries (Leakey, 1999a). Developing propagation techniques with high success rates and multiplication rates iscrucial for the production of superior planting stock for farmers and for the domestication process. IRAD (Institute de la Recherche Agricole pour le Development), and ICRAF (International Centre for Research in Agroforestry) are currently researching into improvements of existing propagation methods in Cameroon and Nigeria (Leakey *et al.,* 2000).

2.2.2 **Nursery**

Ground nursery and movable nurseries can be used to germinate the seeds. However, movable nurseries such as plastic bags, baskets, or perforated trays are recommended instead of ground nurseries due to some advantages movable nurseries have over ground nurseries.

i. Labour cost incurred during digging up for transporting is avoided

ii. Risk of transporting shock is reduced.

iii. Space economy (Eze and Okafor, 1984).

If movable nurseries are used, then the plastic bags should not be smaller than 15mm gauge, 12 cm diameter, and 25 cm deep. The plastic bags should be filled with saw dust, while 1 cm on top should be left as catchment area during watering (NAERLS Bulletin, 1999).

2.2.3 Seed selection for planting of *Irvingia* species

Tree – ripened fruits (which fell by themselves) are selected by the farmer for nursery germination. Fruits that were plucked from the tree are not suitable for nursery germination. The fleshy mesocarp (pulp) is carefully cut out, and the stony endocarp is washed using sand and water (Okafor and Okolo, 1974). Allowing the pulp rot naturally, and then washing in water will reduce the germination percentage by about 20%. The nuts are dried in a shade for one or two days and then, planting can take place within two or three days after extraction.Prolonged drying for more than a week results in decreased seed viability and sun drying should be avoided completely (Shiembo *et al*., 1996).

Benlate or other potent fungicides can be used to treat the nuts before sowing, to avoid the incidence of damping-off. The polybags arecm moved to a shade after sowing, and arranged in 10 x 100 cm or 20 x 50 cm, and watered daily. Over-watering should be avoided to prevent water logging and damping off disease (Eze and Okafor, 1984). Nitrogen: Phosphorus: Potasium(NPK) fertilizer dissolved in water should be applied regularly, especially when nutrient deficiency symptoms such as chlorosis are noticed. One kilogram (1 kg) NPK may be applied in about 6 doses for 2000 seedlings, and it is expected that germination will start 3 weeks after planting, and may continue for another four weeks.

2.2.4 Budding of *Irvingia* seedlings

At ten to twelve (10-12) months after germination, the seedlings are ready for budding. Budding is done at about 15-20 cm height using the inverted “T” cut. The rootstocks to be budded should not be less than 0.5 cm in diameter at a height of 15 cm from the soil surface. Healthy, mature flowering trees should be the source of the budwood, and the rainy season is the recommended period for budding operations (NAERLS Bulletin, 1999).

2.2.5 Land preparation for field planting of *Irvingia* seedlings

The land is prepared for field planting between November and March. This includes clearing, felling of unwanted trees, cross-cutting, packing and burning. After burning, marking out of the planting sites follows, where 7m x 7m is recommended, giving a population of about 200 stands in a hectare. Planting holes are then dug measuring 60 cm x 60 cm x 45 cm. The holes are filled with well decomposed organic matter, and top soil two weeks before transplanting. Inorganic fertilizers are not to be put into planting holes prior to transplanting of seedlings, as this may harm the young seedlings (Eze and Okafor, 1984).

2.2.6 Transplanting of *Irvingia* seedlings

At ten to twelve (10 – 12) months after budding, seedlings are ready for transplanting. Field planting of the seedlings can be done 20 – 24 months after sowing in the nursery. Once the rains stabilize in the early part of the rainy season, transplanting can be done, which falls between May – June in the rain forest zone and June – July in the Savannah. It is advisable to transplant seedlings with the ball of earth around the roots of the plant, and if budded seedlings are planted, regular checking and pruning off of all side shoots from the stock is necessary (Eze and Okafor, 1984).

2.2.7 Fertilizer requirements of *Irvingia*plants species

Fertilizer can be applied one year after field establishement. If the planting holes are enriched with well decomposed organic matter and top soil, chemical fertilizers might not be required within the first year of establishment. Within the next year, that is 1 – 4 years after planting, and if the soil is fairly rich, an application of 0.25kg per stand for NPK (15:15:15; 10:10:20) or NPK:Mg (12:12:17:2) or any other compound fertilizer that contains NPK may be applied. Fertilizer application is better done during the rainy season. Where the soil is poor in nutrients,

then a higher dosage of 0.5 kg per stand is recommended. The plants are ring weeded before broadcasting the fertilizer around each stand once a year (NAERLS Bulletin, 1999).

2.2.8 **Topping of *Irvingia* plants species**

This is a management practice whereby all the growing shoots of the plant are shaped by clipping (i.e pruning or trimming). This is referred to as a "taming" process. Traditionally, constant bush burning and farming activities take care of "topping", and it has quite a number of advantages, such as giving the tree, the desired "dome" shape, instead of the conical shape that it is naturally inclined to. It also exposes more leaves to sunlight for enhanced photosynthetic activities leading to more fruit formation. It encourages early flowering, as well as controlling the height of the plant. The first topping is recommended between the second and third year, before the end of the rains. This is achieved by cutting back all the growing shoots, and should be repeated yearly for the next 3 – 4 years, (Alston, 1976, NAERLS, 1999).

2.2.9 Irrigation of *Irvingia* plant

Seedlings that are transplanted in May/June or June/July depending on location may be adversely affected by the following dry season (November – March), more so if the dry season starts early and extends to March – April of the following year. Mulching round the stands with grass and 20 – 25 litres of water per tree per week this period is recommended, as it will enhance survival rate and early productivity (precocity) (Eze and Okafor, 1984).

2.2.10 Replacement of Dead plants

Plants that do not germinate, are to be replaced by the next rainy season, and is to be maintained till all the missing stands are filled up. However, the cause of death of the dead plants should be ascertained before replanting into the planting holes. The planting site and planting materials should be "cleaned up" if the cause of death is either a disease or pest (NAERLS Bulletin, 1999).

2.2.11 Diseases and their control

Damping-off and leaf spot are nursery diseases of*Irvingia.*Damping off disease of *Irvingia* is caused by two fungi, namely *Pythium* spp. and *Phytophtora* spp. Damping is a very common feature whenever the *Irvingia* nuts are planted in unsterilized soils, and the seedlings remain susceptible till the cotyledons drop 3 – 4 weeks after planting.

Damping off can be controlled by avoiding water logging conditions in the field or nursery. Bordeaux mixture (copper sulphate mixed with lime) should be sprayed on the affected plant at the rate of 2kg/ha at 0.4 g of powder per litre of water. Complete wetting of affected parts will give efficient treatment. Dithane M-45 or Furadan could equally be used only on seedlings that were infected after the cotyledons had opened and the first 2 leaves appear light green in colour.

For leaf spot diseases, there are two kinds, which are (1) chlorotic to necrotic spots that are caused by two fungi namely *Rhizoctonia* spp.and *Colletotrichum* spp.and (2) Reddish purple spots caused by *Rhizoctonia* spp. The same control measures for damping off diseases can be used to control leaf spot diseases (Eze and Okafor, 1984).

2.2.12 Pests of *Irvingia* species seedlings

Young *Irvingia* seedlings are often attacked by grasshoppers, caterpillars, scale insects etc. Mature plants occasionally come under the attack of leaf miners and fruit borers. These pests are controlled by spraying them monthly with any broad spectrum insecticide like Ultracide, Gamalin 20, Rhonalin 20EC and Mocap 10G. Purple blotches on leaves of *Irvingia wombolu* delays onset of fruits, and the causative agents are the armoured scale insects. Any potent insecticide sprayed monthly will bring about its control (NAERLS Bulletin, 1999).

Rodents can attack newly transplanted stands of *Irvingia*, destroying them in the process. For the control of rodents, use of chlorophacinone, a rodenticide is recommended. Traps could also be set, to keep off rodents from newly established fields (NAERLS Bulletin, 1999).

Parasitic plants are plants that grow on another plant, obtaining their nutrition from the first plant, and this in turn, hurts the host plant. In *Irvingia*, the mistletoe (*Visum* spp.) which is a green parasitic shrub attaches itself to the *Irvingia* plant in a ball-like bush. This parasitic plant systematically kills the host plant from branch to branch anywhere it attaches itself, until the entire host plant dies.As a control measure, it is advisable to physically remove and burn the parasite or to sever the affected branch from the tree.

Weeds are a natural phenomenon against the farmer's dominion, so for *Irvingia* plantation, there should be ring weeding 1m from the base of the parts. Where*Irvingia* trees have been interplanted with food crops, the entire field should be clean weeded as the need arises, at least twice annually. On the other hand, if the field is solely *Irvingia* plants without any intercropping, then the inter row (as the need arises) should be slashed to keep down weeds.

In the dry season, bush fires is a regular feature, and this can destroy a newly established *Irvingia* field. It is therefore recommended to cut a fire trace 6 m wide round the plantation during dry season to create a fire corridor round the plantation each dry season (Eze and Okafor, 1984).

2.2.13 Flowering and fruiting of *Irvingia* trees

Wild *Irvingia* trees usually reach maturity and begin flowering at the age of 10 – 20 years (Moss, 1995; and Ladipo *et al.*, 1996). However, with improved cultural practices, much earlier fruiting has been reported at 4 – 5 years e.g. Ladipo *et al.* (1996) described trees that produce fruit at age six. Kang *et al.* (1994) have reported that *Irvingia gabonensis* is more productive than *Irvingia*

wombolu at the same age and in the same location. They also observed that branching intensity seems to be a major factor in determining productivity.

Irvingia trees are insect pollinated (Tutin and Fernandez, 1993). Ladipo *et al.* (1996) stated that very little is known about the mating systems and gene flow of *Irvingia* species, except that *Irvingia gabonensis* is known to have hermaphroditic flowers. They also reported uncertainty about the level of outbreeding in this species, but if a study indicating 100% outbreeding is correct (Ladipo *et al.*, 1996), it implies that *Irvingia gabonensis* is highly heterozygous.

The phenology of these two species differs considerably. *Irvingia gabonensis* generally flowers in February – March (Agwu and Akanbi, 1985), and fruits in the rainy season July – September (Ladipo *et al.,* 1996), while *Irvingia wombolu* flowers in October (Okafor, 1975) and fruits in the dry season around January – March (Okafor, 1975; Ndoye *et al.,* 1998). There is also variation in flowering and fruiting times over geographic range, where in most areas, *Irvingia* species fruit once a year, but biannual fruiting is also common (Tutin and Fernandez, 1993), and some trees only produce fruit every other year (Ladipo *et al.,* 1996).

Irvingia gabonensis can fruit twice per year in Nigeria, once in Janaury – February, and then again in June – August, and within these fruiting seasons, trees fruit earlier or later,depending on the region (Ladipo *et al.,* 1996). There are also reported cases where cultivated trees fruit in both May – July and September – October (Okafor, 1975). There are two peaks for flowering, one in January, and one in October, which rightly corresponds to the peak of the dry and rainy seasons. The fruiting times are however less well defined into seasons, indicating that fruit is available on one of two species nearly all year round, where *Irvingia wombolu* appears to have two fruiting periods from January – February, and May – November. *Irvingia gabonensis*, on the other hand

is reported to fruit in all months except July and September, peaking in April (Ainge and Brown, 2001).

The flowering and fruiting process consists of a series of sequential stages, all of which must proceed unhindered for a successful fruit harvest (Sedgley and Griffin, 1989), and the key steps are floral initiation, flower development, pollination, fruit set, fruit development and seed set. Fruit set may depend on the availability of pollen and the efficiency of pollinators, but seed set, which is the development of viable seeds from the ovules of pollinated flowers depends on endogenous factors, and their interaction with the environment. All these factors are under genetic control, but can be manipulated by management factors. The success of seed set affects the variation in seed viability, and thus germination (Sedgley and Griffin, 1989; Ladipo *et al.,* 1996).

2.3 Harvesting of *Irvingia* Species Fruits

Fruit harvesting has to be undertaken at the optimum time to prevent the harvest of immature fruits (Ladipo, 1999). When *Irvingia* fruits mature, they fall to the ground, and are collected for processing, but they can equally be plucked manually or mechanically, and picking of ripe *Irvingia* fruits is an organized annual event in areas where *Irvingia* grows in the wild on communal lands (Eze and Okafor, 1984).The gathering of *Irvingia* fruits is usually done by women, except when climbing is necessary (Ndoye *et al.,* 1998), though Vabi and Tchamou (1997) reported that tribesmen in the Korup National Park, Cameroon, join their wives in collecting the fruits when they are not trapping.In some cases, children and women are restricted to the compound farms and to nearby village forests, where they make daily runs to collect fruits from specified trees (Ladipo, 2000).

Young adult males are the ones involved in long-term field collection. They go into the forest for two weekly fruit collection trips, process the fruits in the bush in order to reduce the load they will carry back to the villages at the end of the collection trips (Ladipo, 2000). The wild forests contribute about 60% of *Irvingia* fruits for fresh eating, as well as their kernels, the compound farm gardens yield 10%, while the outlying fields contribute 30%, and in high forest areas, many families depend on this enterprise for survival (Ladipo, 2000).

2.4 Processing of *Irvingia* Kernels

Several methods have been reported for obtaining the kernels from *Irvingia* fruits. Traditionally, the fruits are piled up in heaps, and left to ferment for some days, before the seeds are extracted (Ejiofor *et al*., 1987). However, the extraction of the kernels from the fruits can be done by splitting open the hard endocarp with machetes, when the fruits are fresh, or with the use of truncheons or hard stones when the fruits are sundried or fermented. The seed shell splits open along the longitudinal line of weakness, exposing the kernels wrapped in a dark brown testa (Ladipo, 1999). The kernels are dried further to remove all moisture, and can then be stored or processed ready for use in food (Ejiofor, 1994; Joseph, 1995), or for direct sale in units of 5, 10, 25 kg sacks on the wholesale market (Ladipo, 1999). It is important that the kernels are fully dry, unless they are to be used immediately, because fresh kernels quickly get discoloured, and turns mouldy (Ainge and Brown, 2001), and this is a major determinant of quality (Ladipo, 1999). Post-harvest attack by insect pests can seriously affect products market acceptability.

Methods of extraction also differ between countries. The kernels of *Irvingia* in South West Nigeria are usually extracted in the fresh state, then dried before storage and sale, whereas in Cameroon, dry stage extraction which requires very little additional drying, involving 3 – 4 women, is preferred (Ladipo *et al.*, 1996; Ayuk *et al.*, 1999; Ladipo, 1999). Ladipo *et al.* (1996)

warned that all the methods used for kernel extraction are difficult, hazardous, and time-consuming.

