KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

SCHOOL OF GRADUATE STUDIES

COLLEGE OF SCIENCE

DEPARTMENT OF BIOCHEMISRTY AND BIOTECHNOLOGY

KNUST

DETERMINATION OF AFLATOXIN-PRODUCING FUNGI STRAINS AND LEVELS OF AFLATOXIN B1 IN SOME SELECTED LOCAL GRAINS

BY

EBRIMA AA JALLOW

SEPTEMBER, 2015

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A THESIS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY, KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY (KNUST), IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF PHILOSOPHY (MPhil) DEGREE IN BIOCHEMISTRY

SEPTEMBER, 2015

DECLARATION

I hereby declare that this dissertation, which I now submit for the assessment of the Programme of study leading to an award of Master of Philosophy (MPhil) degree in Biochemistry is entirely my work. I certify that to the best of my knowledge, this work has not been previously submitted for an award of any other degree of the University except where due acknowledgement has been made in the text.

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DEDICATION

Praises and thanks to the Almighty Allah, the creator of the heavens and the earth, the Lord of mankind for his decree towards the success of this project.

To my new born angel Rahima Jallow, my son Muhammad Jallow and also to my loving wife Mariam Sowe, I say thank you for being the pillar in my life. Finally and most importantly to my beloved Mother Wuran Bah, whose constant prayers pave the way for the success. And to all the rest of my family and friends. May Allah bestow his mercy on us all.

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ABSTRACT

Aflatoxins are bisfurans that are polyketide-derived, toxic, and carcinogenic secondary metabolites produced by some species of *Aspergillus* and other fungi on food crops and feed. The versatility of these fungi to different factors determine the infestation and colonization of the substrate, the type and amount of aflatoxin produced. Aflatoxin B1, is classified as the most toxic of the aflatoxins, responsible for not only causes great economic loss but also is the most potent naturally occurring chemical liver carcinogen known. Random and replicated samples of groundnut, maize, beans and rice were purchased from the Kumasi Central Market and analyzed for their aflatoxin levels using High Performance Liquid Chromatography (HPLC). The contamination levels found ranged from trace amount to 31.11ppb with groundnut registering the highest aflatoxin content. A further microbial culture examination revealed that most of the crop samples especially groundnut and maize were susceptible to various species of aflatogenic *A. flavus, A. paracitius, A. tamarii, P. expansum, Mucor hiemalis, A. niger P. citrinum, Moniliella* and other pathogenic fungi. Colony forming units per gram (CFU/g) from the microbial cultures ranged from 4.3×10^6 to 2.1×10^3. However, a poor correlation existed between the aflatoxin contamination level and the CFU/g per sample. Furthermore, molecular assessment of aflatoxin producing fungi in the grains samples involving five pairs of universal and eight specific aflatoxin primers were carried out. A consistent correlation could not be made between the molecular analysis and microbial results. Just as in *A. versicolor*, four universal primers 0817F/1196R, U1/U2 and FF2/FR1 and ITS1/IST2 and one specific aflatoxin producing fungi primer Nor1/Nor2 was able to show positive bands on *A. versicolor*. Which means these particular fungi, have the gene to produce aflatoxin however it could not produce detectable aflatoxin by the HPLC.

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LIST OF ABBREVIATIONS

Ab-Ag	antibody-antigen
AF(s)	Aflatoxin(s)
$AFB_{1:}$	Aflatoxin B_1
$AFB_{2:}$	Aflatoxin B_2
$AFG_{1:}$	Aflatoxin G_1
$AFG_{1:}$	Aflatoxin G_2
$AFM_{1:}$	Aflatoxin M_1
$AFM_{2:}$	Aflatoxin M_2
a_w:	Water activity
CAES:	Compressed Air Energy Storage
CDC:	Centers for Disease Control and Prevention
CFU:	Colony Forming Unit
CTBA:	California Thoroughbred Breeders Association
DNA:	Deoxyribonucleic acid
dNTPs;	Deoxynucleotide triphosphates
EDTA:	Ethylenediaminetetraacetic acid
EU:	European Union
FAO:	Food and Agriculture Organization
FDA:	Food and Drugs Authority
Fhb:	Flabohemoglobins
HPLC:	High Performance Liquid Chromatography
HCl:	Hydrogen Chloride
IARC:	International Agency for Research on Cancer
IRRI:	International Rice Research Institute
IgA:	Immunoglobulin A
IITA:	International Institute of Tropical Agriculture
MEA:	Malt extract agar
NOR-1:	None-derived orphan receptor

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PCR: Polymerase Chain Reaction

PDA: Potato dextrose agar

SDS: Sodium dodecyl sulfate

ST: Sterigmatocystin

Taqpol: Tag polymerases (Thermostable DNA polymerases)

TLC: Thin layer chromatography

UV: Ultraviolet

USAID: United States Agency for International Development

US FDA: United States Food and Drug Administration

VGC: Vegetative Compatibility Groups

WHO: World Health Organization

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CHAPTER ONE
1.0 INTRODUCTION

1.1 BACKGROUND INFORMATION

Aflatoxin, a word derived from *Aspergillus fla*vus *toxin* is naturally occurring mycotoxins that is produced by *Aspergillus flavus* and *Aspergillus parasiticus* species of fungi. Aflatoxin is a highly toxic secondary metabolite that contaminates a number of crops causing a great economic loss (Cary *et al*., 2000; CAST 2002). Several other moulds and fungi species also produce aflatoxin and among them include *A. nomius, A. pseudotamarii* and *A. bombycis* (Peterson *et al.,* 2001). The biosynthetic pathway of aflatoxin in *A. flavus* and *A. parasiticus* are similar and well characterized (Cary *et al.,* 2000; Yu *et al.,* 2004).

Aflatoxin is produced in certain foods and feeds and undoubtedly the best known and most intensively researched mycotoxins in the world. It has been associated with various diseases, such as aflatoxicosis in livestock, domestic animals and humans throughout the world (Bhatnagar *et al.*, 2003). The occurrence of aflatoxin is influenced by certain environmental factors, hence the extent of contamination will vary with geographic location, agricultural and agronomic practices, and the susceptibility of crop commodities to fungal invasion during pre-harvest, storage or processing periods. (Bhatnagar *et al.*, 2003).

Food security on the African continent has worsened and deteriorating in recent decades. The proportion of the malnourished population has remained dominant in most Sub-Saharan African countries (FAO, 1996). To eradicate or mitigate this situation, an increase in the productivity of agricultural food crops especially groundnut, maize, rice millet and beans are not only necessary as these crops form part of the staple foods and the main cash crops of most of these countries but must go hand-in-hand with better farming practices and post-harvest handling.

These agricultural produce are very prone to aflatoxin contamination, particularly during growth periods, harvesting, threshing, and drying (Siriacha *et al.,* (1989). Contamination can also occur when grains are poorly stored which can cause pest infestation and poor conditions leading to an accelerated growth rates of *Aspergillus* and other fungi species (WHO, 2006).

Aflatoxin-producing species of *Aspergillus* are common and widespread in nature. These crops are particularly susceptible to infection by *Aspergillus* following prolonged exposure to a high-humid environment, or damage from stressful conditions (Udoh, 2000).

1.2 PROBLEM STATEMENT

Mycotoxins affect nutritional and economic value of staple foods and cash crops especially in developing countries including those in Africa. Of the many mycotoxins, aflatoxin is of major concern especially countries where agricultural practices are not strictly controlled, human and animal exposure to mycotoxins is very high (Wagacha *et al.,* 2008). Attention is only paid to meet export criteria while the effects of aflatoxin on health of the local consumers is not prioritized. The contamination of foods with aflatoxin has in recent times created a great alarm on food security in Africa (Leslie, 2005). It has caused massive economic losses on export and import markets and diseases such as impaired immune system, cancer and stunted growth in infants (Williams *et al.,* 2004).

In Ghana, groundnut, maize and other cereals and legumes are sold in the open market with less or no regulation of quality. Most of the contaminated foods find their way into households and restaurants and patronizeded by unsuspecting consumers. The assessment of the levels of aflatoxin in food crops and the identification of fungi responsible for their contamination will inform policy makers to improve upon proper handling to reduce the toxin in foods.

1.3 MAIN OBJECTIVE

To evaluate Aflatoxin-producing fungi and levels of Aflatoxin B1 in selected cereals and legumes.

1.4 SPECIFIC OBJECTIVES

1. To determine the level of aflatoxin B1 in groundnut, maize, beans and rice from Kumasi Central Market in Ghana using HPLC.

2. To determine the strains of filamentous fungi (moulds) on the food stuffs using microbial culture techniques.

3. To confirm the identity of the aflatoxin-producing fungi isolates on the food stuffs using PCR.

1.5 JUSTIFICATION

There is a great need to study and understand the various fungi strains that produce mycotoxins such as aflatoxin in foods. Although knowledge about the level of aflatoxin in foods is important, identifying the fungal strains responsible for the toxin is better approach in solving aflatoxin related food poisoning. The risks associated with aflatoxin are inadequately communicated to farmers and consumers particularly in Sub-Saharan Africa, and also for policy makers to formulate regulatory frame works including interventions to reduce if not stop food contamination with these toxins. This study will also add clarity and support to existing knowledge and aid as a reference material for further studies in research areas.

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CHAPTER TWO
2.0 LITERATURE REVIEW

2.1 HISTORY OF AFLATOXIN

In the 1960s more than 100,000 young turkeys on poultry farms in England died in the course of a few months from an unknown disease that was termed "Turkey X disease" (Alleroft *et al*., 1961). This disease was characterized by loss of appetite, lethargy, and weakness of the wings.

Studies made in the 1960s regarding the nature of the contaminant of the peanut meal suggested that it might be of fungal origin. A careful survey of the early outbreaks showed that they were all associated with feeds from a Brazilian peanut meal added into the animal's ration. An intensive investigation of the suspected peanut meal using thin layer chromatography (TLC) was undertaken and it was quickly found that the peanut meal was highly toxic. During analysis of the peanut meal four kind of dots appeared on the plates, and when illuminated with an ultra violet light, two dots emitted a blue light and two emitted a green light. The fungus was later identified as the toxin-producing fungus and named *Aspergillus flavus* in 1961 and the toxin was also called Aflatoxin by virtue of its origin, where 'A' stands for *Aspergillus,* 'fla' for *flavus* and 'toxin' for poison (Craufurd *et al*, 2006).

The occurrence of aflatoxin contaminated foods and food products vary with geographic location, agricultural and agronomic practices (Cotty *et al*., 2006). The susceptibility of food product to fungal attack occurs during pre-harvest, transportation, storage, and processing of the foods**.** Aflatoxin contamination of food products is a common problem in tropical and subtropical regions of the world especially in the developing countries such as those in Sub-Saharan Africa with poor agricultural practices and where the environmental conditions of warm temperatures and humidity favour the growth of fungi (Thrasher, 2012). The various food products contaminated with

aflatoxins include cereals such as maize, sorghum, pearl millet, rice and wheat; oilseeds example groundnut, soybean, sunflower and cotton; spices like chilies, black pepper, coriander, turmeric and zinger; tree nuts such as almonds, pistachio, walnuts and coconut; and milk and milk products. (Lopez, 2002).

2.2 *ASPERGILLUS* GENERA AND SPECIES

Aspergillus exhibit immense ecological and metabolic differences (Perrone *et al.*, 2007). They are filamentous cosmopolitan microbes that can be isolated from soil, plant or animal debris and indoor environment and reproduction is by asexual reproduction (Geiser, 2009). Members belonging to this genus of spore-forming fungi appear like an aspergillum (a device used in the Catholic Church to sprinkle Holy water) in structure hence the name *Aspergillus* as coined in 1729 by Micheli, (Asan, 2004). Some among the genera are indeed useful, for example, *Aspergillus terreus* produces lovastatin, a potent cholesterol-lowering drug. Other Aspergilli secrete antibiotics (e.g. penicillin and cephalosporin), antifungals (e.g. griseofulvin), and anti-tumour drugs (e.g. terrequinone A) (Keller *et al*., 2005; Hoffmeister *et al*., 2008). Others are used as commercial food additives such as kojic acid and in microbial fermentation (Thom and Church, 2001). *Aspergillus niger* perhaps has been widely used as a major source of citric acid, accounting for over 99% of global production of citric acid as well as the production of enzymes (Archer *et al.*, 2008). *A. oryzae* is also widely used in soybean and rice fermentation (Chang and Ehrlich, 2010).

Several reports (Henry *et al.*,2002; Chun *et al.*, 2007; Davison, 2010) have in various aspects, addressed the economic and medical importance of *Aspergillus* genera. Undoubtedly, some are useful but others are toxigenic by way of their secondary metabolism which produces mycotoxins including carcinogen. Others are pathogenic (diseases causing) in animals by way of

their food/feed primary contamination. Of the known species, about 60 are pathogenic causing diseases generally referred to as aspergillosis (Bozkurt *et al*., 2008).

Members belonging to this fungal genera that are of problematic or pathological importance to animals are *Aspergillus fumigatus, A. flavus, A. paraciticus, and A. clavatus* (Zirbes and Mila, 2008), while others are agricultural saprophytes or parasites, colonizing and causing degradation of many agricultural commodities (Klich *et al.*, 2002) which reduce the quality of these products resulting in serious economic losses including livestock production.

2.3 AFLATOXIN–PRODUCING FUNGI

As with fungi in general, *Aspergillus* taxonomy is complex and ever evolving. The genus is easily identified by its characteristic conidiophore (a conidium-bearing hypha or filament), but species identification and differentiation is complex, it is traditionally based on a range of morphological features (Cotty *et al*., 1994). Macro- morphological features which are observed include conidial and mycelial colour, colony diameter, colony reverse colour, production of exudates and soluble pigments, presence of sclerotia and cleistothecia. Micromorphology characterization is mainly dependent on seriation, shape and size of vesicle, conidia and stipe morphology, presence of Hülle cells, and morphology of cleistothecia and ascospores (Klich, 2002). *Aspergillus* Sub-genus *Circumdati* Section Flavi, also refered to as the *Aspergillus flavus* group, has attracted worldwide attention for its industrial use and toxigenic potential. Section *Flavi* is divided into two groups of species. The aflatoxigenic species such as *A. flavus, A. parasiticus*, as well as several other produce aflatoxin, including, and *A. nomius, A. pseudotamarii* and *A. bombycis* (Peterson *et al.*, 2001). These group cause serious problems worldwide in agricultural produces. The other section includes the non-aflatoxigenic species

such as *A. oryzae* which is genetically indistinct from *A. flavus. A. oryzae* is used as a starter culture for the preparation of fermented foods and alcoholic beverages, and is an important source of many enzymes, such as glucoamylase, alpha-amylases and proteases used for starch processing, baking, and brewing worldwide (Machida *et al.,* 2008). *A. sojae* and *A. tamarii* are traditionally used for production of fermented foods in Asia (Kumeda *et al.,* 2001).