During the 1995 *Irvingia* collections in Gabon, a tree (G28) was found in Bibas, in the north of Gabon, whose nuts split open naturally. The owner of the G28 tree was awareof the advantage of its self-splitting fruits (Ladipo *et al.,* 1996). Some fruits were collected from it, and divided into three (3) groups; one was processed and spread out to dry, while the other two groups were sent to Onne in Nigeria, and M'Balmayo in Cameroon for germination, and the establishment of living genebanks.About 72 hours later, 30 out of the 33 seeds had already split open, exposing intact cotyledons. After 70 – 72 hours, 93% of the seeds from G28 had split open, while none of the seeds from 6 other accessions in Gabon split at all. This useful trait, is an early splitting of the hard endocarp, a process that usually takes place much later during germination (Ladipo *et al.,* 1996).

2.5 Storage of *Irvingia* Seeds

The seeds may be stored in calabashes, pots or sacks at room temperature for upward of 6 – 8 months. The kernels (cotyledons) are the most important commodity of commerce from the *Irvingia* plants, and are classified as oil crop or oil seed as the kernels contain as much as 54 – 67% oil (Nkwatoh *et al.,* 2010), *Irvingia* kernels can be stored in four ways. One of the ways is to gather the fruits and allow the mesocarp to get rotten, then the rotten mesocarp are squeezed to release the seeds. These seeds are then sundried for a while to drain. After draining, the seeds are put in synthetic bags and hung over a fire place for storage. The seeds are removed whenever they are needed, and sundried for one or two days, and then sold. Another way of storing the seeds is to first sun-dry the squeezed seeds for it to drain and later spread on a platform built out of bamboos over a fire place in the kitchen. They are left in that condition until when

needed,hence they are removed, cracked and sundried for a few hours before being used. The third method is to gather the fruits either under the tree, or by the side of mud houses, for the mesocarp to rot. This leads to the production of a slimy substance that acts as gum that helps to hold the seeds as they are pasted on the outside walls of local mud houses. The seeds dry there on the wall, before they are removed and cracked as the need arises for either consumption or marketing purposes. The fourth stage involves drying the cracked kernels to acceptable market level. However, if they are not to be sold immediately, they are packaged in cement bags, tied firmly and put on a platform over a fire place for storage. These bags are changed periodically and the kernels are sundried to reduce humidity and hence check molding.

Stored *Irvingia* seeds keep for up to a year (Ndoye *et al.,* 1997), but are susceptible to pests, such as the merchant grain beetle (*Oryzaephilus mercator*) which lays its eggs between the testa and cotyledons of the seed, or in cracks in the cotyledons, so that the larvae can consume the cotyledons when they hatch (Dudu *et al.*, 1998). One way to tackle this challenge is to remove the testa completely in order to reduce the number of preferred oviposition sites, but this may further expose the kernels to attacks from other pests. The seeds therefore, need careful handling to prevent cracks, so as to prevent the grain merchant beetle from infesting the kernels. Pest-free kernels will give high value, but damage reduces market value. The beetle has along lifespan, and multiplies fast in number, hence early detection will prevent huge losses. Diethyl ether extract of *Irvingia gabonensis* could be used to attract the beetle either for detection, or to attract it away from stored oil seeds, including *Irvingia* seeds (Dudu *et al.*, 1998).

2.6 Yield of *Irvingia* Kernels

An average kernel yield of 25kg/stand of *Irvingia*is considered good. Kernel yields could be up to 30 – 35kg/plant in good years, and could go as low as below 20kg in bad fruiting years

(Ladipo, 2000). Estimates show that over 750,000 tonnes of fresh fruits are collected annually from the high forest zones in Nigeria from all sources. This figure excludes the imported, processed kernels into Nigeria from other West and Central African Countries. Their inclusion will yield over 12,000,000 tonnes of *Irvingia* kernels that are marketed in Nigeria, representing about 40% of West Africa total production (Ladipo, 2000).

2.7 Quality Control of *Irvingia* Kernels

The kernels of *Irvingia* are the most important product of the *Irvingia* tree (Ainge and Brown, 2001), hence increase in trade will require quality standardization which is central to setting prices for producers and traders. Ladipo (1999) suggests four quality classes (A – D) for *Irvingia* kernels, based firstly on visual characters. He noted that when the fruits are harvested in the immature state, extracted kernels are greenish, thin and shrunken. The kernel colour should be whitish cream on the inside and dark brown on the outside after drying. The kernels should have a uniform colour, instead of being patchy. Other visual characters include kernel sizes, where larger uniform kernels are generally preferred. There is also thickness or wholeness of kernels, where damaged kernels usually break easily, especially at the drying stage, while immature kernels are prone to shrinkage, and usually irregular in shape. There is also the issue of insect pest damage to kernels, fungal damage to kernels that lead to discoloration of the kernels. Fungal damage is often caused by poor handling during extraction due to high humidity and inadequate drying, which promotes fungal growth. Colour changes can vary from brown to black depending on the stage of infection (Ladipo, 1999). Moisture content of kernels is also key as more effective drying helps prevent pest and disease attack. Other qualities like oil content is important, as kernels dried directly using heat from naked fire causes the oily ingredient in the kernels to melt out, and this stains the face of the kernels in due course, resulting in dark-brown

oily unattractive product (Nkwatoh *et al.*, 2010). There is also the issue of flavour and viscosity (drawability or sliminess), as the more viscous *Irvingia wombolu* is preferred to the less viscous *Irvingia gabonensis* in cooking. These factors determine the value of the kernels to the consumer. It has also been observed that *Irvingia gabonensis* and *Irvingia wombolu* kernels are often mixed due to the abundance of *Irvingia gabonensis*, but because, they are less desirable than *Irvingia wombolu* kernels, this reduces the overall value (Ladipo, 1999), hence the suggestion that there is the need to emphasize separation of the two species to maintain high market prices.

2.8 Marketing of *Irvingia*Kernels

The *Irvingia*trees are a valuable source of income for West and Central African farmers, as their fruits are sold, but the kernels remain the most important product, which fetch a price several times higher than the fruits (Ayuk *et al.*, 1999). Both the producers and the traders all benefit from the trade financially. The kernels are traded locally, regionally and internationally.

Ladipo (1999) reported that the market for the kernels and products was worth in the region of US$50 million. The market has grown beyond that since then. Fresh fruits of *Irvingia wombolu* and *Irvingia gabonensis* are marketed locally where they are produced, and are traded to non – producing areas. Naturally, prices are higher in non – producing areas of Nigeria, than in the producing areas (Agbor, 1994). In producing areas, *Irvingiagabonensis* fruits are more expensive than *Irvingia wombolu* fruits, however, in non – producing regions, the situation is the reverse.Agbor (1994) reports that there was an increase in the value of fruits between 1986 and 1994. He reported that the prices in the northern parts of Nigeria are quite high due to lack of storage facilities, and high cost of transportation, and not due to low yield. In the Korup National Park, in Cameroon, transporting *Irvingia* fruits to the Suburban market is difficult because of the

poor state of the roads during the peak season, thereby reducing the income that accrued to the farmers (Vabi and Tchamou, 1997). *Irvingia wombolu* kernels are more highly cherished for cooking than *Irvingia gabonensis*, yet in the market, there is no distinction between the two species, in terms of price (Ndoye *et al.*, 1998), but the introduction of quality control will change this. The price farmers get for the fruits and kernels of *Irvingia* vary from the location of the market from source, as well as, seasonal availability (Ayuk*et al.*1999). Leakey (1999a) also reports that there is variation in price of kernels in West Africa with the season, between £1 (US$1.6) and £3 (US$ 4.8) per kilogram.

Ndoye *et al.* (1998) examined the sales value of *Irvingia* spp. in 28 markets in the humid forest zone of Cameroon, and found that over 29 weeks, the total value of sales was 34, 633, 100 CFA Francs (US$70,000). This translated into 30% marginal gains of the total value of sales by the traders. These high market values were reported in Rio Muni in Equatorial Guinea, where Sunderland and Obama (1997) reports that *Irvingia* seeds are sold more widely than any other forest product.

Initially, produce is bought from the farmers by country buyers, who in turn sell to wholesale distributors, who in turn sell to retailers. Several intermediaries are involved before the product reaches the retail market, hence retail price has to be high to accommodate what each middleman takes(Agbor,1994). The demand for *Irvingia*products exists both in urban areas, as well as in the country side, and much of the trade focuses on moving produce from rural areas into the towns and cities. Where the demand in a country cannot be met, produce is imported from neighbouring countries (Ainge and Brown, 2001).

The international trade in *Irvingia* kernels is more profitable for traders (Ndoye *et al.*, 1998). There is no reliable statistics for the volume of international trade, but the trade routes for

Irvingia kernels are widely known. Cameroon is a major producer, exporting to Gabon and Equitorial Guinea in particular (Ndoye *et al.,* 1998; Sunderland and obama, 1997). Equatorial Guinea in turn supplies kernels to Gabon (Yembi, 1997; Sunderland and Obama, 1997). In West Africa, the main exporters are Cameroon, Nigeria and Cote d'ivoire, trading to Gabon, Nigeria, Liberia and Sierra Leone. The demand in Southern Nigeria alone for kernels is around 80,000 tonnes per year (Ndoye *et al.,* 1997), hence Nigeria serves as both a source and destination for trade.

Irvingia kernels are also exported to other continents, as processed kernels are also exported from Africa to the UK and the USA (Ladipo, 1999). Tabuna (1997), reported that the trade to European markets for Africa nonwood forest products (NWFPs) in France and Belgium particularly to areas where African immigrants abound, mainly from Cameroon and Congo. The trade name for *Irvingia* kernels in Europe is "Malombo". He estimated that, there are about 100,000 potential consumers in this European market. This trade also empowers traders in the destination countries.

2.9 Genetic Variation of *Irvingia* Species

There is a huge amount of variation within the two *Irvingia* species that can be exploited for selection of superior breeding specimens. Tchoundjeu *et al.* (1997), list variations inthe fruit (quality, taste and size) as well as in the size of the kernels, the timing of fruit production and in the maturation process as important characters that could be improved upon. There is also the high likelihood of favourably altering the season and pattern of fruiting and increasing tree yield, such as *Irvingiagabonensis* (Leakey and Newton, 1994). The assessment of genetic variation in tree, fruit and kernel properties is in progress, and the knowledge of the farmers is very important in the selection of trees with potentially suitable genotypes for further breeding. This is because

the farmers are very knowledgeable about the individual *Irvingia* trees on their land, and this knowledge will greatly aid tree selection. Farmers are able to identify which trees fruit early and those that constantly bear large fruits each year (Moss, 1995; Ladipo, 2000).

2.10 Growth and Yield Potentials Due to Genetic Variationin *Irvingia* Species

In order to improve *Irvingiagabonensis* and *Irvingia wombolu* as agroforestry trees and potentially domesticate them, the natural variation between trees should be recognized and utilized genetically – determined characters only are of interest, since only these are heritable. The *Irvingia* plants in existence now have been described as slow growing, and poor in yield (Akubor, 1996), and slow maturation is an impediment to the choice of *Irvingia* plants for planting, but they still have great potential for improvement due to the extent of natural variation in the species. These variations have been revealed partly from observations of existing trees, and partly from genebank specimen studies (Ladipo *et al.*, 1996).

Three live genebanks for *Irvingiagabonesis* and *Irvingia wombolu* have been created, that have seeds considered by farmers in Gabon, Cameroon and Nigeria to be from superior trees, and the germplasm collections are at M'Balmayo (Cameroon), and Ibadan and Onne (Nigeria) (Tchnoundjeu *et al.,* 1997). Knowledge of the genetic variation of the *Irvingia* species gained from the studies of these collections will allow genotypic selection of individuals for vegetative propagation, to promote the domestication process (Ladipo *et al.,* 1996). The genetic studies of diversity in *Irvingia* species can be done using seven nuclear cleaved amplified polymorphic sequences, found by Lowe *et al.* (1998), and are suitable for phylogeographic analysis. Leakey *et al.* (2000) report that Lowe and his co-workers have found the centre of genetic diversity for each *Irvingia*species, which for *Irvingia gabonensis* is around Ebolowa in Southern Cameroon, while *Irvingia wombolu* is in South-East Cameroon, and Western Nigeria.

Some of the characteristics that farmers want to see improvedinclude fruit quality and yield, earlier maturation of the trees (precocity), a longer period of fruit availability, and reduced tree height or dwarfing (Leakey, 1999a). Okafor (1997) lists the desirable characteristics of *Irvingia gabonensis* and *Irvingia wombolu* from Okafor (1990) as follows: fruit size, fruit yield, flavor, lack of fibrousness, short time to reproductive maturity (precocity), wide range of products, and high quality and value.Another trait that could be bred into the *Irvingia* plant is the self-cracking seeds character found in the tree in Bibas Northern Gabon.(Ladipo*et al.*,1996), as this trait would have enormous benefits for farmers, such as saving them the huge time and labour expended in splitting open the hard endocarp, as well as the risk or danger involved in using sharp knives or machetes to crack the seeds open, as finger – trimming and inflicting of wound has occurred sometimes (Nkwatoh *et al.,* 2010).

2.11 Uses of *Irvingia* Species

Every aspect of the *Irvingia* species plant and plant products is useful. The trees have positive effects on the soil in which they grow, like helping to reduce soil bulk density, and increasing levels of organic carbon, and exchangeable Potassium and Magnesium ions, hence they are suitable as agroforestry trees in a multi-storey crop set-up (Kang *et al.,* 1994). The leaves and bark have medicinal properties (Agbor, 1994). The fresh fruits are consumed by both man and animals, while industrially, the fruit pulp of *Irvingia gabonensis* is used in the making of jam, jelly and juice (Ejiofor, 1994; Okolo,1995) and high quality wine comparable to the German wine in colour, flavour, sweetness and general acceptability, having 8.12% alcohol content (Akubor, 1996).

In traditional religion, some tribes use the split endocarp in divination, giving a favourable omen if one falls flat and the other cover side up (Abbiw, 1990). The kernels are extensively

consumed. When milled, it is used to make the "dika or Odika" bread or cake in Central Africa, particularly in Northern Gabon (Moss, 1995; Ndoye *et al.,* 1997), while in Cameroon, this paste is known as "etima" (Ndoye *et al.,* 1997). In Nigeria, as well as in several other countries, it is also milled to serve as sauce or soup thickener for the famous draw soup, known as "Ogbono soup" (Ohochuku, 2005a,b; Matos *et al.,* 2009). The soup made from *Irvingia wombolu* kernels is more viscous or slimy, about 91.05% than the soup made from *Irvingia gabonensis* which gives about 64.01% drawability (Festus and Nwala, 2012).

The kernels of the *Irvingia* species can also be industrially put to other uses, such as extracting the kernel fat, since *Irvingia* is an oil seed (Ainge and Brown, 2001), to get cooking oil, margarine, perfume, soap and pharmaceuticals (Joseph, 1995). He noted that the de-fatted kernels residue still possesses the consistency and thickening properties required for soup-making, hence no waste by-products from the fat extraction process, as both fat and residue are still useful. They are also useful as a binder in food and pharmaceuticals (Joseph, 1995). The extracted polysaccharides from *Irvingia* kernels possess the potentials as an industrial gum (Ndjouenkeu *et al.,*1996).

Irvingia species kernels are highly valued for their health and medicinal benefits, with their seed extract leading to significant reduction in bodyweight among obese people in Cameroon (Duguma *et al.,* 1990; Ndoye *et al.,* 1997; Ngondi *et al.,* 2005). The kernels are rich in antioxidant phytochemicals, such as alkaloids, flavonoids, glucosides, saponins and tannins, with positive influence on human health (Ashihara*et al.,* 2008; Quideau *et al.,* 2011). It is very useful in the treatment of cancer, cardiovascular diseases, cataracts, and brain immune dysfunction due to the presence of these antioxidant phytochemicals (Ames *et al.,* 1993; Vinson *et al.,* 1998; Quideau*et al.,* 2011).In terms of trade, *Irvingia* kernels are a veritable commodity of trade and

commerce, both locally and internationally (Okafor, 1985; Shiembo *et al.*, 1996; Leakey *et al.*, 2003).Mechanically damaged kernels however, are predisposed to insect and microbial attacks (Dennis, 1990) which reduce its market value, hence caution is required in its harvesting, processing, transportation and storage, in order to prolong its shelf life, and ensure quality (colour, shape, oil content, flavor, etc) (Ladipo, 1994).

2.12 FactorsPredisposing *Irvingia*Kernels to Fungal Attack

Some of the factors that predispose *Irvingia* kernels to fungal attack are:

Method of splitting the fruits in order to extract the cotyledons (kernels): Here, there are different methods of splitting the fruits, depending on the choice of the farmers. Some could decide to use stones, while others could decide to use hammers, and yet there are those who make use of machetes or cutlasses. Any of the methods employed above could bring about mechanical damage to the kernels, and this could in turn, pre-dispose the kernels to fungal attack (Ladipo, 1994).

Treatment conditions: Treatment conditions here refer to how the kernels were dried, whether directly under the sun, or under the shade or above a fire place, may be in the kitchen or hearth or kiln. The moisture content of the kernels ought to be drastically reduced, to about 1 %, in order to prevent or minimize microbial attack during storage (Etebu and Bawo, 2012).

Storage conditions: Farmers use all manner of storage facilities for their kernels, e.g. silos, drums, baskets, sacks, nylon or plastic bags. Incidence and severity of diseased kernels depends on whether these containers were either open or sealed. Etebu and Bawo (2012), have shown that *Irvingia* kernels do better when stored in sealed plastic bags, with the application of 0.9% NaCl, and 3% $KHCO_3$in order to discourage fungal growth and reduce moisture. This method greatly

suppressed the incidence of some pathogenic fungi. However, there are times that the kernels are packed in contaminated containers (Adebayo – Tayo *et al.*, 2006).

Transportation: *Irvingia* trees are cultivated in the forests in the rural areas, where the road network is poor, hence while transporting these kernels to the urban areas for sale, the kernels receive so much shock as a result of the bad bumpy roads. This predisposes the kernels to fungal infestation, which is a fallout of the mechanical damage they had (Wu and Khlangwiset, 2010).

Storage days : The length of storage is a key factor in determining whether the kernels would be infested by microbes, as well as the severity. Various workers have shown in earlier works that incidence of storage disease among fruits increase with increasing storage duration. When mature *Irvingia* fruits are harvested green, with very few patchy specks of dark colouration without rot, after three days, the fruits turn yellow as they ripen. By the fifth day of storage after harvest, the yellow colouration gradually gave way to brownish-black rot disease. By the ninth day of storage after harvest, the severity of the blackrot disease had increased sharply. By the fourteenth day, there was complete rottening and also brownish black in colour. The disease severity progressively increased as the fruits aged through senescence after harvest (D'hallewin and Schirra, 2000, Abd-El-Azizand Mansour, 2006, Etebu, 2012a,b).

2.13 Mycotoxins

Agricultural products are often contaminated with fungi that can produce toxic secondary metabolites referred to as mycotoxins (Lugauskas, 2005). Out of the 300 – 400 compounds that have been recognized as mycotoxins (Mahendra *et al.*, 2012), aflotoxins have assumed economic importance because of their influence on the health of human beings and livestock, as well as the marketability of agricultural products (Etebu, 2012a; Etebu and Bawo, 2012).

Some other mycotoxins are citrinin, ergot alkaloids, 3-nitropropionic acid, fumonisins, ochratoxin (OT) trichothecenes, T-2 and HT-2, diacetoxyscirpenol, nivalenol, diacetoxyscripenol, fusarenon-X. They are all relatively small molecular weight compounds (approximately 700), and the twelve listed above being potentially poisonous to man and livestock when ingested in high doses (Mahendra *et al.*, 2012). For instance, in 1962, England witnessed an unusual veterinary crisis that led to the loss of about 1,000,000 turkey poults. It was this veterinary crisis that led to the proposal of the term "mycotoxin", as the deaths of the poults was traced to mycotoxins (Mahendra *et al.*, 2012).

2.13.1 Aflatoxins

Aflatoxins have been observed to be produced by several fungi species, such as *Aspergillus flavus and Aspergillus parasiticus* (Rizzo *et al.,* 2004; Wu and Khlangwiset, 2010), and these fungi have been shown to grow on *Irvingia* kernels displayed for sale in Nigerian markets (Adebayo-Tayo*et al.,* 2006; Iyayi *et al.,* 2010). Adebayo –Tayo *et al.*(2006) reported the presence of aflatoxin B_1 and G_1 concentrations ranging from 0.2 – 4.0 and 0.3 – 4.2mg/kg respectively in the *Irvingia* kernels sold in four major markets in Akwa Ibom state.

Consumption of high levels of aflatoxin in commercial maize products has been reported to have caused illness among several hundreds of Kenyans in 2004, with about 125 deaths recorded (Lewis *et al.,* 2005; Strosnider *et al.,* 2006). Also, it has been estimated that more than five (5) billion people in developing countries worldwide, are at risk of chronic exposure to aflatoxins through contaminated foods (Strosnider *et al.,* 2006), probably due to lack of or poor or inadequate monitoring and control measures as well as poor preservation.

Ciegler (1977) reported that aflatoxin occur in a variety of crops and animal products, such as meat, milk and eggs. Ciegler (1977) also stated that, aflatoxin production is affected by several

factors, which influence the fungal growth, such as moisture content, relative humidity, temperature, substrate composition, and the presence of competing microorganisms. Aflatoxins are highly carcinogenic, causing hepatoma (cancer of the liver), and have also been associated with acute hepatitis in man, mostly in the developing world (Krogh, 1992; Prasad, 1992; Eaton and Gallagher, 1994). Aflatoxins showed toxic, carcinogenic, mutagenic and teratogenic effect in laboratory animals (Abdel-Wahhab *et al.,* 2005; Abdel-Wahhab *et al.,* 2006).

Aflatoxins have been reported to have been detected in grapes and musts in France (Sage *et al.*, 2002), in edible nuts and nut products, milk and milk products (Prasad *et al.*,2001). Singh (1983), reported that, out of 342 samples of different fruits and spices obtained from commercial centres, 95 of them tested positive for aflatoxin contamination.

One major problem with aflatoxin contamination is that, cooking the food substance thoroughly will have no effect on the potency of the toxic materials, as aflatoxins have been found to be heat stable, with a melting point of between 268 – 269^0C (Frazier and Westhoff, 1988). Since aflatoxins are ubiquitous in the food and feeds substances consumed locally, there is therefore the need to monitor their levels, so that they do not exceed the World Health Organization (WHO) standard for food samples, which is 20 µg/kg (Oladejoh and Adebayo-Tayo, 2011). The maximum aflatoxin B_1 (AFB_1) residue limit of 20 µg/kg is what is permitted in Nigerian foods (Bankole *et al.,* 2006).Aflatoxins belong to the strongest natural occurring carcinogenous substances. Aflatoxin B1 appears nearly in all cases together with Aflatoxin B2, G1 and G2, and it is the analyte with the highest toxic significance, and is commonly found in corn, peanuts, Brazil nuts, cotton seeds and pistachios.Aflatoxin M1 is produced as a metabolite of aflatoxin B1. It is secreted in milk after feeding of aflatoxin B1 – containing feed to lactating cows.

Aflatoxin M1 is relatively stable towards the pasteurizing process, hence a comprehensive routine check of both the raw material and the final products is necessary.

2.14 ELISA for the Estimation of Aflatoxin

Enzyme-Linked Immunosorbent Assay (ELISA) is a powerful method for detecting and quantifying a specific protein in a complex mixture. It enables the analysis of protein samples immobilized in microplate wells using specific antibodies. Since *Aspergillus flavus* species was among the various fungal species identified in the *Irvingia* kernel samples, and because these fungal species are capable of producing aflatoxins, it became imperative to estimate the aflatoxins via a very sensitive, serological technique, known as ELISA.

Regardless of the method chosen, mycotoxin analysis via ELISA usually involves extraction, clean up, and detection (Pittet, 2005). The purpose of extraction is to remove as much of the mycotoxin from the sample matrix as possible, into a solvent suitable for subsequent clean up and determination. Once the mycotoxin has been extracted from the solid matrix, the liquid extract is cleaned up to remove impurities before the determinative, or operation set up. The clean up involves isolating the toxin from the extract, and is a requirement for most of the major mycotoxins, for example, aflatoxins, thus these are good quantitative methods.

In direct competitive ELISA technique, wells in the ELISA micro litre plate contain a bound antibody against mycotoxins. The detection re-agent is a covalent complex of these mycotoxins and enzyme, usually horse radish peroxidase (HRP) or alkaline phosphate. The reagent is mixed with a sample of the aflatoxin extract (i.e. test sample), and the mixture is placed in the well.

The control bears the mycotoxins-free sample, the mycotoxins – enzyme conjugate can saturate the bound antibody, after additions of a chromogenic substrate results in the development of colour. In the test well, free mycotoxin molecules in the extract compete with the conjugate on

the bound antibody, leading to fainter colour development. Immunoaffinity columns can be used here to purify and concentrate mycotoxin samples (Stark, 2009).

Finally, positive reactions are detected when a colourless substrate usually P-nitrophenyl phosphate, undergoes a chemical change which results in a yellow coloured product due to exposure to the alkaline phosphate that is linked to the antibody. The degree of colour change is indicative of the degree of reactivity that the ELISA reader apparatus reads.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This survey of *Irvingia* species kernels was conducted in 2014,on five (5) major markets around Zaria in Kaduna State, North Western Nigeria, covering two (2) Local Government Areas, namely:

1. Zaria Local Government Area, which is where the ancient city wall is situated, as well as the seat of the Emir of Zazzau.
2. Sabon Gari Local Government Area, where Ahmadu Bello University, Samaru Zaria is located.

Zaria is located on latitude $11^{0}10^{1}$N, and longitude $7^{0}38^{1}$E (Owonubi and Olorunju, 1985). It is about 670 m above sea level, and about 640 km from the Atlantic shores of Nigeria. Zaria has an annual rainfall and average temperature of 1055mm and 24.55^{0}C respectively (Owonubi and Olorunju, 1985). (See Appendix II for relative humidity).

According to National Population Commission Census of2006, it has a population of 692,069. It is a cosmopolitan town, boasting of all manner of individuals from all walks of life, and diverse occupations, such as traders, artisans, civil servants, public servants, students and the clergy.The laboratory aspect of this work, that is culturing, isolation and microscopy of fungi on *Irvingia* kernels was carried out in the Department of Microbiology, Ahmadu Bello University, Zaria.

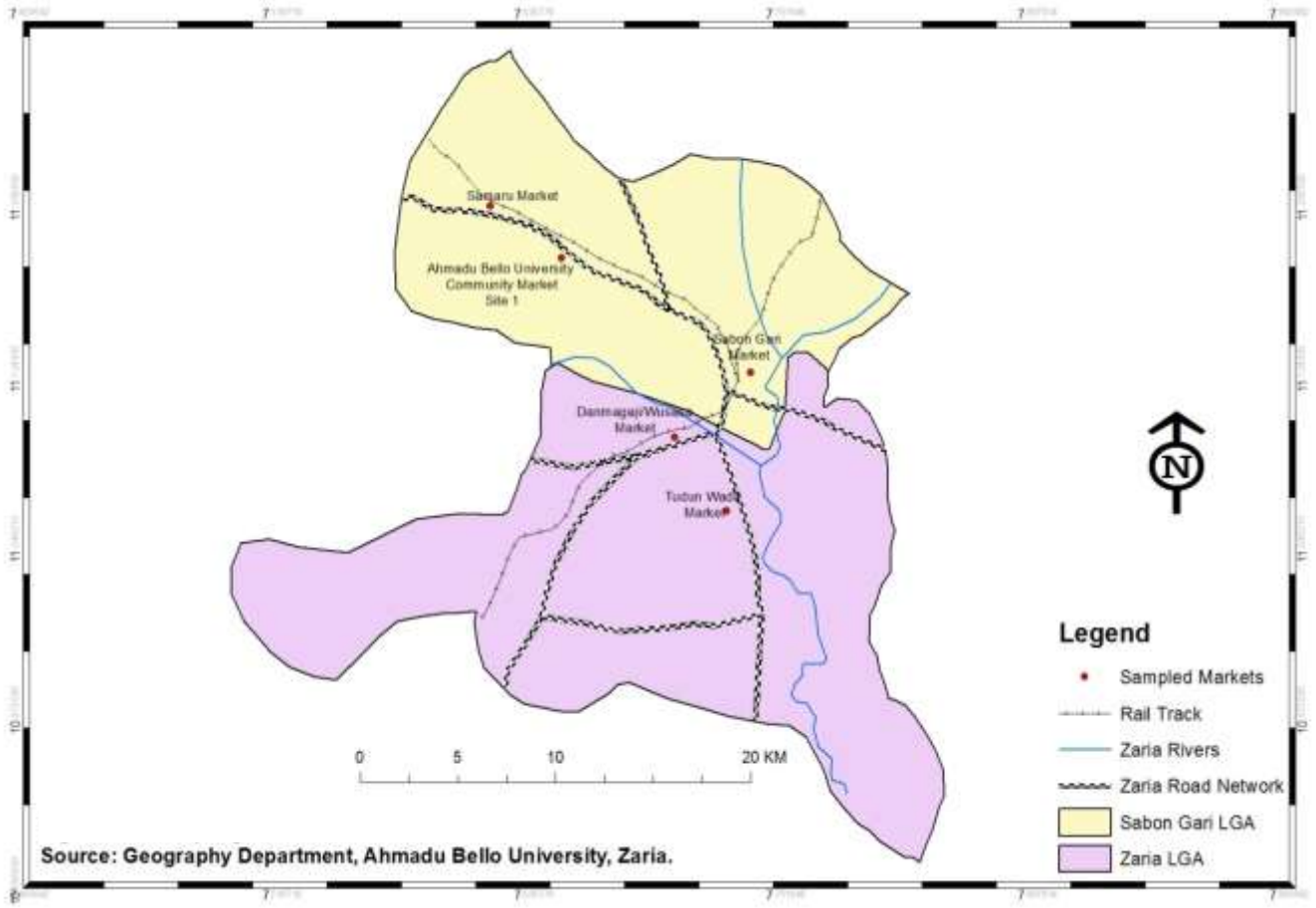


Figure 1: Map of Zaria showing locations of surveyed markets

3.2 Sample Collection

The *Irvingia* species kernels were purchased from five(5) markets, Ahmadu Bello University Community Site 1, Samaru and Sabon Gari markets, in Sabon Gari Local Government Area; TudunWada Agoro and Dan Magaji/Wusasa markets in Zaria Local Government Area. From each market, by selective random sampling, 60g of each *Irvingia* spp.kernels were purchased fromfour (4) sellers that were patronized per market. The sample collection was done thrice (3), February and March, April and May and finally in July to August and not necessarily from the same sellers.The relative humidity (see Appendix II) of the sampling periods ranged between 15 and 73. The samples were collected into transparent labeled plastic bags.