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Previously, *A. flavus* was known to be producing only AFB1 and AFB2, while *A. paraciticus* produced AFB1, AFB2, AFG1 and AFG2 (Horn *et al.,* 1996). With some recent studies (Cotty and Cardwell 1999; Cardwell, 2002; Varga *et al.,* 2009), the question of which isolate produces what AFs is rather complex. Other fungi belonging to the *Aspergillus* genera have been found to produce AFs (Pitt, 1993; IARC, 2002) Table 1 but they both have different aflatoxigenic profile (El Khoury *et al*., 2011) based on DNA (nor1) sequence and AFLP fingerprint analyses (Barros *et al*., 2007; Petterson, 2009).

Table 1: Taxonomy of some Aspergillus species with the type of aflatoxin

Fungi	**AF(s) produced**	**References**
A .flavus	B and G	Varga *et al*., (2009)
A. parasiticus	B and G	Cardwell (2002); Cotty and Cardwell (1999)
A. nomius	B and G	Saito *et al*., (1989); Pitt (1993); Ito *et al*., (2001)
A. bombycis	B and G	IARC (2002), Peterson *et al*., (2001)
A. Ochraceoroseus	B and G	Frisvad (1997); Klich *et al.,* (2000); Keller *et al.,* (2000)
A. australis	B and G	IARC (2002)
A. tamarii	B	Ito *et al*., (2001)
A. pseudotamarii	B	Ito *et al*., (2001)

2.3.1 Identification and characterization of *A. flavus* and *A. parasiticus*

A. flavus and *A. parasiticus* belong to the same group of classification in the fungi world, as shown in Table 2 differing only at the species level, hence their names *flavus* and *parasiticus* (Klich and Pitt, 1988).

Table 2: Classification of fungi

Kingdom	*Fungi*
Phylum	*Ascomycota*
Class	*Eurotiomycetes*
Order	*Eurotiales*
Family	*Trichocomaceae*
Genus	*Aspergillus*
Species	*Aspergillus* Flavus *and Aspergillus parasiticus*

A. flavus is the most notorious species linked with aflatoxin contamination of food crops and feeds (Cotty *et al.,* 1994; Cotty, 1997). In addition to aflatoxin B1and B2, *A. flavus* also produces many other mycotoxins such as cyclopiazonic acid, kojic acid, beta-nitropropionic acid, aspertoxin, aflatrem and aspergillic acid (Goto *et al*., 1996).

Another aflatoxin-producing fungi species, *A. parasiticus* has also been related with the infection of agricultural commodities particularly groundnuts in the US (Horn, 2005), West Africa (Ismail, 2001). *A. flavus* is more invasive than *A. parasiticus* when both species are together in the soil.

Although both *A. flavus* and *A. parasiticus* species have been considered to be strictly asexual and lack the ability to undergo meiosis (Geiser *et al*., 1996), a recent study by Horn *et al* (2009) revealed that sexual reproduction of *A. flavus* occurs between compatible sex strains that belong to different Vegetative compatibility Group (VCG). They can be easily differentiated phenotypically and genotypically by expert scrutiny aside from being separated by their

morphology, mycotoxins profile and molecular characters (Walsh *et al*., 2008). They cause the same disease known as *aspergillosis* and produce AFs (Patterson, 2009).

The knowledge of vegetative compatibility groups between different lineages is of particular interest in asexual fungi. Since the VCGs subdivide the population into groups that can exchange genetic information via heterokaryosis and the parasexual cycle (Brooker *et al*., 1991). Fungi lineages that are capable of anastomosis (fusing) and forming stable and functional heterokaryons (multinucleate cell that contains genetically different nuclei) are known as sexually or vegetative compatibility group —VCG (Leslie, 1993). Two individuals that have the same alleles (a variant form of a gene) at all het loci (ability to form a productive heterokaryon with a genetically dissimilar individual is controlled by specific loci termed *het loci*) belong to the same VCG. Vegetative compatibility can be used to estimate genetic diversity and may limit the potential for heterokaryosis and asexual gene flow (Bayman and Cotty, 1991).

The biosynthetic pathway of aflatoxin is well delineated. Several genes involved in the synthesis of aflatoxin have been characterized. Two of these are the aflR gene and apa-2 from *A. parasiticus* which are involved in regulation of transcription of several pathway genes and the omt-1 gene, which is involved in the conversion of *strigmatocystin* to O- *methylsterigmatocystin* (Bhatnagar *et al*., 2003).

Aspergillus flavus soil populations contain isolates from two morphologically distinct sclerotial size variants, termed the L-strain (also called *A. flavus* Group IB) (Geiser *et al*., 2009) for isolates with average sclerotial size greater than 400 μm and the S-strain (Group IA) for isolates with sclerotial size less that 400 μm (Cotty, 1997). Both S (small) and L (large) strains were found where maize is highly cultivated. On culture media grown in the dark, S-strain isolates

yield higher levels of aflatoxins, and further sclerotia, and less conidia, however, atoxigenic S-strain isolates are seldomly found in the natural environment (Orum *et al.*, 1997).

2.4 AFLATOXIN

Aflatoxins are secondary metabolites that are closely related heterocyclic compounds produced mainly by the genus *Aspergillus* which grow naturally in foodstuffs. They are able to generate a wide variety of toxic effects in vertebrates, including man (Coulombe, 1991). Toxigenic fungi may contaminate a wide range of agricultural commodities and foodstuffs in most different phases of production and processing, from cultivation to transport and storage. Aflatoxins show high chemical stability and may persist in the foodstuff even after fungi were removed by common manufacturing and packaging processes (Chu, 1991).

The four main aflatoxins display a decreasing potency in the order AFB1 > AFG1 > AFB2 > AFG2. This order of toxicity indicates that the double bond in terminal furan of AFB1 Figure 1 is a critical point for determining the degree of biological activity of the mycotoxins (Hall and Wild, 1994). The "B" and "G" refer to the blue and green fluorescent colours respectively produced under ultraviolet light on thin layer chromatography (TLC) plates, while the subscript numbers "1" and "2" indicate major and minor compounds respectively according to the migration distances on the plates.

In 1966, it was observed that the metabolic products of aflatoxins M1 and M2 were first isolated from milk of lactating animals fed on Mouldy grains contaminated with aflatoxin, hence, the M designation (Holzapfel *et al.,* 1966). The aflatoxin M1 and M2 are mammalian bioconversion products of AFB1 and AFB2 respectively and are originally isolated and identified from bovine milk (Garrido *et al.*, 2003). After entering the mammalian body (human or animals), aflatoxins are

metabolized by the liver cytochrome P450 enzymes to a reactive epoxide intermediate which becomes more carcinogenic, or be hydroxylated and become the less harmful aflatoxins M1 and M2 Figure 1.

2.4.1 Chemical structure of Aflatoxins

The skeleton of aflatoxin B1 is the structural analogue of all the other types of aflatoxins. AFB1, AFG1 and AFM1 differ from AFB2, AFG2, and AFM2 respectively by the double bond at the phenol on the far left.

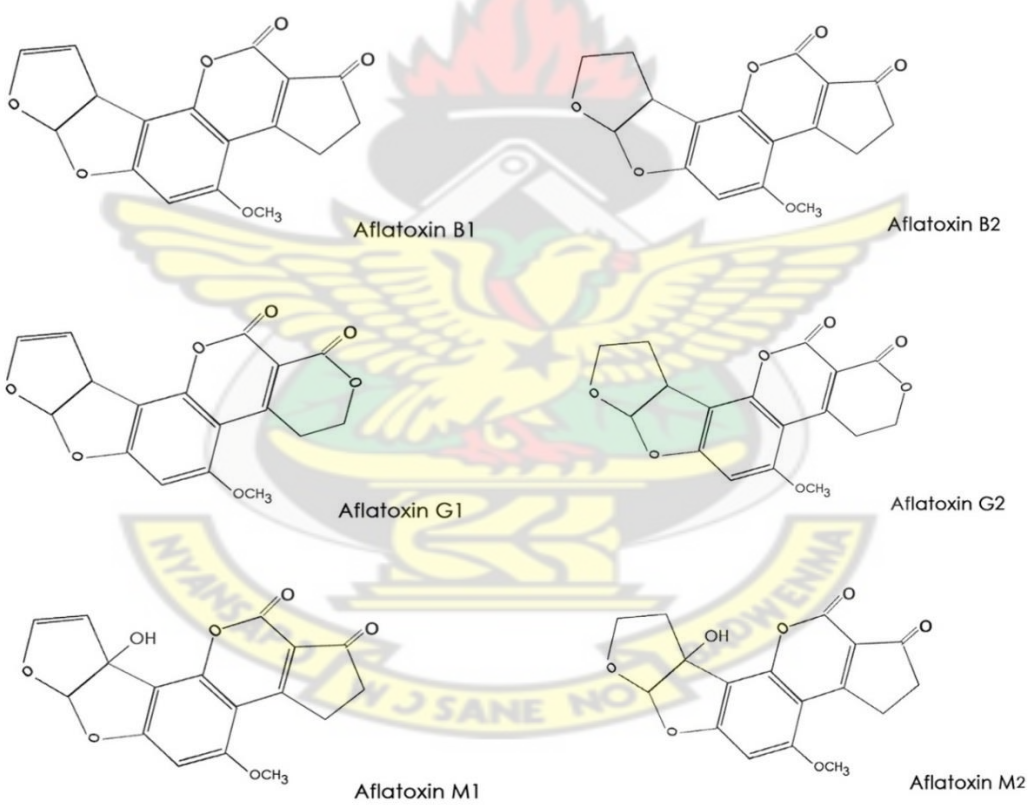

Figure 1: Chemical structures of aflatoxin B1, B2, G1, G2, M1 and M2.

2.4.2 Properties of aflatoxin

Aflatoxin is colourless, odorless, and tasteless and thus very difficult to detect. Since very minute quantities are toxic and cannot be seen by the naked eye, contamination cannot be judge based on the appearances of agricultural produce or commodities, hence laboratory testing is the only required method for assurance. However, certain indicators like crops or grains with severe mold growth, rotten, discolored, unusual or offensive smell or taste can give a high suspicion of contamination.

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Table 3: Properties of aflatoxins

PROPERTY	AFBI	AFB2	AFG1	AFG2
Molecular formula	$C_{17}H_{12}O_6$	$C_{17}H_{14}O_6$	$C_{17}H_{12}O_7$	$C_{17}H_{14}O_7$
Molecular weight	312	314	328	330
Crystals	Pale yellow	Pale yellow	Colorless	Colorless
Melting point (° C)	268-269 (D)	287-289 (D)	244-249 (D)	237.40 230SA
Fluorescence under UV light	Blue	Blue	Green	Green
Solubility	Soluble in water and polar organic solvent. Normal solvent methanol, water: acetonitrile (9:1), trifluoroacetic acid, methanol: 0.1N HCl (4:1), DMSO and acetone			

D= Decomposition, Source: Cole and Cox (1981).

2.4.3 Aflatoxin biosynthesis

Aflatoxins (AFs) are bisfurans Figure 1 that are polyketide-derived, toxic, and carcinogenic secondary metabolites produced by some fungi growing on corn, peanuts, cotton seed, and tree nuts (Payne and Yu, 2010). While biosynthesis of these toxins has been extensively studied *in vitro*, much less is known about what causes the fungi to produce AFs under certain environmental conditions and only on certain plants. It is not yet known why wheat, soybean, and sorghum are resistant to AF contamination in the field whereas, under laboratory conditions *A. flavus* is able to colonize these plant tissues and produce AFs (Cleveland *et al*., 2009). AF and most secondary metabolites are hydrophobic organic compounds, therefore compartmentalization of hydrophobic substrates has been seen as a common feature of secondary metabolite production (Sirikantaramas *et al*., 2009). Recently, evidence has been obtained that the enzymes involved in AF biosynthesis are organized into a specialized peroxisomal vesicle where different oxidative steps occur after formation of the polyketide. In general, the metabolism or biotransformation of xenobiotics (chemicals foreign to the organism) is a process aimed at converting the original molecules into more hydrophilic compounds readily excretable in the urine (by the kidney) or in the bile (by the liver) (Chanda *et al*., 2009).

At molecular level, major biochemical steps and genetic components of AF biosynthesis have been clarified or elucidated only in the last two decades (Yu *et al.,* 2004). Bhatnagar *et al*., (2002) describing the biochemistry and genetics pathway of AF formation as a complicated process involving many levels of transcriptional and post-transcriptional control (Abbas *et al*., 2009; Chanda *et al*., 2009; Georgianna and Payne, 2009; Schimidt-Heydt *et al.*, 2009). The current acceptable AFB1 biosynthetic pathway is as follows;

Acetate →polyketide → norsolorinic acid (NOR) →averantin (AVN) → Hydroxyaverantin (HAVN) → averufin → hyroxyversiocolorone → versiconal hemiacetal acetate → *versiconal* → versicolorin B → versicolorin A → *dimethyl- sterigmatocystin* → sterigmatocystin → O-methylsterigmatocystin → AFLATOXIN B1 (Yu *et al.*, 2004).

2.5 FACTORS FAVOURING AFLATOXIGENIC FUNGAL GROWTH AND AFLATOXIN PRODUCTION

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Fungal growth and aflatoxin contamination are the consequence of interactions among the fungus, the host and the environment (Dighton, 2003). The appropriate combination of these factors determine the infestation and colonization of the substrate, and the type and amount of aflatoxin produced. A suitable substrate is, however, required for fungal growth and subsequent toxin production, and although the precise factor that initiate toxin formation is not well understood. Aflatoxin is formed as a result of fungal growth in agricultural commodities. Once formed, they are extremely stable and persist long after the fungi have been eliminated and the contaminated commodity remains toxic and injurious to those who consume it (Cotty *et al*., 2008). The toxins are produced under specific conditions compared to those required for normal fungal growth (Varga *et al.*, 2009). Similarly, specific crop growth stages, poor fertility, high crop densities, and weed competition have been associated with increased mould growth and toxin production (Cotty *et al*., 2008). Strains of fungal communities associated with crops heavily influence the severity of aflatoxin contamination (Grubisha and Cotty, 2010). The contamination process is complex and may start in the field where crops first become infected by Aspergilli that reside in the soil and on decaying plant residues.

2.5.1 Physical factors affecting aflatoxin production

Plant physiological stress and insect damage create a conducive environment for fungal growth. Unseasonal rains at harvest periods and dense plant population also increase susceptibility of crops to *Aspergillus infections* (Cotty *et al*., 2008). Chances of contamination may still continue even after crop maturation and harvest, and when the crop is exposed to high humidity both in the field, and during storage. It was also observed that storage of the grain after heavy rains increased the chance of spoilage (Cotty *et al*., 2008). In order for *Aspergillus* to grow, a relative humidity of about 80% is required (Moreau, 1979). Aflatoxin contamination levels may rise and new form of infections become established until crops or produce are finally consumed (Cotty *et al.*, 1994). In order to estimate risks associated with communities of aflatoxigenic resident on crops, both aflatoxin-producing potentials and frequencies of occurrence need to be considered. The moulds frequently form spores to protect themselves from unfavorable environmental conditions such as low relative humidity and low water content. (Probst *et al*., 2011).