3.3 Culture Media

Two commercially available media were used in this work. These were Potato Dextrose Agar (PDA), which is a general purpose culture media, and Sabouraud Dextrose Agar (SDA), which is a modification of Dextrose Agar developed by the French dermatologist Raymond A. Sabouraud in the late 1800's to support the growth of fungi that cause infections of the skin, hair or nails, collectively referred to as dermatophytes (Sabouraud, 1896a,b). SDA differs from PDA in that it contains peptones, while PDA contains potato. Sabouraud Dextrose Agar (SDA) is a selective mediua that is formulated to allow growth of fungi, and inhibit the growth of bacteria. SDA media is able to inhibit bacterial growth by having an acidic pH of 5.6, and this was even before the current practice of adding an appropriate antibiotics to discourage bacterial contamination. High acid concentration in SDA inhibit all bacterial growth (Sonnenwirth and Jarrett, 1980).

Potato Dextrose Agar (PDA), (HKM culture media HCM 050, Guangzhou, PRC), is composed of dehydrated potato infusion, and dextrose that encourage luxuriant fungal growth. Agar, here, serves as the solidifying agent (McIntosh, 2012). Then 0.1M HCl was used in drops to bring the

final pH to 6.5. The 0.1M HCl volume should be insignificant in order not to alter the volume from 39g/litre.

PDA formular/litre

Potato infusion from 200g	4g
Dextrose	20g
Agar	15g
Total	39g

Final pH: 6.5 $\pm$ 0.2 at 28$^{o}\pm$ 2^{0} C

3.3.1 PDA media preparation

In one litre of distilled water, 39g of the medium was suspended, heated over a Bunsen flame with frequent agitation, and allowed to boil for one minute to completely dissolve the medium/contents. The solution was autoclaved at temperature of 121^{0} C for 15 minutes, at a pressure of one (1) atmosphere (15 PSI).After removing from the autoclave, allowed to cool for 10 minutes. Five hundred (500 mg) streptomycin sulphate was added into the molten solution to serve as antibiotics. The PDA medium was used to culture the cotyledons and testa.

3.3.2 Sabouraud Dextrose Agar (SDA)

Sabouraud Dextrose Agar (SDA), is a peptone medium supplement with dextrose to support the growth of fungi. SDA is used for culturing pathogenic commensal fungi, as well as yeasts (Sonnenwirth and Jarrett, 1980). Since no one medium is enough to culture fungi, SDA was used to sub-culture the different fungi that grew on the PDA, for pure cultures

SDA formula in gram per litre (g/l)

Pancreatic digest of casein	5 g
Peptic digest of Animal Tissue	5 g
Dextrose	40g
Bacteriological Agar	15g
Total	65g

Final pH 5.6 $\pm$ 0.2 at room temperature (28^{o} $\pm$2^{0} C)

About 65g of the medium was suspended in one litre of distilled water, mixed well, and dissolved by heating to boil, with frequent agitation. After heating for one minute and dissolving the solution, it was sterilized in an autoclave at 118-121^{0}C for 15 minutes. This was followed by the addition of 500mg streptomycin antibiotic while the solution was still in a molten state. If all the solution was not used at that moment, the remainder was stored in the refrigerator at 8 – 15^0 C until when needed.

3.4 Isolation of fungi from cotyledons and testa

The kernels were removed from the sealed, transparent plastic bags, were surface-sterilized by immersing in 1% Sodium hypochlorite for two (2) minutes, and rinsed in three (3) changes of sterile distilled water. Kernels were picked up with sterile forceps and with the aid of sterile blades, testa were carefully scrapped off the cotyledons.

Using sterile blades and forceps, barecotyledons were cut into smaller sizes, about 4mm. Testa (5mg each), and pieces of *Irvingia*cotyledons were plated on the 100ml Potato Dextrose Agar (PDA) media, each petri-dish, measuring 9cm in diameter, and incubated at room temperature (28^o $\pm 2^0$ C) for three (3) days. This was done in triplicates. The incubation was done in a dark drawer (Etebu *et al.,* 2005).On the fifth day, fungi colonies were sub-cultured into fresh, sterile petri-dishes containing SDA, in order to obtain pure cultures. These were also given five days to grow at room temperature (Jha, 1995), process was repeated whenever more than a single colony of fungi was observed in the petri-dishes, until pure cultures were obtained.

3.5 Identification of isolated fungi

All the various species of fungi isolated were identified, both macroscopic and microscopic features, and their various characteristics studied, (i.e) colour, texture, form of hyphae, form of conidia, presence of conidiophores, shape of conidial heads (Barnett and Hunter, 1972; Larone,

1987; Fawole and Osho, 1995; Samson *et al.*, 2000; Klich, 2002; James and Natalie, 2013). The pure cultures were inoculated into slant bottles containing SDA, to serve as reservoir, and kept under low temperatures inside the refrigerator (8-15^{0}C), to arrest their growth.The microscopic identification was aided by appropriate taxonomic keys (Barnett and Hunter, 1972; Boerema *et al.*, 2004; James and Natalie, 2013).

3.6 ELISA Assay Preparation

The determination of aflatoxin content was done at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria.

The ELISA kit was used according to the manufacturer's (Beacon, Germany) protocol. The materials used include: one plate containing 12 test strips of 8 wells each (96 wells in all), vacuum-packed in aluminized pouch with indicating desiccant; 5 vials, each containing 2 ml of aflatoxin calibrators corresponding to 0, 2.0, 7.5, 25 and 100 µg/l of aflatoxin; 1 vial containing 8ml of aflatoxin-HRP enzyme conjugate; 1 vial containing 8ml of Rabbit anti-aflatoxin anti-body; 1 vial containing 14ml of substrate, 1 vial containing 14 ml of stop solution (1N HCL); distilled water; methanol (ACS grade); graduated cylinder (at least 100 ml); glass ware for sample extraction and extract collection; Pipette with disposable tips capable of dispensing 50 µl; multichannel pipette (8 channel capable of dispensing 50 and 100 µl); paper towels or equivalent absorbent material; microwell plate reader with 450 nm filter (Stat Fax Model 303 plus); high speed blender, and timer.

The extraction solution was prepared by carefully measuring 20 ml of distilled water for each 100 ml being prepared, and transferred to a clean glass container with tight-fitting lid. Then 80 ml of methanol was carefully measured for each 100 ml being prepared and poured into the

container. The glass container was covered and swirled to mix completely and stored tightly sealed to minimize evaporation.

For sample preparation, 50 g of each whole *Irvingia*kernel (not cotyledons and testa separately) samples were weighed and transferred to a clean blender jar with tight fitting lid (250 ml), 100 ml of 80% methanol/water was added to the jar, it was blended for 1 minute. A minimum of 10 ml of the solution was filtered through a filter paper. 5 ml of each extract was diluted with 20 ml of distilled water, and mixed thoroughly.

3.7 Assay Procedure

For the assay proper, the calibrators and samples were run in duplicate in order to improve assay precision and accuracy. The reagents and sample extracts were allowed to reach room temperature prior to running the test.

The test wells were placed into a microwell holder, 50 ml of enzyme conjugate were dispensed into each test well, a pipette with disposable tips were used to drop 50 ml of calibrators and samples to the appropriate test wells, each time using a clean pipette tip for each. Fifty(50 ml) of antibody solution was dispensed into each test well, and the plate was placed on an electrical shaker and shaken gentlyfor one minute to mix contents, and thereafter, the test wells were incubated for 10 minutes at room temperature ($28^{o}\pm2^{0}$ C). The contents of the wells were dumped or emptied into an appropriate waste container. The wells were filled with laboratory quality distilled water to overflowing, and dumped or emptied. This process of filling the wells with distilled water and dumping was repeated four times (4x), for a total of five washes. After the last wash, the inverted wells were tapped onto absorbent paper to remove the last of the wash solution. One hundred microliter (100 µl) of stop solution was dispensed into each test well, with

the plate rack shaken gently to mix. The absorbance of the wells at 450 nm were read and recorded immediately using Optic Ivymen ® -system 2100- plate reader.

3.8 Statistical Analysis

Percentages and means of fungal colonieswere calculated,t - testwas used to test significant difference between data from testa and cotyledons, *Irvingiagabonensis* and *Irvingiawombolu*. Data obtained was subjected to Analysis of Variance (ANOVA),and Duncan Multiple Range Test (DMRT) was used to separate the treatmentmeans when significant at 5 % level of probability.

CHAPTER FOUR

4.0 RESULTS

4.1 Fungal Species Isolated

Nine different fungal species were isolated from the cotyledons and testa of both *Irvingia gabonensis* and *Irvingiawombolu* (Table 1). These include *Absidia corymbifera, Aspergillus flavus, Aspergillusfumigatus,Aspergillus niger, Aspergillus versicolor, Mucor* sp., *Penicillium* sp., *Phoma herbarum* and*Rhizopus stolonifer*. All these nine species were isolated from *Irvingia wombolu*.However, only five fungal species were isolated from *Irvingia gabonensis* samples namely, *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Penicillium* sp.and*Rhizopus stolonifer.*

4.2 Systematic description of fungi isolated from *Irvingia* species cotyledons and testa

4.2.1 Macroscopic and microscopic features of *Absidia corymbifera*

The colonies were coarse, having woolly gray surface on both SDA and PDA. Their growth was rapid, covering the 9 cm agar surface within 96 hours, at room temperature ($28^{o}\pm 2^{0}$ C). The fluff resembled gray cotton candy, reverse was white in colour (Plate III a and b)**.**

The hyphae were non - septate, sporangiophores arose on the stolon between the rhizoids. Sporangiophores were branched, forming a conical apophysis just below the columella, sporangia were relatively small, and slightly shaped like a pear (Plate III c).

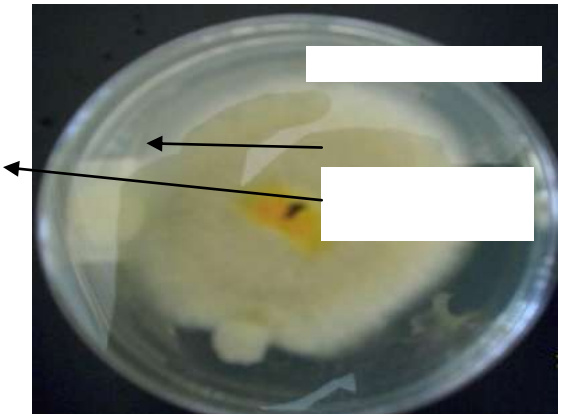
Table 1: Fungi Isolated from *Irvingia gabonensis* and *Irvingia wombolu*

Fungal species	*Irvingia gabonensis*	*Irvingia wombolu*
Absidia corymbifera	-	√
Aspergillus flavus	√	√
Aspergillus fumigatus	√	√
Aspergillus niger	√	√
Aspergillus versicolor	-	√
Mucor sp.	-	√
Phoma herbarum	-	√
*Penicillium*sp.	√	√
Rhizopus stolonifer	√	√

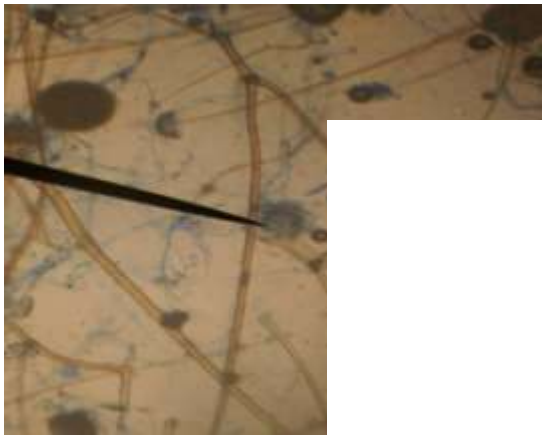
key: √ = Present

\- = Absent

(a) Upper surface of *Absidia corymbifera*, pure culture



(b) Reverse side of *Absidia corymbifera*, pure culture



sporangiophore

stolon

(c) Microscopic features of *Absidia corymbifera* (× 40)

Plate III: *Absidia corymbifera*

4.2.2 Macroscopic and microscopic features of *Aspergillus flavus*

The colonies appeared regularly shaped on both PDA and SDA and grew rapidly, covering petri-dish 9cm in diameter within 120 hours at room temperature ($28^{o}\pm 2^{0}$ C). They appeared velvety, yellow to green or brown on the surface, while the reverse appeared red-brown (Plate IV a andb).Conidiophores of variable length, which were rough, pitted and spiny. Conidia covered the entire swollen vesicle. (Plate IV c).

4.2.3 Macroscopic and microscopic features of *Aspergillus fumigatus*

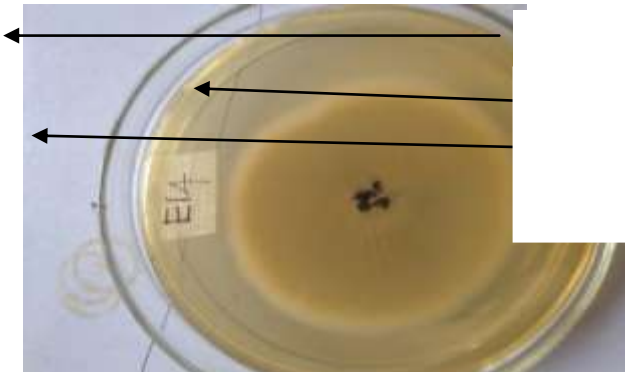
The fungus grew rapidly, covering the surface of the 9 cm diameter petri-dish within 72 hours. The surface of the colony was at first white, then turning dark greenish to gray. The reverse was white to tan. It had a velvety texture (Plate V a and b).The conidiophores were unbranched, smooth and short, enlarged at the tip, forming a swollen vesicle, bearing chains of round conidia (Plate V c).