The ability of members belonging to the *Aspergillus* genera to attack and colonize agricultural commodities is largely due to their versatility (Pardo *et al*., 2005). The usual temperature for colonization is in the range of 10-35 ^{0}C. At 37 ^{0}C (98.6F) the optimum temperature for the growth of *A. Flavus* and *A. parasiticus* is therefore in most cases favored by hot conditions (Mónica and Leda, 2002). Although fungus readily grows within the temperature ranges of 25-42 ^{0}C (77-108 F), and they even grow at ranges from 12-48 ^{0}C (54-118 F), this high temperature contribute to their pathogenicity on animals (Payne, 1998).

Water activity (a_w), is the amount of free humidity in a crop or product and is the water vapour pressure of the substance divided by the vapour pressure of pure water at the same temperature.

Water activities beyond 0.70 at 25 degrees Celsius (77 ° F) provides a conducive environment for the growth of *Aspergillus flavus* and *Aspergillus parasiticus* (Recommended International Code of Practice, 2003)

It has been reported that *Aspergillus* species produce more AF/ST at acidic pH as compared to alkaline pH (Keller *et al.,* 1997). The pH effect on colonization of *Aspergillus* is of limited value, since the fungi secondary metabolism alters pH during their growth (Wheeler *et al*., 1991). In contrast to studies by Keller et al., (1997) a more recent study by Delgado-Virgen and Guzman-de-Pena (2009) showed an increase in AF/ST production in alkaline pH with respect to the level produced at acidic pH. This view is also supported by Wheeler *et al* (1991) who stated that *Aspergillus* species are more tolerant to alkaline than acidic conditions.

2.5.2 Chemical factors affecting aflatoxin production

Fungi require exogenous materials to form their biomass (Dighton, 2003). It has been shown that AF is produced from glucose derived acetyl-CoA (Buchanan and Lewis, 1984; Shantha and Murthy, 1981). Simple carbon sugars like glucose, sucrose, fructose or sorbitol enhance AF production, while complex carbon sources like peptone, galactose, xylulose, mannitol and lactose are not conducive to AF biosynthesis (Calvo *et al.,* 2002). Nitrogen metabolism affects mycotoxin production, however, the effect of nitrogen varies depending on the nitrogen source utilized. Organic nitrogen sources induce AF production, whereas nitrate as the sole nitrogen source in the medium is non-conducive to AF production (Georgianna and Payne, 2009). The availability of these chemicals as nutrients can enhance growth of these fungi.

At low levels, nitric oxide (NO) is an important signaling molecule controlling numerous cell functions in higher eukaryotes (Wendehenne *et al.,* 2001, Romanov *et al*., 2008; Roselli *et al*., 1998; Masuda *et al*., 2001; Kim *et al*., 2007; Lamattina *et al.,* 2003). Until recently, however, there were no studies of NO as a signaling molecule in fungi. In *A. nidulans,* two flabohaemoglobins, FhbA and FhbB, have been reported (Schinko *et al.,* 2010). A recent study by Baidya *et al.,* (2011) showed a link between NO and morphological development and ST production in *A. nidulans*. This was the first report of the effect of NO in morphogenesis and secondary metabolism in the fungal kingdom (Baidya *et al.,* 2011).

Other compounds such as ethylene and CO_2 have been described to reduce AF production (Cary *et al*., 2009). Decreasing O_2 concentration of air to 10% depresses aflatoxin production, but only at O_2 levels less than 1% are growth and aflatoxin production completely inhibited (Roze *et al*., 2007). Other molecules such as 2-ethyl-1-hexanol stimulate mycotoxin biosynthesis or affect it in a dose-dependent manner, such as the case of 2-buten-1-ol (Roze *et al*., 2007). Interestingly, ethylene and 2-ethyl-1-hexanol have also been found to be produced by *Aspergillus* specie, such as *A. parasiticus* and *A. nidulans,* where they could play a role as signal molecules (Roze *et al*., 2004; Roze *et al.,* 2007).

2.5.3 Biological factors affecting aflatoxin production

Numerous microbes have been considered as biological pest agents and can serve as a determinant for fungal attack and colonization of agricultural produces. A large number of substrate can influence fungal growth (Chang *et al.,* 2004), however, the nutrients required for growth must already be present in the growth medium. Fungal species grow well at relative substrate moisture levels of 50 to 75% (Pardo *et al.,* 2005). Most often *Aspergillus* can derive all its survival and

growth needs from its substrate such as in the case of sawdust, mulched hay, wood chips seed hulls, etc. which generally contain enough nitrogen and carbohydrate for rapid growth (Job, 2004).

Insects, rodents, water and wind can serve as vectors for aflatoxigenic fungal colonization of feed (Makun *et al*., 2012). Insects and rodents excrete on feed or agricultural produces thus increasing the moisture content in the produces which enhances microbial contamination. They may also act as agents together with water and wind to spread fungal spore resulting in cross contaminations. The genotype and physiological adaptation of aflatoxigenic fungi to an environment could also be a determining factor to their growth and colonization (Bhatnagar *et al*., 2008).

2.6 HUMAN EXPOSURE TO AFLATOXIN

In developing countries, many individuals are not only food insecure, but also are chronically exposed to high levels of mycotoxins in their diet. Some of the highest and most persistent human exposures to aflatoxin occur in West Africa, where nearly 99% of the children were positive for an aflatoxin biomarker (Gong *et al.,* 2004). Maize consumption is an important source of aflatoxin exposure for these children (Egal *et al., 2005).* Humans and animals get exposed to aflatoxins by two major routes. Firstly, by direct ingestion of aflatoxin-contaminated foods or ingestion of aflatoxins carried over from feed into milk and milk products like cheese and powdered milk as well as other animal tissues. Secondly, by inhalation of dust particles of aflatoxins especially AFB1 in contaminated foods in industries and factories (Coulombe, 1994).

After entering the body, the aflatoxins are absorbed across the cell membranes where they reach the blood circulation. They are distributed in blood to different tissues and to the liver, the main organ for metabolism. Epidemiological studies carried out in several parts of Africa and Asia indicate a correlation between exposure to aflatoxins and primary liver cancer (Ramesh and Siriguri, 2003).

The risks associated with exposure to aflatoxins are enhanced by simultaneous exposure to hepatitis B and possibly hepatitis C viruses (Ngindu *et al.,* 1982).

2.7 HEALTH CONSEQUENCES OF AFLATOXIN

Acute exposure to high levels of aflatoxin leads to aflatoxicosis, any diseases caused by aflatoxin consumption, which can result in rapid death from liver failure. Evidence of acute aflatoxicosis in humans has been reported worldwide especially in some third world countries such as Taiwan, Uganda, India, Kenya and many others. Chronic primary aflatoxicosis is a results from ingestion of low to moderate levels of aflatoxin. The effects are usually subclinical and difficult to recognize. Some of the common symptoms are impaired food conversion and slower rates of growth with or without the production of an overt aflatoxin syndrome, (WHO and CDC 2005).

The chronic forms of aflatoxicosis include:

(1**)** Teratogenic effects associated with congenital malformations

(2) Mutagenic effects where aflatoxins cause changes (mutations) in the genetic code, altering DNA and leading to chromosomal breaks, rearrangement of chromosome pieces, gain or loss of entire chromosomes, or changes within a gene. (Ezekiel *et al*., 2011).

(3) The carcinogenic effect in which the carcinogenic mechanisms have been identified such as the genotoxic effect where the electrophilic carcinogens alter genes through interaction with DNA and thus becoming a potential for DNA damage.

Aflatoxins appear to be much more prevalent than previously anticipated, with a large proportion of foods and a high percentage of the population in Africa affected (Oladele 2014). The adverse effect of chronic exposure of aflatoxins on human health and nutrition has been ignored even though it has serious effects on children's growth. Development of chronic effects of aflatoxin

has been reported to impair normal body immune function (Turner *et al*., 2003), either by reducing phagocytic activity or reducing T cell number and function.

Aflatoxin B1, classified as the most toxic of the aflatoxins, is the most potent naturally occurring chemical liver carcinogen known. Specific P450 enzymes in the liver metabolize aflatoxin into a reactive oxygen species (aflatoxin-8, 9-epoxide), which may then bind to proteins and cause acute toxicity (aflatoxicosis) to DNA and induce liver cancer (Wild and Gong 2010; Wu and Khlangwiset, 2010).

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A possible explanation of the transmission rates of aflatoxin is suggested by the exposure of human fetuses to maternal aflatoxin (Denning *et al*.,1990;Wild *et al*.,1991) and by the fact that aflatoxin is also secreted in human mothers' milk (Zarba *et al.*, 1992; Saad *et al.,* 1995). Presence of aflatoxin traces were found in *sera,* maternal intravenous blood, breast milk, and umbilical cords of patients in the maternity wards in The Gambia (Miele *et al*., 1996). In animals, exposure of the foetus and via milk has been shown to have significant effects on the immune competence of progeny (Silvotti *et al*., 1997; Mocchegiani *et al*., 2001).

Aflatoxins have also been reported to interfere with nutrition in a prescribed-amount-response relationship between exposure to aflatoxin and rate of growth in infants and children (Gong *et al*., 2004). Turner *et al*. (2003) detected aflatoxin albumin adducts in 93% of sampled children (6-9 years) in The Gambia and proved that effect of aflatoxin can diminish immuoglobin A (IgA) in saliva. Aflatoxin also cause nutrient modification like vitamin A (Pimpukdee *et al*., 2004), Zinc and Iron shortage (Doyle *et al.,* 1977) in animals and thus making them unavailable for the normal body physiology and hence leads to nutritional deficiencies. Aflatoxins have been reported to cause digestive system effects such as diarrhoea, vomiting, intestinal haemorrhage,

liver necrosis and fibrosis (Harriet, 2003). The contamination of foods and feeds with aflatoxin can cause serious consequences in human and animal health. Aflatoxins have also been linked with *kwashiorkor* and *marasmus* in children in most of the sub-Saharan countries. Many people in these countries experience chronic aflatoxicosis associated with long-term exposure to low or moderate levels of aflatoxin in the food supply chain (Adhikari *et al*., 1994).

In Africa, a correlation between aflatoxin and infectious diseases has not been consistent. Hendrickse *et al.,* (1989) investigated the connection of aflatoxin in malnutrition and found out that children with *kwashiorkor* were less prone to malaria. In The Gambia, children with malaria were found to have higher aflatoxin-albumin adduct concentration (Allen *et al.,* 1992). In a study conducted by Williams, 2011, reported that in The Gambia and Ghana where there is a high aflatoxin biomarker status, people are very prone to active malaria.

2.8 SOCIO- ECONOMIC EFFECT OF AFLATOXIN CONTAMINATION

The exportation of agricultural cash crops mainly groundnuts from the third world countries, to especially the EU has lost momentum in the last two decades, leading of a drastic economic fall from producer countries (Bhat and Vashanti, 1999; Otzuki*et al.*, 2001). The EU aimed to reduce importation of food crops especially groundnut and maize by 64% from African countries; Chad, Egypt, The Gambia, Mali, Nigeria, Senegal, South Africa, Sudan and Zimbabwe, This will cost about US $750 million losses in trade per year (Diaz Rios and Jaffee, 2008).

The economic losses associated with AFs contamination is not only limited to discarding of foods or feeds but trade rejection, loss of income, loss of livelihoods, health-care costs and veterinary expenses, cost of food/feed borne diseases surveillance and monitoring (WHO, 2006).

2.9 INCIDENCE OF AFLATOXIN POISONING THROUGH FOOD CONSUMPTION

One of the first major documented reports of aflatoxins in humans occurred in western India in 1974. About 108 persons among 397 people affected, died from aflatoxin poisoning (Krishnamachari *et al.*, 1975). Another incident of aflatoxin poisoning occurred in Kenya in July 2004 leading to the death of 125 people among the 317 reported illnesses due to consumption of aflatoxin contaminated maize (corn) (Lewis *et al*., 2005; Probst *et al.*, 2007). Acute toxicosis is not the only concern. The world health authorities warn that low doses with long-term dietary exposure to aflatoxins is also a major risk as it can lead to hepatocellular carcinoma (Bressac, 1991; Hsu *et al.*, 1991; Fung, 2004; Wogan, 1992). International Agency for Research on Cancer (IARC) has designated aflatoxin as a human liver carcinogen. (IARC, 2002). This food poisoning problem is rarely observed in the US in humans but does occasionally occur in animals. The most notable case involved the reported death of over 100 dogs in 2006 that had consumed tainted dog feed (US FDA, 2006).

2.10 STRATEGIES FOR PREVENTION AND CONTROL OF AFLATOXIN CONTAMINATIONS

Most countries are signatories to *Codex Alementarius* (WHO/FAO documents that deal with food quality in traded commodities) and pledge to limit exposure of their people to aflatoxins. . However, they also seek to minimize the economic consequences of attaining this health goal and to use the means accessible for health on the highest-priority problem. There are many approaches investigated to manage aflatoxin contamination. Strategies tested include the use of resistant and/or tolerant varieties, insect management practices. (Recommended International Code of Practice, 2003).

2.10.1 Creating awareness of aflatoxin effects on humans

Increased awareness of the carcinogenic effect of aflatoxins has now been created among consumers in the developed world. This customary approach to preventing exposure to aflatoxin has been to ensure that foods distributed have the lowest practical aflatoxin limits. This has been achieved only with strictly enforced regulation in the developed countries. The majority of farmers, traders and consumers in Africa are not in fact aware of the effect of aflatoxin intoxication in human and animal's health. Therefore, the need to disseminate information to these people by various means or methods, including radio and television programs and extension workers on aflatoxin and its hazardous effect. Public awareness should be a priority to various National food safety agencies of each country in collaboration with their agricultural sector on safety measures to at least minimize aflatoxin contamination at every phase of production.

2.10.2 Prevention through pre-harvest handling

Pre-harvest control of aflatoxin contamination must take into consideration all the varied environmental and agronomic factors that influence pod and seed infection by the aflatoxin-producing fungi, and subsequent aflatoxin production. These factors can vary considerably from one location to another, and between seasons. To be effective in reducing aflatoxin contamination, diverse agricultural practices should be employed.