4.2.4 Macroscopic and microscopic features of *Aspergillus niger*

The fungus grew rapidly on both PDA and SDA, covering the 9 cm diameter petri-dish surface within 72 hours at room temperature ($28^{o}\pm 2^{0}$ C). The colony which was initially white/yellow, then turning black, while the reverse was white to yellow (Plate VI a andb).

The long, smooth, erect and hyaline unbranched conidiophores narrowed slightly towards the base. Each conidiophores terminated in globose vesicle covered by conidia. (Plate VI c).

(a) Upper surface of *Aspergillus flavus*, pure culture

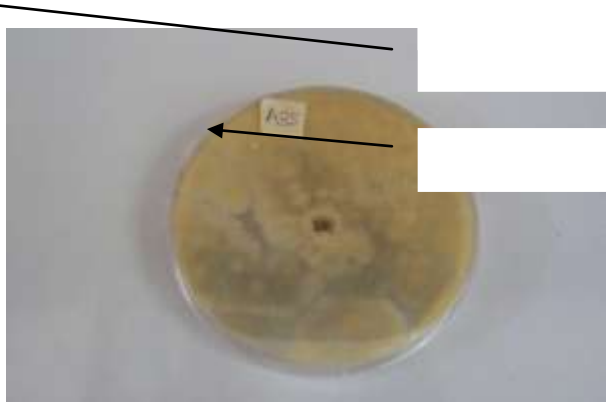


(b) Reverse side of *Aspergillus flavus*, pure culture

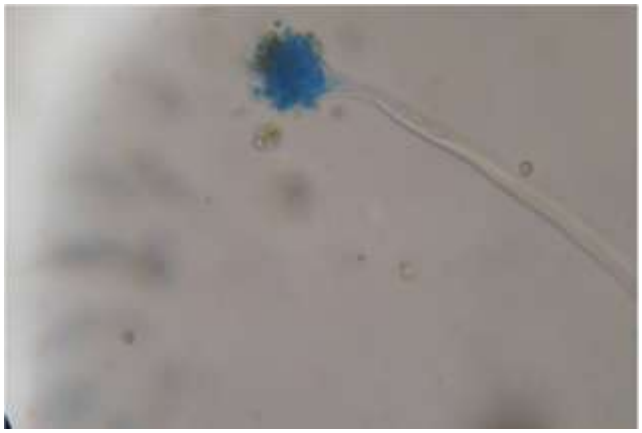
features of *Aspergillus flavus*(× 40)

Plate IV: *Aspergillus flavus*.

(a) Upper surface of *Aspergillus fumigatus*, pure culture



(b) Reverse side of *Aspergillus fumigatus*, pure culture



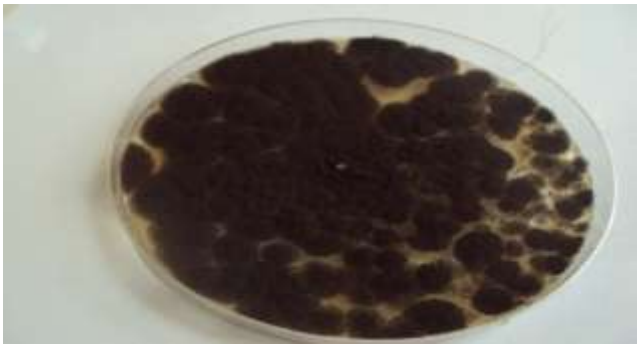
conida

swollen vessicle

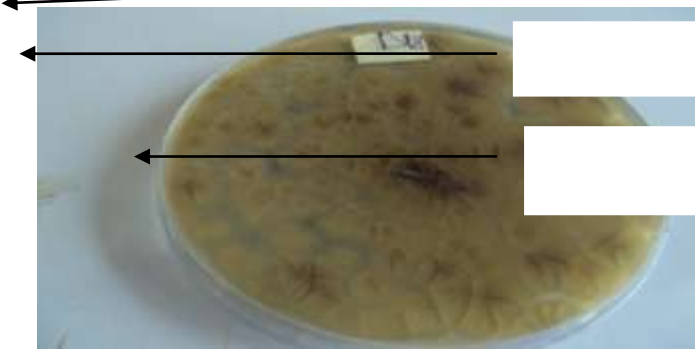
conidiophore

(c) Microscopic features of *Aspergillus fumigatus*(× 40)

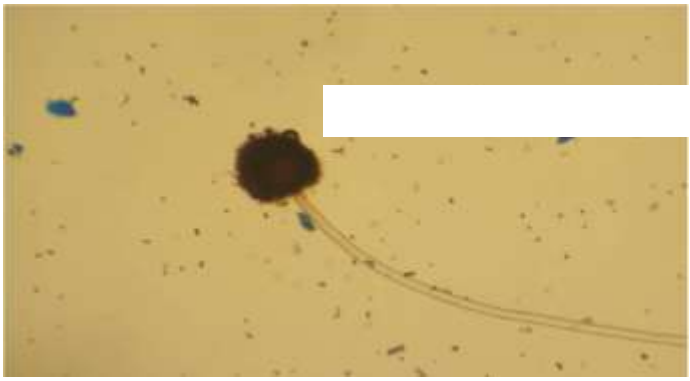
Plate V: *Aspergillus fumigatus*



(a) Upper surface of *Aspergillus niger*, pure culture



(b) Reverse side of *Aspergillus niger*, pure culture



(c) Microscopic features of *Aspergillus niger*(× 40)

Plate VI: *Aspergillus niger*

4.2.5 Macroscopic and microscopic features of *Aspergillus versicolor*

The growth rate was rapid, covering the 9 cm diameter petri-dish agar surface within 72 hours. The colony surface was at first white, yellow, orange, tan, green and occasionally pinkish, on both PDA and SDA. The reverse of the petri-dish was white, but yellow, orange or red coloration were also encountered. The texture was velvety (Plate VIIa and b).

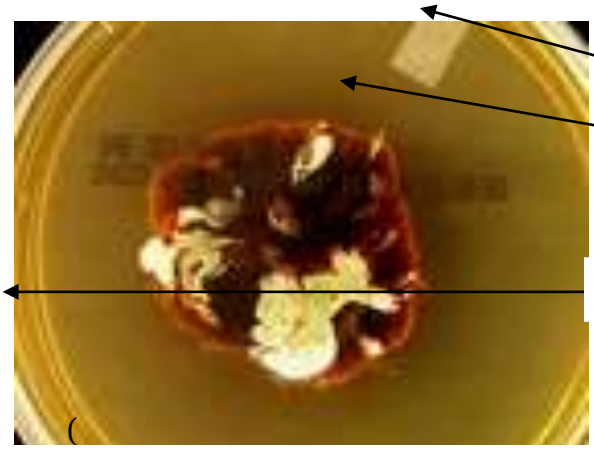
The hyphae had smooth and unbranched conidiophores. The tips of the conidiophores were enlarged, forming a swollen vesicle, covered by chains of round conidia that were loosely radiate. (Plate VIIc).

4.2.6 Macroscopic and microscopic features of *Mucor* sp.

The growth of this fungus on both PDA and SDA was rapid, after 96 hours, it had covered the entire surface of the 9 cm diameter Petri-dish, at room temperature ($28^{o}\pm 2^{0}$ C). The colony covered the agar surface with fluff resembling white cotton candy initially, but later turned grayish to greenish brown, while the reserve side was white throughout (Plate VIII a and b).

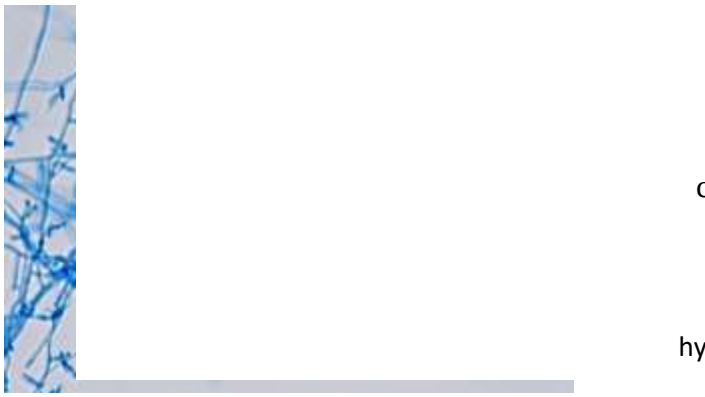
Sporangiophores are long and often branched, bearing terminal, round, spore-filled sporangia. Rhizoids were absent (Plate VIII c).

(a) Upper surface of *Aspergillus versicolor*, pure culture



(

(b) Reverse side of *Aspergillus versicolor*, pure culture

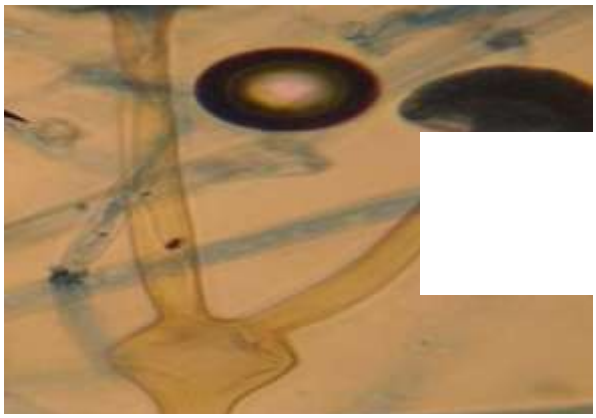


(c) Microscopic features of *Aspergillus versicolor*(× 40)

Plate VII: *Aspergillus versicolor*.

(a) Upper surface of *Mucor* sp., pure culture

(b) Reverse side of *Mucor* sp., pure culture



branched sporangiophore

(c) Microscopic features of *Mucor* sp., (× 40)

Plate VIII: *Mucor* sp.

4.2.7 Macroscopic and microscopic features of *Penicillium* sp.

The growth was rapid, covering the entire agar surface within 96 hours at room temperature ($28^{o}\pm2^{0}$ C). The surface of the colony was at first white, became very powdery and bluish green, having a white border on both PDA and SDA. The reverse side was white (Plate IXa andb).

The conidiophores were branched, with secondary branching known as metulae. Flask-shaped phialides that bore unbranched chains of round conidia were arranged in whorls on the metulae. The fungus had the characteristic “penicillus” or “brush” appearance (Plate IXc).

4.2.8 Macroscopic and microscopic features of *Phoma herbarum*

The growth was rapid, covering the 9cm diameter petri-dish within 120 hours at room temperature ($28^{o}\pm 2^{0}$ C) on both PDA and SDA. The colony was powdery/velvety, spreading and grayish brown. The reverse side was black (Plate X a and b)The hyphae had large dark and round pycnidia (Plate X c).

4.2.9 Macroscopic and microscopic features of *Rhizopus stolonifer*

The growth was rapid, covering the entire agar surface within 96 hours at room temperature on PDA and SDA. The colony grew densely, resembling cotton candy. The colonies appeared white initially, turning gray to yellowish brown, while the reverse side was white (Plate XI a andb).

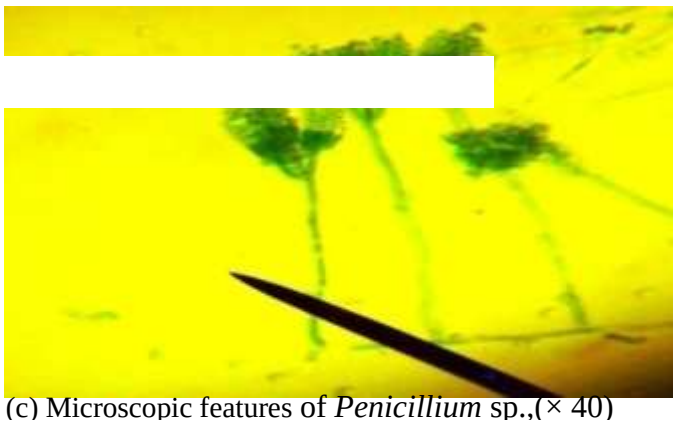
Numerous stolons run among the mycelia, linking groups of long, unbranched sporangiophores. Root-like structures (rhizoids) were produced at the point where the stolons and the sporangiophores meet. The sporagiophores were long and terminated with a dark, round sporangium (Plate XI c)

(a) Upper surface of *Penicillium* sp., pure culture

(b) Reverse side of *Penicillium* sp., pure culture

phialide

metula

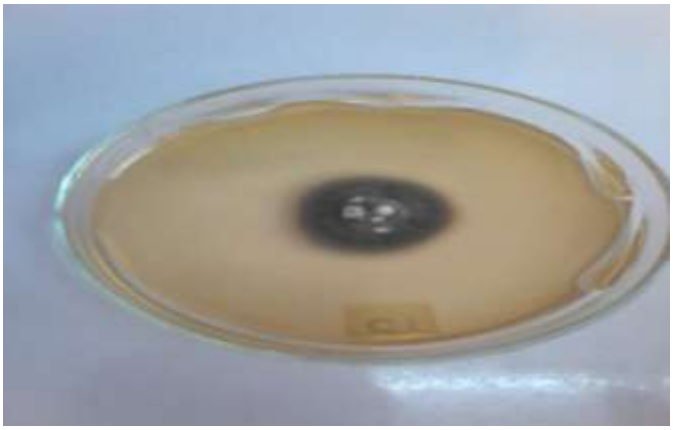


conidia

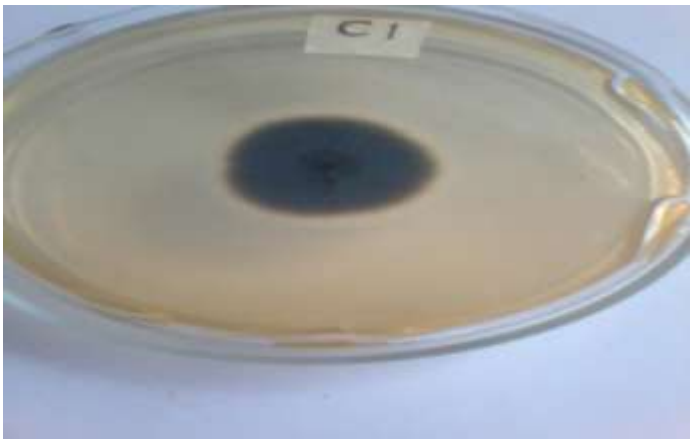
conidiophore

(c) Microscopic features of *Penicillium* sp.,(× 40)

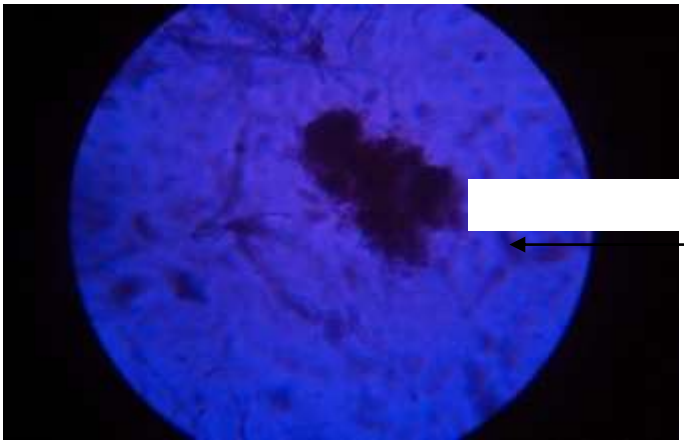
Plate IX: *Penicillium*sp.