2.10.3 Good Agronomical Practices

Good agricultural farming practices such as rotating crops, proper weed controlling techniques, cultivating mould-resistant varieties. A research conducted in Texas, demonstrated a much lower aflatoxin levels in crops grown during winter months than in summer months, as soil temperatures had a great influence on *A. flavus* growth (Accinelli *et al.*, 2007). In addition, they rotated crops, including corn (maize), cotton, and sorghum, between experimental fields and found that all crops showed reduced levels of aflatoxin contamination during winter months, however maize/corn consistently showed higher levels of aflatoxin than the other tested crops. Since cotton and sorghum are much less prone to aflatoxin contamination than corn, these findings suggest that rotating prone crops such as maize and groundnut with less susceptible ones such as cotton, sorghum, or other plants, may allow for a healthier growth environment and generally decrease aflatoxin exposure (Jaime-Garcia *et al*., 2010).

2.11.0 BIOLOGICAL CONTROL OF AFLATOXIN CONTAMINATION OF CROPS

Aflatoxin cannot be readily removed from contaminated foods by detoxification. Therefore, there is interest in developing a biological control method that can increase crop safety by decreasing toxin content through the displacement of toxigenic isolates using atoxigenic isolates of the same or other species (Cotty *et al.*, 2006).

2.11.1 Competitive displacement by atoxigenic *Aspergillus* strains

Pest management researchers have begun development of more natural methods in agriculture, primarily in the development of biocontrols, through the use of products such as “AflaSafe”, now used in Africa (Wu *et al.*, 2010). There are several strains of *A. flavus*, however, not every strain of *A. flavus* produces aflatoxin, some strains are atoxigenic, (nonpoisonous when consumed), (Dorner, 2009). When atoxigenic strains are applied to crops, the strategy is based on and aimed

at the atoxigenic strains to competitively overcome naturally toxigenic strains in the same niche and compete for space, crop substrates and nutrients required for aflatoxin production and not necessarily for growth. Generally, the application of atoxigenic strains results in a significant decrease in or elimination of the toxigenic strains that produce aflatoxin. Research has been done on peanuts (Horn *et al*., 2009) and corn or maize (Dorner *et al.,* 2009; Probst *et al.,* 2011) and have proven reductions of 77 to 98 % in aflatoxin contamination in peanuts with the application of atoxigenic strains. Biocontrols, used in place of traditional chemical pesticides, are ecologically harmless and derived from natural means and may include beneficial insects, plant extracts, or the introduction of other natural organisms.

2.11.2 Plant extracts as fungal disinfectant

Some essential oils such as anise, boldus, mountain thyme, clove, griseb, and poleo extracted from certain plants have been confirmed to be a suitable alternatives to chemical pesticides and fungicides (Chulze, 2010). These essential oils can be functional as a vapour, making application particularly suitable for use in closed storage (Chulze, 2010). Neem (*Azadirachta indica* Juss.) leaf extracts (Bhatnagar and McCormick 1988), extracts of spices (Olojede *et al.,* 1993) and extracts of the herb *Amorphophallus campanulatus* Decne (Prasad *et al.*, 1994) have been variously reported to completely inhibit aflatoxin production from strains of *A. parasiticus* and *A. flavus* respectively.

Methyleugenol is a naturally occurring substance present in essential oils and fruits used for flavouring nonalcoholic beverages, chewing gum, and candies. In a study, the use of methyleugenol spray demonstrated to be a significant inhibitor of *A. flavus* growth on 56% of peanut agar meal (Sudhakar *et al*., 2009). Methyleugenol also suppressed growth in the peanut pods and kernels. Although field testing should be conducted for verification, the use of naturally

occurring methyleugenol spray may be a valued substitute to chemicals in preventing the growth of *A. flavus* on stored peanut crops (Sudhakar *et al*., 2009).

2.12.0 PREVENTION OF FUNGAL INFESTATION IN FOOD THROUGH POST-HARVEST HANDLING

There are several strategies for post-harvest to eliminate or limit the spread of aflatoxin contamination throughout an entire harvest. These measures include food processing, better storages, drying rapidly by mechanical means, sorting contaminated nuts, washing or cleaning with water, better packaging system, use of appropriate storage systems to reduce mycotoxins (Nautiyal and Joshi, 1991).

2.12.1 Drying of food samples

Good quality grains with desired features and fungi free and other microorganisms is highly recommended for marketing and consumption. There are various drying methods available with modern equipment that acquire energy from the sun. Temperature and air velocity, rate of drying, drying efficiencies, kernel quality, air power, fuel source, fixed costs, and management are factors to consider for effective drying (FAO, 1992). Siriacha *et al.*, (1989) found that if shelled grain are immediately sun-dried, the chance of contamination was reduced by 14.5% compared with that of undried shelled maize. The heat does not eliminate aflatoxin exposure, but eliminating excess moisture in harvested grains is an effective method of inhibiting mould and other fungal growth. Heat drying is effective at limiting the spread of *A. flavus* and other harmful fungi which can produce mycotoxins (Magan *et al.*, 2007).

2.12.2 Smoking of food

The use of insecticides and fungicides in Africa is limited by their availability in remote rural areas (Wu, 2010). African farmers especially in the Gambia, often use methods such as smoking to reduce moisture content and insect damage. The efficiency of smoking in controlling insect infestation is comparable to that of Actellic, (an organophosphate insecticide for the control of insect pests in stored grains) that is, Pirimiphos-methyl (Daramola, 1986). Hell *et al*., (2000) highlighted the efficiency of smoking but caution that if not properly done it alters both the looks (colour) and the flavour of the product.

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2.12.3 Physical separation of suspected contaminated grains or nuts and hygiene

The distribution of aflatoxin on a maize cob or in a grain lot is very heterogeneous with large quantities of the toxin concentrated in just a few or a small percentage of the kernels. Usually heavily molded and or damaged kernels contain high amounts of aflatoxin (Whitaker, 2006). Therefore sorting out physically damaged and infected grains (based on their colouration, disfigured shapes, shriveled and reduced size) from the undamaged lot can reduce aflatoxin levels by 40-80% (Park, 2002). Sorting can be done manually or with electronic sorters, which are used to reduce aflatoxin contamination in peanuts or grains. Remnants of previous produce should be properly discarded. Destroying infested crop residues are basic sanitary measures that reduces grain deterioration in field and in storage. Cleaning storage areas prior to filling those with the new harvest reduced aflatoxin levels according to Hell *et al.,* (2000).

2.12.4 Good storage strategies

Thorough sanitation is the first and most effective step toward preventing insect infestation. Storage areas should be clean and tight enough to keep out insects and to keep in fumigant

gasses when applied. Longer-term storage in all forms historically have required proper drying before storage (Navarro *et al.,* 2010).

Research has shown that fluctuations of weather especially temperature and humidity have a serious effect on storage silos and buildings. This can lead to a higher threat to already harvested crops. Besides causing potentially widespread damage and crop spoilage, measures on how to decrease moisture levels in storage building should be made available and affordable (Safe Ultra Hermetic) to mitigate fungal growth, which in turn can lead to the production of mycotoxins in food stores (Chulze, 2010).

Silo bags are waterproof and have a certain level of gas-tightness, the oxygen and carbon-dioxide levels is influenced by the balance between respiration, the loss of carbon-dioxide, and the entrance of oxygen into the bag. For long term storage, this impermeability can lead to fungal growth. Thus, silo bags may be used as a short-term storage method when necessary (Udoh, 2000).

The following post-harvest practice if suggested to farmers with information on various methods will undoubtedly reduce aflatoxin contamination:

• Identify grains that are mouldy or have damaged shells

• Use mats to dry grains to avoid humidity during the drying process.

• Judge the thoroughness of sun drying grains.

• Use natural fiber bags for storage.

• Store bags on high platforms instead of on the floor.

• Use insecticide.

Any one of these methods has been demonstrated to have an impact on aflatoxin contamination in the local population, however when used as a package these measures reduced post-harvest exposure in the food chain by more than half (Wu *et al.,* 2010).