(a) Upper surface of *Phoma herbarum*, pure culture



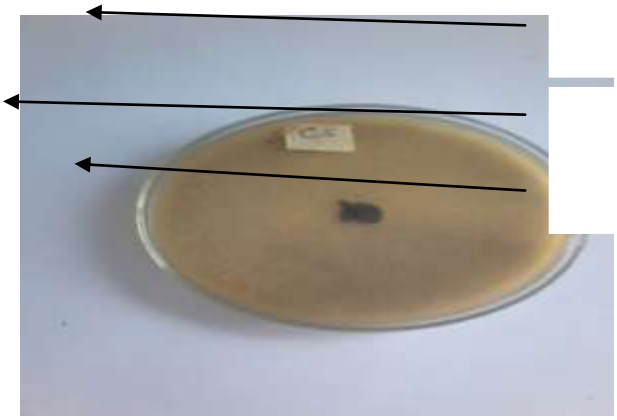
(b) Reverse side of *Phoma herbarum*, pure culture



(c) Microscopic features of*Phoma herbarum*, (× 40)

Plate X: *Phoma herbarum*

(a) Upper surface of *Rhizopus stolonifer*, pure culture



(b) Reverse side of *Rhizopus stolonifer*, pure culture

stolon

spore – filled sporangium

sporangiophore

rhizoid

opic features of *Rhizopus stolonifer*,(× 40)

Plate XI: *Rhizopus stolonifer*

4.3 Number of fungi colonies obtained from cotyledons and testaof *Irvingia gabonensis* and *Irvingia wombolu*

From the cotyledon of *Irvingia gabonensis* and *Irvingia wombolu*, 211(49.88 %) discrete fungal colonies were isolated, while 212 (50.12 %) were isolated from the testa (Table 2). There was no significant difference (P> 0.05) between the total number of fungi colonies on the cotyledons and testa of *Irvingia gabonensis* and *Irvingia wombolu.*The cotyledons of *Irvingia gabonensis* had lower 36 (50.70%) discrete fungi colonies, than the cotyledons of *Irvingia wombolu* which had 175 (49.72 %). The testa of *Irvingia gabonensis* also had lower 35 (49.30 %) discrete fungi colonies, while the testa of *Irvingia wombolu* had 177 (50.28 %) discrete colonies of fungi. There was no significant difference (P> 0.05) between the total number of fungal colonies isolated from both the cotyledons and testa of *Irvingia gabonensis* and *Irvingia wombolu* (Table 2).

The cotyledons of *Irvingia gabonensis* had higher 36 (50.70 %) discrete colonies isolated from them, than the testa of *Irvingia gabonensis* 35 (49.30 %) isolated from them. There was no significant difference (P>0.05) between the number of fungal colonies isolated from the cotyledons and the testa of *Irvingia gabonensis*.

Rhizopus stolonifer had the highest percentage occurrence 25 (69.44 %) in the cotyledons of *Irvingia gabonensis* followed by *Aspergillus niger* with 8 (22.22 %), while *Penicillium* sp. had the least with1 (2.78 %) occurrence. On the testa of *Irvingia gabonensis*, *Aspergillus niger* had 16 (45.71 %) followed by *Aspergillus flavus* 14 (40.00 %) while the least was *Aspergillus fumigatus* with 1 (2.86 %) occurrence (Table 2).

The cotyledons of *Irvingia wombolu* had lower discrete colonies of fungi 175 (49.72 %) isolated from them, than the testa which had 177 (50.28 %) discrete colonies isolated from them. There was no significant difference (P> 0.05) between the fungal colonies isolated from the cotyledons, and the testa of *Irvingia wombolu* (Table 2).

Table 2: Mean number of fungi colonies and their percentages from *Irvingia gabonensis* and *Irvingia wombolu*cotyledons and testa, 2014

***Irvingia* species**	**Fungal Isolates**	**Number of discrete colonies and their percentages (%)**							
		Cotyledons				**Testa**			
		1st	**2nd**	**3rd**	**Total**	**1st**	**2nd**	**3rd**	**Total**
Irvingia gabonensis	*Aspergillus flavus*	0 (0.00)	1 (4.55)	1 (12.50)	**2 (5.56)**	2 (25.00)	3 (27.27)	9 (56.25)	**14 (40.00)**
	Aspergillus fumigatus	0 (0.00)	0 (0.00)	0 (0.00)	**0 (0.00)**	1 (12.50)	0 (0.00)	0 (0.00)	**1 (2.86)**
	Aspergillus niger	1 (16.67)	5 (22.73)	2 (25.00)	**8 (22.22)**	3 (37.50)	6 (54.55)	7 (43.75)	**16 (45.71)**
	Penicillium sp.	0 (0.00)	1 (4.55)	0 (0.00)	**1 (2.78)**	2 (25.00)	0 (0.00)	0 (0.00)	**2 (5.71)**
	Rhizopus stolonifer	5 (83.33)	15 (68.18)	5 (62.50)	**25 (69.44)**	0 (0.00)	2 (18.18)	0 (0.00)	**2 (5.71)**
Total		**6 (16.67)**	**22 (61.11)**	**8 (22.22)**	**36**	**8 (22.86)**	**11 (31.43)**	**16 (45.71)**	**35**
Irvingia wombolu	*Absidia corymbifera*	0 (0.00)	0 (0.00)	1 (1.92)	**1 (0.57)**	0 (0.00)	0 (0.00)	0 (0.00)	**0 (0.00)**
	Aspergillus flavus	12 (21.05)	0 (0.00)	6 (11.54)	**18 (10.29)**	17 (29.83)	26 (41.94)	16 (27.59)	**59 (33.33)**
	Aspergillus fumigatus	3 (5.26)	6 (9.09)	2 (3.85)	**11 (6.29)**	2 (3.51)	1 (1.61)	1 (1.72)	**4 (2.26)**
	Aspergillus niger	23 (40.35)	22 (33.33)	14 (26.92)	**59 (33.71)**	30 (52.63)	18 (29.03)	28 (48.28)	**76 (42.94)**
	Aspergillus versicolor	0 (0.00)	0 (0.00)	0 (0.00)	**0 (0.00)**	0 (0.00)	1 (1.61)	0 (0.00)	**1 (0.57)**
	Mucor sp.	0 (0.00)	0 (0.00)	0 (0.00)	**0 (0.00)**	0 (0.00)	1 (1.61)	0 (0.00)	**1 (0.57)**
	Penicillium sp.	3 (5.26)	0 (0.00)	0 (0.00)	**3 (1.71)**	2 (3.51)	0 (0.00)	0 (0.00)	**2 (1.13)**
	Phoma herbarum	0 (0.00)	0 (0.00)	0 (0.00)	**0 (0.00)**	1 (1.75)	0 (0.00)	0 (0.00)	**1 (0.57)**
	Rhizopus stolonifer	16 (28.07)	38 (57.58)	29 (55.77)	**83 (47.43)**	5 (8.77)	15 (24.19)	13 (22.41)	**33 (18.64)**
Total		**57 (32.57)**	**66 (37.71)**	**52 (29.71)**	**175**	**57 (32.20)**	**62 (35.03)**	**58 (32.77)**	**177**
Grand Total		**63 (29.86)**	**88 (41.71)**	**60 (28.44)**	**211**	**65 (30.66)**	**73 (34.43)**	**74 (34.91)**	**212**

Key:

1st = Feb – March (1st sampling period)

2nd = April – May (2nd sampling period)

3rd = July – August (3rd sampling period)

Bracket = percentages (%)

Rhizopus stolonifer had the highest occurrence 83 (47.43 %) in the cotyledons of *Irvingia wombolu*, followed by *Aspergillus niger* 59 (33.71 %), while *Absidia corymbifera* 1 (0.57 %) had the least occurrence. *Phoma herbarum, Aspergillus versicolor* and *Mucor* sp. were not isolated (i.e. 0 %) from the cotyledons of *Irvingia wombolu* at all. On the testa of *Irvingia wombolu*, *Aspergillus niger* had the highest occurrence 76 (42.94 %), followed by *Aspergillus flavus* 59 (33.33 %), while the least occurring fungi were *Phoma herbarum*, *Aspergillus versicolor*, and *Mucor* sp.which had 1 (0.57 %) occurrence each (Table 2). *Absidiacorymbifera* was not found (i.e. 0 %) on the testa of *Irvingia wombolu* at all. *Phomaherbarum*, *Aspergillusversicolor* and *Mucor* sp. were not isolated (i.e 0 %) from the cotyledons of *Irvingiawombolu* at all.

4.4 Mean number of fungi colonies from *Irvingia wombolu*per market

The data shows that the dominant fungi species in all the markets were *Aspergillusniger*, *Rhizopusstolonifer* and *Aspergillusflavus*(Table 3), while the occurrence of the other fungi species was very low or zero. The number of fungi colonies on *Irvingia wombolu* cotyledons and testa in the various markets are comparable.The mean number of fungi colonies isolated from the cotyledons and the testa of *Irvingia wombolu* samples obtained from Samaru market were 11.0 each, giving a total of 22.0. The cotyledons yielded four (4) fungal species;*Aspergillus niger* (4.0), *Aspergillus flavus* (0.7), *Rhizopus stolonifer* (6.0) and*Aspergillus fumigatus* (0.3) were isolated. The testa also had four (4) fungal species isolated from them;*Aspergillus niger* (4.7), *Aspergillus flavus* (5.3), *Rhizopus stolonifer* (0.7) and *Aspergillus versicolor* (0.3).

The mean number of fungi colonies isolated from the cotyledons and the testa of the *Irvingia wombolu* samples obtained from Ahmadu Bello University Community Market site 1 were 12.0 each, giving a total of 24.0 (Table 3).

Table 3: Mean number of fungi colonies from *Irvingia wombolu* from five markets in Zaria, 2014

Kernel part	Fungi isolated	Samaru Market	ABU Community Market	Sabon Gari Market	Tudun Wada Market	Dan Magaji/Wusasa
Cotyledons	*Absidia corymbifera*	0.0	0.0	0.3	0.0	0.0
	Aspergillus flavus	0.7	3.0	2.0	0.7	1.7
	Aspergillus fumigatus	0.3	1.3	0.0	0.0	0.0
	Aspergillus niger	4.0	4.4	3.7	5.0	2.3
	Penicillium sp.	0.0	0.0	1.0	0.0	0.0
	Rhizopus stolonifer	6.0	3.3	4.7	6.3	8.0
		11.0	**12.0**	**11.4**	**12.0**	**12.0**
Testa	*Aspergillus flavus*	5.3	5.0	3.3	2.3	3.7
	Aspergillus fumigatus	0.0	1.0	0.0	0.3	0.0
	Aspergillus niger	4.7	6.0	5.7	5.7	3.3
	Aspergillus versicolor	0.3	0.0	0.0	0.0	0.0
	Mucor sp.	0.0	0.0	0.0	0.0	0.3
	Penicillium sp.	0.0	0.0	0.7	0.0	0.0
	Phoma herbarum	0.0	0.0	0.3	0.0	0.0
	Rhizopus stolonifer	0.7	0.0	2.0	3.7	4.7
		11.0	**12.0**	**12.0**	**12.0**	**12.0**
	Total	**22.0**	**24.0**	**23.4**	**24.0**	**24.0**

The cotyledons had four (4) fungal species isolated from them; *Aspergillus niger* (4.4), *Aspergillus flavus* (3.0), *Rhizopus stolonifer* (3.3), and *Aspergillus fumigatus* (1.3). The testa had three (3) fungal species isolated from them; *Aspergillus niger* (6.0), *Aspergillus flavus* (5.0) and *Aspergillus fumigatus* (1.0). Table 3 shows that the mean number of fungi colonies isolated from the cotyledons and testa of the *Irvingia wombolu* samples obtained from Sabon Gari market were 11.4 and 12.0 respectively, giving a total of 23.4. The cotyledons had four (4) fungal species isolated from them; *Aspergillus niger*(3.7), *Aspergillus flavus* (2.0), *Rhizopus stolonifer* (4.7) and *Penicillium* sp. (1.0). The testa had five (5) fungal species isolated from them; *Aspergillus niger* (5.7), *Aspergillus flavus* (3.3), *Rhizopus stolonifer* (2.0), *Penicillium* sp.(0.7) and *Phoma herbarum* (0.3).The mean number of fungi colonies isolated from the cotyledons and testa of the *Irvingia wombolu* samples obtained from Tudun Wada market were 12.0 each, giving a total of 24.0 . The cotyledons had three (3) fungal species isolated from them; *Aspergillus niger* (5.0), *Aspergillus flavus* (0.7) and *Rhizopus stolonifer* (6.3).The testa had four (4) fungal species isolated from them; *Aspergillus niger* (5.7), *Aspergillus flavus* (2.3), *Rhizopus stolonifer* (3.7), and *Aspergillus fumigatus* (0.3). Table 3 shows that, the mean number of fungi colonies isolated from the cotyledons and the testa of *Irvingia wombolu* samples obtained from Dan Magaji/Wusasa market were 12.0 each, giving a total of 24.0. The cotyledons yielded three (3) fungal species; *Aspergillus niger* (2.3), *Aspergillus flavus* (1.7) and *Rhizopus stolonifer* (8.0). The testa had four (4) fungal species isolated from them; *Aspergillus niger* (3.3), *Aspergillus flavus* (3.7), *Rhizopus stolonifer* (4.7), and *Mucor* sp. (0.3).

4.5 Mean number of fungi colonies on *Irvingia gabonensis* from five markets in Zaria

The number of fungi colonies in both cotyledons and testa of *Irvingia gabonensis* from Sabon Gari market was higher than that of the other markets (Table 4). This was followed by that of

Samaru, Tudun Wada and Dan Magaji/Wusasa markets in descending order. Ahmadu Bello University (A.B.U) Community market had non isolated from there because, no *Irvingia gabonensis* samples were obtained from there, hence no isolation of fungi took place. *Aspergillusniger*, *Aspergillusflavus* and *Rhizopusstolonifer* occurred more on the cotyledons and testa of *Irvingiagabonensis* from each market basis than the other fungi species.The mean number of fungi colonies isolated from the cotyledons and the testa of *Irvingia gabonensis* samples obtained from Samaru market were three (3) each, giving a total of six (6.0). The cotyledons had three (3) fungal species isolated from them, namely; *Aspergillus niger* (0.7), *Rhizopus stolonifer* (2.0) and *Penicillium* sp. (0.3).The testa also had three (3) fungal species isolated from them, *Aspergillus niger* (2.0), *Aspergillus flavus* (0.3) and *Penicillium* sp. (0.7) (Table 4).

The mean number of fungi colonies isolated from the cotyledons and the testa of *Irvingia gabonensis* samples obtained from Sabon Gari market were six (6.0) each, giving a total of twelve (12.0). The cotyledons had three (3) fungal species isolated from them, namely; *Aspergillus niger*(1.0), *Aspergillus flavus (*0.3) and *Rhizopus stolonifer* (4.7). The testa had four (4) fungal species isolated from them;*Aspergillus niger* (2.3), *Aspergillus flavus* (3.0), *Rhizopus stolonifer* (0.4) and *Aspergillus fumigatus* (0.3) (Table 4).

4.5.1 Mean number of fungi colonies isolated from Tudun Wada market samples

The mean number of fungi colonies isolated from the cotyledons and the testa of *Irvingia gabonensis* samples obtained from Tudun Wada market were three (3.0) and two (2.0) respectively, giving a total of five (5.0). The cotyledons had three (3) fungal species isolated from them; *Aspergillus niger* (0.7), *Aspergillus flavus* (0.3) and *Rhizopus stolonifer* (2.0). The

testa had three (3) fungal species isolated from them; *Aspergillus niger* (1.4), *Aspergillus flavus* (0.3) and *Rhizopus stolonifer* (0.3) (Table 4).

4.5.2 Mean number of fungi colonies isolated from Dan Magaji/Wusasa market samples

The mean number of fungi colonies isolated from the cotyledons and the testa of *Irvingia gabonensis* samples obtained from Dan Magaji/Wusasa market were one (1.0) each, giving a total of two (2). The cotyledons had two (2) fungal species isolated from them; *Aspergillus niger* (0.3) and *Rhizopus stolonifer* (0.7). The testa had three (3) fungal species isolated from them;*Aspergillus niger* (0.3),*Aspergillus flavus* (0.3) and *Rhizopus stolonifer* (0.4) (Table 4).

Table 4: Mean number of fungi colonies on*Irvingia gabonensis*from five markets in Zaria, 2014

Sample part	Fungi isolated	Samaru Market	ABU Community Market	Sabon Gari Market	Tudun Wada Market	Dan Magaji/Wusasa
Cotyledons	*Aspergillus flavus*	0.0	0.0	0.3	0.3	0.0
	Aspergillus fumigatus	0.0	0.0	0.0	0.0	0.0
	Aspergillus niger	0.7	0.0	1.0	0.7	0.3
	Penicillium sp.	0.3	0.0	0.0	0.0	0.0
	Rhizopus stolonifer	2.0	0.0	4.7	2.0	0.7
	Total	**3.0**	**0.0**	**6.0**	**3.0**	**1.0**
Testa	*Aspergillus flavus*	0.3	0.0	3.0	0.3	0.3
	Aspergillus fumigatus	0.0	0.0	0.3	0.0	0.0
	Aspergillus niger	2.0	0.0	2.3	1.4	0.3
	Aspergillus versicolor	0.0	0.0	0.0	0.0	0.0
	*Mucor*sp.	0.0	0.0	0.0	0.0	0.0
	*Penicillium*sp.	0.7	0.0	0.0	0.0	0.0
	Phoma herbarum	0.0	0.0	0.0	0.0	0.0
	Rhizopus stolonifer	0.0	0.0	0.4	0.3	0.4
	Total	**3.0**	**0.0**	**6.0**	**2.0**	**1.0**
	Grand Total	**6.0**	**0.0**	**12.0**	**5.0**	**2.0**

4.6 Comparison of means of colonies of fungal isolates from the *Irvingia* species samples across the five markets

Comparison of means of colonies of fungal isolates from the *Irvingia* species samples across the five markets is presented inTable 5. It shows that there were no significant differences (P>0.05) between the means of the fungal isolates from *Irvingia gabonensis* cotyledons and testa, apart from *Rhizopus stolonifer*, where the means of isolates from the cotyledon was significantly diferent from the mean of isolates from the testa.

For *Irvingia wombolu* samples, *R. stolonifer* was the highest, and showed and showed significant difference (P<0.05) from the testa. *Aspergillus niger*and *A. flavus*isolates from the cotyledons and testa showed significant differences (P<0.05), the remaining six isolates showed no significant differences (P>0.05) between the means of the cotyledons and testa.

Table 5: Comparison of means of colonies of fungal isolates from the *Irvingia* species samples across the five markets

	Irvingia gabonensis		*Irvingia wombolu-*	
Species	Cotyledon	Testa	Cotyledon	Testa
Aspergillus corymbifera	0.00±0.00[b]	0.00±0.00[b]	0.07±0.26[d]	0.00±0.00[d]
Aspergillus flavus	0.13±0.35[b]	0.93±1.22[b]	1.60±1.72[c]	3.93±1.91[b]
Aspergillus fumigatus	0.00±0.00[b]	0.07±0.26[b]	0.33±0.72[d]	0.27±0.46[d]
Aspergillus niger	1.20±1.90[a]	1.80±2.62[a]	3.80±1.70[b]	5.20±2.18[a]
Aspergillus versicolor	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[d]	0.07±0.26[d]
Mucor sp. .	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[d]	0.07±0.26[d]
Penicillium. sp.	0.07±0.26[b]	0.13±0.52[b]	0.20±0.76[d]	0.13±0.52[d]
Phoma herbarum	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[d]	0.07±0.26[d]
Rhizopus. Stolonifer	1.67±2.38[a]	0.20±0.41[b]	5.80±3.28[a]	2.07±2.28[c]

Means with the same superscript acrossthe rowsforeachspecies are not significantly different (P> 0.05),using DMRT at 5 % level.

4.7 Comparison of the means of the various fungal isolates obtained from the two *Irvingia* species across the five markets

Table 6 gives a comparison of the means of the various fungal isolates obtained from the two *Irvingia* species across the five markets. The data on the fungal isolates from *Irvingia gabonensis* samples obtained from Samaru market,showed that *A. niger* had significantly higher number of colonies than the other species. This was followed by that of *R. stolonifer.* However, there were no significant differences (P>0.05) in the number of colonies of the other fungal species.

The data on the fungal isolates from *Irvingia wombolu* samples obtained from Samaru market showed that the highest number of colonies was from *A. niger*is comparable with *R. stolonifer* and *A. flavus.* All other fungal species (most of which were not isolated) had significantly lower number of colonies (Table 6). No *Irvingia gabonensis* samples were obtained from A.B.U. Community market, hence no fungal isolates were obtained, therefore, no significant differences (P>0.05) recorded.

The fungal isolates from *Irvingia wombolu* samples obtained from A.B.U Community market, showed that colonies of *A. niger*and *A. flavus* were significantly higher than that of the other fungal species. These were followed by that of *R. stolonifer* and *A. fumigatus* . The remaining five fungal species were not isolated, hence no differences between their means (Table 6).

The fungal isolates from *Irvingia gabonensis* samples obtained from S/Gari market showed that there were no colonies of *A. corymbifera, A. versicolor, Mucor* sp., *Penicillium* sp.and *Phoma herbarum*found.. *Rhizopus stolonifer* had the highest number of colonies, but was not significantly (P>0.05) different from *A. niger*and *A. flavus.* These were however significantlyhigher(P<0.05) than thatfrom *A. fumigatus.*

The fungal isolates from *Irvingia wombolu* samples obtained from S/Gari market showed that the higher number of colonies of *A. niger* was not significantly different (P>0.05) from that of *R. stolonifer* and *A. flavus.* The colonies observed from other fungal species were not significantly different (P>0.05) from those not isolated (Table 6). The fungal isolates from *Irvingia gabonensis* samples obtained from Tudun Wada market showed no significant differences (P>0.05) between their means throughout.

No*A.corymbifera, A. versicolor, Mucor* sp., *Penicillium* sp., and *P. herbarum* was isolated from *Irvingia wombolu* samples obtained from Tudun Wada market.*Irvingia wombolu* samples obtained from Tudun Wada market showed that the number of colonies of *A. niger* and *R. stolonifer* were significantly higher (P<0.05) than that of *A. flavus* and *A. fumigatus* (Table 6).

The few fungal isolates from *Irvingia gabonensis* samples obtained from Dan Magaji/Wusasa market were only those of *R. stolonifer,A. niger* and *A. flavus,* and the number of colonies from them were not significantly different (P>0.05) from those without isolates. The data from *Irvingiawombolu* samples obtained from Dan Magaji/Wusasa market showed that only *R. stolonifer, A. niger,A. flavus* and *Mucor* sp. were found. The number of colonies of *R. stolonifer* was significantlyhigher (P<0.05) than that of the other fungal species isolated (Table 6).

Table 6 Comparison of means of colonies of fungal isolates from the *Irvingia*species samples for the five different markets

Fungal Species	Samaru		Comm MKT		S/G MKT		T/W MKT		D/M/W	
	I. gabonensis	***I. wombolu***	***I. gabonensis***	***I. wombolu***	***I. gabonensis***	***I. wombolu***	***I. gabonensis***	***I. wombolu***	***I. gabonensis***	***I. wombolu***
A. corymbifera	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.17 ± 0.41^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}
A. flavus	0.67 ± 1.21^{c}	3.00 ± 2.76^{a}	0.00 ± 0.00^{a}	4.00 ± 2.10^{a}	1.17 ± 1.47^{a}	2.67 ± 2.50^{a}	0.67 ± 0.82^{a}	1.50 ± 1.38^{b}	0.17 ± 0.41^{a}	2.67 ± 1.63^{b}
A. fumigatus	0.00 ± 0.00^{c}	0.17 ± 0.41^{b}	0.00 ± 0.00^{a}	1.17 ± 0.75^{b}	0.17 ± 0.41^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	0.17 ± 0.41^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}
A. niger	4.33 ± 2.66^{a}	4.33 ± 1.63^{a}	0.00 ± 0.00^{a}	5.17 ± 2.04^{a}	2.17 ± 2.48^{a}	4.83 ± 2.64^{a}	0.67 ± 1.03^{a}	5.33 ± 1.97^{a}	0.33 ± 0.52^{a}	2.83 ± 1.33^{b}
A. versicolor	0.00 ± 0.00^{c}	0.17 ± 0.41^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}
***Mucor*.sp.**	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}	0.17 ± 0.41^{c}
***Penicillium*sp.**	0.50 ± 0.84^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.83 ± 1.33^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}
P. herbarum	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.17 ± 0.41^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}
R. stolonifer	1.00 ± 1.27^{b}	3.33 ± 3.72^{a}	0.00 ± 0.00^{a}	1.67 ± 2.73^{b}	2.50 ± 3.39^{a}	3.33 ± 3.98^{a}	0.67 ± 1.21^{a}	5.00 ± 2.53^{a}	0.50 ± 0.84^{a}	6.33 ± 2.582^{a}

Means with the same superscript down the column for each market and sample, are not significantlydifferent (P>0.05) using DMRT

Key: Comm Mkt - A.B.U Community Market

S/G Mkt - Sabon Gari Market

T/W Mkt - Tudun Wada Market

D/M/W - Dan Magaji/Wusasa Market

4.8 Mean aflatoxin levels for *Irvingiawombolu* and *Irvingia gabonensis*kernel samples from the five markets.

Irvingiawombolu kernel samples, obtained in February/March from Tudun Wada market had the highest mean aflatoxin content, followed by Samaru market samples, while the A.B.U community market site I samples recorded the least. However, this difference was not significant. The kernels obtained in April/May from Tudun Wada, showed the highest aflatoxin content which was similar to that from Sabon Gari and Samaru markets.

Kernels from Dan Magaji/Wusasa and A.B.U. community markets showed significantly lower aflatoxin content, compared to that of others. However, in July/August the aflatoxin content of kernels from the five markets was comparable, although the sample from Sabon Gari market recorded the highest concentration, followed by that from A.B.U. community market, while the least was recorded in the *Irvingia* sample from Tudun Wada market (Table 7)

The overall means for the three sampling periods however, showed that kernels from Sabon Gari recorded the highest, which was comparable with that from Samaru and Tudun Wada markets, while the least recorded from Dan Magaji/Wusasa was similar to that from ABU community market. On the other hand, the overall means for the three sampling periods showed that Feb/March recorded the highest, followed by July/August, and lastly, the least aflatoxin content from those obtained in April/May (Table 7).

Irvingiagabonensis kernel samples obtained in February/March from Tudun Wada market had the highest aflatoxin content that was significantly higher than that of the other markets. This was followed by that obtained from Samaru market. The least aflatoxin content from those of Dan Magaji, Wusasa was significantly lower than that from other markets, as they were all significantly different from one another.

Table 7: Aflatoxincontent of kernels of two *Irvingia* species from five markets in Zaria

Irvingia Species	Sampling period	Markets Aflatoxin content (µg / kg)					Mean	Standard error (±)
		Tudun Wada	Dan Magaji / Wusasa	Sabon Gari	Samaru	A.B.U., Community Market		
Irvingiawombolu	Feb/March	95.80±5.16[a]	89.15±12.05[a]	89.81±8.21[a]	93.50±7.15[a]	82.52±28.01[a]	90.16±13.25	3.42
	April/May	94.48±7.24[a]	33.96±7.82[b]	92.92±14.11[a]	88.77±2.77[a]	58.95±31.10[b]	73.82±28.09	7.25
	July/August	79.43±17.73[a]	87.72±22.18[a]	104.08±12.46[a]	78.74±5.26[a]	93.78±10.85[a]	88.75±15.94	4.12
	Mean	89.91±10.04	70.28±14.02	95.60±11.59	87.00±5.06	78.42±23.32		
Irvingiagabonensis	Feb/March	128.68±0.00[a]	101.49±0.00[d]	108.36±0.00[c]	113.7±0.00[b]	NA	113.06±10.45	3.02
	April/May	110.36±0.00[c]	47.17±0.00[d]	128.03±0.00[a]	111.36±0.00[b]	NA	99.23±32.24	9.31
	July/August	72.98±0.15[a]	82.90±11.20[a]	88.69±29.75[a]	NA	NA	81.52±17.32	5.77
	Mean	104.01±0.05	77.19±3.73	108.36±9.92	75.02±0.00			

Means with the same superscriptin each row are not significantly different (P> 0.05), using DMRT

Key:

NA = Not Available

The A.B.U. community market had no *Irvngia gabonensis* samples at all for each of the sampling dates (Table 7). The *Irvingia gabonensis* kernel samples obtained in April/May, from Sabon Gari market had the highest aflatoxin content which was significantly higher than that of the other markets. This was followed by that obtained from Samaru market. The least aflatoxin content from those of Dan Magaji/Wusasa was significantly lower than that from the other markets, as all the samples from the four markets were significantly different from one another.

Irvingia gabonensis kernel samples obtained in July/August from Sabon Gari market had the highest aflatoxin content which was not significantly higher than that of the other markets. This was followed by that obtained from Dan Magaji/Wusasa market, and lastly the samples obtained from Tudun Wada market. Samaru market had no *Irvingiagabonensis* samples for July/August sampling date.

The overall means for the three sampling periods however, showed that kernels from Sabon Gari recorded the highest, which was comparable with that from Tudun Wada market. While the least recorded was from Samaru market, which was comparable to that of Dan Maggaji / Wusasa market. On the other hand, the overall means for the three sampling perids showed that Feb / March recorded the highest, followed by April / May, and the least aflatoxin content was those obtained in July / August (Table 7).

CHAPTER FIVE

5.0 DISCUSSION

Irvingia kernels are nutritionally rich, medicinally highly valued and economically lucrative, both locally and internationally (Ladipo, 1999). However, studies have shown that a major setback in the sales and consumption of *Irvingia* kernels is their susceptibility to post-harvest spoilage by fungi, which also have potential health risks to the consumer (Etebu and Bawo, 2012).

Nine different fungi species isolated in this study from*Irvingiagabonensis* and *Irvingiawombolu* cotyledons and testa include: *Absidia corymbifera, Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus versicolor, Mucor* sp. *Penicillium* sp., *Phoma herbarum, and Rhizopus stolonifer.* Among these, *Aspergillus niger, Rhizopus stolonifer, Aspergillus flavus, Penicillium and Mucor* sp.had earlierbeen isolated from *Irvingia* kernels in storage and are recognized to be among the most common group of fungi that infect grains after harvest, and grow on them during storage (Agrios, 1978). This is in agreement with the reports of Adebayo-Tayo *et al.* (2006), Iyayi *et al.* (2010), Aboloma and Ogunbusola (2012) and Etebu and Bawo (2012) who isolated different fungal species from *Irvingia*species kernels displayed for sale to consumers in some Nigerian markets.Etebu and Bawo (2012) isolated eight species of fungi from*Irvingia* kernels in Amassoma, Bayelsa State, namely:*A. niger, Rhizopus stolonifer, A. flavus, Penicillium* sp., *Mucor*sp.,*Candida tropicalis, Phythophthora* sp. and *Fusarium oxysporum* in descending order of abundance. Their findings are similar to the ones isolated from this study, except that *Candida tropicalis, Phytophthora* sp. and *Fusarium oxysporum* were not isolated from this study. Also, *Absidia corymbifera, A. fumigatus, A. versicolor* and *Phoma herbarum* were isolated from this present work, which they did not isolate. This may be due to environmental/climatic differences as they did their work in Bayelsa state, (South South),

Nigeria. Another difference betweentheir work and this present study is that they worked on intact kernels (i.e. testa not separated from cotyledons), whereas in this work, the testa was cultured separately from the cotyledons.

The fungi isolated here have also been found to be associated with a variety of other crops as reported by several workers (Adebayo-Tayo *et al*., 2006; Amadi and Adeniyi, 2009; Akintobi *et al*., 2011 and Fagbohun *et al.,* 2011).

This study revealed that more *Aspergillusniger*(57.86%), and *A. flavus* (78.49%), were isolated from the testa, than that from the cotyledons (42.14%) and (21.51%) respectively. However, *Rhizous stolonifer* were isolated more from the cotyledons (75.52%) than from the testa (24.48%). Hence, there was significant difference between the cotyledon and testa.

The result showed that *Aspergillusniger* had the highest (37.59%) percentage occurrence of all the fungi isolated, and was closely followed by *Rhizopus stolonifer* (33.81%). The dominance of *Aspergillus*spp. here is in agreement with Visconti *et al.* (2001) who reported that *Aspergillus*spp. are the pioneer fungal colonizers in seed-borne infection of*Sorghum*before other species of fungi arrive. More *Aspergillusniger* were isolated from the testa, than from the cotyledons. One could therefore infer that *Aspergillusniger* is better adapted to the testa, probably due to the fact that the testa is the external and most accessible part of the kernel, than the cotyledons. For *Rhizopus stolonifer* however, the fungus is more abundant on the cotyledonsthan the testa, probably due to adaptation.

The third most occurring fungus was *Aspergillusflavus*. Most of the *Aspergillusflavus* isolates were from the testa, while the cotyledonshad fewer. It is also evident that *Aspergillusflavus* colonized the testa more than the cotyledons, andthis could be due to adaptation,bearing in mind that *Aspergillusflavus* is potentially aflatoxic (Wu and Khlangwiset, 2010). Therefore, it appears potentially harmful to mill *Irvingia* kernels for consumption, without removing the testa.

One of the fungi isolated from this study of *Irvingia* cotyledons and testa have the tendency to habour aflatoxin, which if ingested by man or animals, could lead to serious health challenges, and this fungiwas*Aspergillus flavus.* Wu and Khlangwiset (2010) reported that aflatoxins are produced primarily by the fungi *Aspergillusflavus* and *Aspergillusparasiticus*.

Certain post-harvest conditions have been implicated as pre-disposing factors that lead to fungal attack and aflatoxin production in contaminated food, and some of these post-harvest conditions include storage conditions and duration,transportation, and processing methods (Wu and Khlangwiset, 2010).

Storage is a very vital process in the entire *Irvingia* kernels business as usage of contaminated containers enhances fungal contamination. Common salt prevents the growth of spoilage microbes by: decreasing the amount of water in foods available for microbial growth and chemical reactions (Potter and Hotchkiss, 2012; Fennema 1996), kills microbial cells through osmotic shock (Davidson, 2001), retards microbial growth by interfering with cellular enzymes (Shelef and Seiter, 2005). Etebu and Bawo (2012), reported that sun-drying and adding 0.9% common salt (NaCl) to *Irvingia* kernels and then sealing in polythene bags reduced post-harvest disease incidence. Failure to adopt these measures might account for why all the samples in this study recorded fungal contamination, though in varying proportions. The storage of agricultural products, especially grains in sealed containers has been recommended because storing grains in tight containers causes a drastic reduction in oxygen, and an increase in carbon IV oxide (CO_2) which helps to reduce both insects and fungal activities (Soffe, 2011).

Irrespective of species, *Irvingia*kernels in storage are attacked by similar fungal species, as this study has revealed. This corroborates the reports of Adebayo-Tayo *et al.,* (2006), Etebu and Bawo (2012). Etebu (2012a,b) asserted that fungal population increased with increase in storage

period. Also, Obetta *et al*. (2011) who worked on selected fruits and vegetables showed that fungal and bacterial populations increased correspondingly as storage days increased.

Most of the kernels are produced in remote areas that have poor road network, hence before these kernels are brought to the commercial centres, they would have suffered mechanical damage due to the bad roads, hence weakening the kernel's resistance to fungal attack. Other factors that affect myctotoxin production include the fruit or vegetable type and cultivar, geographical location, climate, pre-harvest treatments and method of harvest (Golan and Paster, 2008).

All the samples from the five markets subjected to Enzyme Linked Immunosorbent Assay (ELISA) showed aflatoxin presencewhich varied with market, species and season.

All the samples from the five markets showed aflatoxin concentrations higher (33.96 - 128.68µg / kg) than the World Health Organization (WHO) approved standard of 20 µg/kgfor food samples, (Bankole and Joda, 2004; Oladejo and Adebayo-Tayo, 2011). This corroborates the reports of Adebayo-Tayo*et al.* (2006) and Iyayi *et al.* (2010), who showed that the *Irvingia* kernels sold in Nigerian markets are not safe for human consumption.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Sample collection was done thrice; February – March, April – May, and lastly, July – August. In order to isolate the fungal species, the cotyledon and testa were separated and cultured in PDA media, while the sub-culturing of the emergent fungi was done in SDA media in order to obtain pure cultures.

From the five markets (A.B.U Community, Dan Maggai/Wusasa, Sabon Gari, Samaru and Tudun Wada) sampled within Zaria that provided *Irvingia gabonensis* and *Irvingia wombolu* samples, nine species of fungi were isolated from both the cotyledons as well as the testa.Five fungi species were isolated from *Irvingia gabonensis*samples,namely *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Penicillium*sp.,and *Rhizopus stolonifer*whereas nine species were isolated from *Irvingiawombolu*, namely, *Absidia corymbifera*, *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus versicolor, Mucor* sp.,*Penicillium*sp., *Phoma herbarum*,. and*Rhizopus stolonifer.*

Out of these, *Aspergillusflavus*was known to produce aflatoxins, hence ELISA was carried out to ascertain if the *Irvingia* kernels were aflatoxic.The ELISA results showed that,all the *Irvingia* kernels had aflatoxin concentrations higher than the World Health Organization (WHO) approved standard of 20µg/kg (Bankole *etal.*,2004; Oladejoh and Adebayo-Tayo 2011). Hence, these kernels might not be safe for human consumption.

6.2 Conclusion

Kernels of*Irvingia gabonensis* and *Irvingia wombolu*obtainedfrom markets within Zaria metropolis are contaminated by several fungi of post-harvest origin.These are*Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Rhizopus stolonifer* and *Penicillium* sp. *and Rhizopus stolonifer*were isolatedfrom *Irvingiagabonensis*, while from *Irvingia wombolu, Absidia corymbifera, Aspergillus flavus, Aspergillusfumigatus, Aspergillusniger, Aspergillus versicolor, Mucor* sp., *Penicillium* sp., *Phoma herbarum, and Rhizopus stolonifer.*One of these, *A. flavus*is anaflatoxin-producing fungus, which makes consumption of contaminated kernels risky to the consumer. The aflatoxin concentrationsranged between33.96 and 104.08µg/kg for *I.wombolu,*aswellas47.17to128.68 µg/kg *for I.gabonensis,which* were quite higher than the WHO standard of 20 µg/kg.

6.3 Recommendations

1. This work revealed that most of the aflatoxin-producing fungi are lodged or domiciled in the testa,therefore,detaching the testa from cotyledons before milling would greatly lower the aflatoxin concentration.
2. Furtherinvestigations using advanced serological and molecular tools on the aflatoxin contents in the cotyledons, testa and other tissues of *irvingia* species seed should be conducted.

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APPENDIX

APPENDIX I: Systematic Description of Fungi Isolated from *Irvingia* Species Cotyledons and Testa

FUNGAL ISOLATE	MACROSCOPIC FEATURES	MICROSCOPIC FEATURES
Absidia corymbifera	Coarse colonies, woolly gray surface on both SDA and PDA. Fluff resembled gray cotton candy. Their reverse was white in colour (Plate III	Wide, non-septate hyphae, sporangiophores arise at points on the stolon that were between the rhizoids, and not opposite

	a andb).	them. sporangiophores are branched. Sporangia relatively small, and slightly shaped like a pear, instead of spherical (Plate III c)
Aspergillus flavus	Rapid growth within one week at room temperature (28 ± 2^0C) colonies were regularly shaped on both PDA and SDA. Appeared velvety yellow to green/brown on the surface, while the reverse appeared red-brown (Plate IVa andb).	Mycelium had conidiophores of variable length, which were rough, pitted and spiny. Phialides were both uniseriate and biseriate, covering the entire vesicle and point out in all directions (Plate IV c).
Aspergillus fumigatus	Rapid growth within 3 days. Surface initially white, then dark greenish to gray later. Velvety texture. Reverse side white to tan (Plate V a and b)	Hyphae septate, with unbranched conidiophores that are smooth and short. Conidiophores enlarged at the tip forming a swollen vesicle covered with flask shaped phialides (formerly known as sterigmata). Phialides are uniseriate, usually only on upper two-thirds of vesicle, parallel to axis of conidiophores. Phialides bear chains of mostly round conidia (PlateVc).
Aspergillus niger	Rapid growth on both PDA and SDA, maturing within 3 days at room temperature. Colony white to yellow at first, then turning black later. Reverse is white to yellow (Plate VI a andb).	Mycelium had profusely branched, hyaline septate hyphae, bearing long, smooth and erect hyaline unbranched conidiophores, narrowing slightly towards the base. Conidiophores terminated in a globose vesicle which bear biseriate flask shaped phialides, covering the entire vesicle, forming a radiate head (Plate VI c.)
Aspergillus versicolor	Rapid growth, maturing within 3 days at room temperature, colony surface first white, then yellow, orange, tan, green, and even occasionally pinkish on both PDA and SDA, within velvety texture. Reverse was white but at times yellow, orange or red (Plate VII a and b)	Hyphae septate, having long, smooth and unbranched conidiophores that arose from a specialized foot cell. Tip of the conidiophores enlarged, forming a swollen vesicle. Biseriate phialides produce chains of mostly round conidia that were loosely radiate (Plate VII c)
Mucor species	Rapid growth on both PDA and SDA, maturing within 4 days at room	Wide, practically non-septate hyphae. Have long, and often

	temperature. Fluff resembling white cotton candy. Surface of colony usually white, but later turned gray to grayish brown. Reverse side was white (Plate VIII a andb).	branched sporangiophores, bearing terminal, round, spore-filled sporangia. Rhizoids were absent (Plate VIII c).
Penicillium species	Rapid growth, covering entire agar surface within 4 days at room temperate. Surface of the colony was first white, then very powdery, and bluish green. The reverse was white (Plate IX a and b)	Septation of hyphae, conidiophores branched with even secondary branching known as metulae. Phialides flask-shaped, and bore unbranched chains of round conidia arranged in whorls on the metulae. Fungus had the characteristic "Penicillus" or "brush" appearance (Plate IX c).
Phoma herbarum	Rapid growth, covering the 9cm diameter petri-dish within 5 days at room temperature. Colony was powdery velvety, spreading and grayish brown. Reverse side black (Plate X a andb).	Hyphae showed septation, with large asexual fruiting bodies (pycnidia), which were round, with openings (ostioles) for conidia dispersal (Plate X c).
Rhizopus stolonifer	Rapid growth, covering entire agar surface within 4 days at room temperature on both PDA and SDA. Colony were initially white, then turning gray to yellowish brown later. Reverse side was white (Plate XI a andb).	Non-septate hyphae, with long, unbranched sporangiophores that terminated in dark, round sporangium. Root-like structures (rhizoids) produced at the point where stolons and sporangiophores met (Plate XI c).

Note: Identification aided by appropriate taxonomic keys (Barnett and Hunter, 1972; Fawole and Osho, 1995; Boerema *et al*., 2004; James and Natalie, 2013)

APPENDIX II: Mean Relative Humidity in Zaria, from January to September, 2014

Source: Institute for Agricultural Research (I.A.R) Meteorological unit, Ahmadu Bello University, Zaria.

	Relative Humidity (%)	
MONTH	10.00 AM	4.00 PM
JANUARY	19	13
FEBRUARY	15	12
MARCH	27	19
RIL	54	39
MAY	67	51
JUNE	73	61
JULY	78	70
AUGUST	82	73
SEPTEMBER	78	71