2.12.5 Processing

Cereal-based products are not necessarily mycotoxins free nor fit for consumption after undergoing processing (Scudamore, 2008). Many cereal-based products are manufactured either by cooking in water under raised temperatures, fermentation, baking, frying, drying, toasting, or extrusion. These approaches can neither guarantee a complete diminished levels of mycotoxins in a food end-product. High temperatures can diminish the threat of aflatoxin contamination though it can also destroy the nutrient value of the crop. Standard home-based cooking processes are usually insufficient to reduce aflatoxin to safe levels (Scudamore, 2008). Proper roasting and blanching of groundnut have a great influence on aflatoxin level Siwela *et al.,* (2011) noted a reduction in levels of aflatoxin up to about 89 %.

2.13.0 CHEMICAL TREATMENTS FOR AFLATOXIN INTOXICATION

There are yet other courses of action to the disposal of contaminated produce and such measures could be considered as a last resort. Actions of insect pave the way for fungal growth, their activities provide heat and moisture or water that make the environment highly conducive for fungal growth (Pardo *et al.,* 2005). Thus, and preventing insect infestation by chemical application may provide adequate grain protection aside from good storage structure and storage hygienes. Chemicals are applied as both preventive and control measures.

2.13.1 Fumigation

Fumigants are toxic gases used to disinfest a commodity in an enclosure which ideally is completely gastight to kill all stages of the insects present in or amongst the grains. Phosphine and methyl bromide are grain fumigants that obtain a more-or-less immediate disinfestation of the commodity and the space enclosing it. However, reinfestation could occur once the commodity is exposed again and the fact that the most effective fumigants are all highly toxic to humans and other non-target organisms (Bhatnagar *et al.,* 2001).

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Degradation of aflatoxin was shown to be effective to 88 % in stored maize using nontoxic chemicals which include sodium carbonate, sodium bicarbonate, potassium carbonate, ammonium carbonate, acetic acid, and sodium propionate which are also safe for use with foods (Shekhar *et al.,* 2009). Rice with low aflatoxin content of about 30ppb was found to be completely degrade by the treatment with citric acid (Safara *et al.,* 2010).

2.13.2 Ammoniation

Ammonia, with chemical formula NH_3 (both an anhydrous vapour and an aqueous solution), is a detoxification reagent which has attracted the widest interest and has been exploited commercially, most especially in the feed industry, for the decomposition of aflatoxin. In a study on artificially contaminated corn, gaseous ammonia in a vacuum-packed for about two weeks, destroyed 90 % (Nyandieka *et al*., 2009). This practice makes disposable crops previously unsafe for intake, now safe for livestock to a degree, however, detoxification of aflatoxin-contaminated food for human consumption may result in undesirable modifications in the nutritious and organoleptic qualities of the food (Das and Mishra, 2000).

2.13.3 Chemoprotection

This is based on manipulating the biochemical processing of aflatoxin to ensure detoxification rather than preventing biological exposure since in reality it is inevitable to avoid contamination. Obviously, areas where there are less enforcement of regulations, humans are commonly exposed to aflatoxins and more so than animals. Chemoprotection either increase an animal's detoxification processes (Kensler *et al.,* 1993) or prevent the production of the epoxide that leads to chromosomal damage (Hayes, 1998). Aflatoxin detoxification has been reported effective against chronic exposure with the use of drugs and supplements in dietary products. (Bolton *et al*., 1993; Wang *et al.,* 1999).

2.13.4 Enterosorption

This is a process where binding agents are added to food to prevent the absorption of the toxin while the food is in the digestive tract. The combined toxin-sorbent is then excreted in the feces. This approach has been used extensively and with great success in the animal feeding industry (Ibrahim *et al.*, 2000; Rosa *et al*., 2001).

Smectite clay (e.g., NovaSil) used as a binding agent on aflatoxin B1 molecules, shielding the toxin from being absorbed into the digestive system pass harmlessly through the body (Dixon *et al.*, 2008; Philip *et al.,* 2008). Similar studies are examining the use of certain bacteria species as binding agent on contaminated feed (Oluwafemi *et al*., 2009).

2.14 AFLATOXIN LEVELS FOR CONSUMPTION AND EXPORT REGULATION

Aflatoxin exposure is mainly a problem in poor and developing countries with poor set up of regulatory authorities in the food chain processing. Because aflatoxin is considered toxic and carcinogenic, about 100 countries have established maximum levels or regulatory limits for

aflatoxin in food and feed products (FAO, 2003). Maximum levels for aflatoxin not only vary in magnitude, but vary with the type of aflatoxin being controlled. Maximum limits can be based upon only aflatoxin B1 (AFB1), total aflatoxins AFT (AFT = AFB1 + AFB2 + AFG1 + AFG2), or a combination of AFB1 and AFT. For example, the European Union has a maximum acceptable limit of 2 and 4 µg/kg for B1 and total aflatoxins respectively (van Egmond *et al*., 2007).

Import and export permits are required if crops and soil samples are shipped outside a country. The crop samples are analyzed for aflatoxin to obtain baseline information on aflatoxin levels from the exporting country. As a result of the random variation related with the sampling, sample preparation, and analytical steps of aflatoxin test procedures, sampling product at the point of exportation does not guarantee that a batch will be accepted by the importer when re-sampled and tested for aflatoxin at the point of receipt (Whitaker *et al.,* 2006). In the same light, it is not possible to determine the true aflatoxin level in a batch with 100% assurance by measuring the aflatoxin concentration in samples taken from the particular batch. As a result, some batches will be misclassified by both the exporter and importer when using sample test results to represent the whole lot which must be above or below a defined regulatory limit.

At a training on mycotoxins (Valencia-Spain, 2012), Two risks are associated with any sampling plan design

(i) there is a possibility that some good lots with aflatoxin levels below a regulatory limit will be rejected by the design sampling plan which is referred to as false positive (i.e. good lots rejected termed the exporter's risk) and

(ii) there is also a probability that some bad lots with aflatoxin levels above a regulatory limit will be accepted by the design sampling plan also referred to as false negative (i.e. bad lots accepted termed imported risk).

Accredited laboratories with well trained staff are needed to determine the levels of aflatoxin contamination throughout the cultivation, harvesting and post-harvesting stages, as well as certify the quality of export products at origin.

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2.15.0 CERTIFIED METHODS FOR AFLATOXIN ASSESSMENT IN GRAINS

To ensure food safety, maximum levels for aflatoxins in food and feed have been set by national and international organizations and various methodologies have been developed for the determination of aflatoxin concentrations in food and feed commodities.

2.15.1 Chromatography

Chromatography is one of the most common methods for quantifying aflatoxin. Later on, new chromatography-based techniques were developed for aflatoxins. Examples of these improvements are Liquid Chromatography (LC), Thin Layer Chromatography (TLC) (Stroka *et al.*, 2000), and High-Performance Liquid Chromatography (HPLC) (Bacaloni *et al.*, 2008) which nowadays is the most commonly used chromatographic technique for detection of a wide diversity of mycotoxins, especially for aflatoxin dericatives (De Rijk *et al*., 2011).

Frisvad and Thrane (1987) described an HPLC method to identify 182 mycotoxins and other fungal metabolites based on their alkylphenone retention indices and diode array spectra. Coupling of HPLC with mass spectroscopy or tandem mass spectroscopy allows for highly

accurate determination of toxin concentrations and compound identification in one analysis (Sobolev, 2007).

Alternatively, fluorescence detection of the unmodified aflatoxins is widely used in HPLC applications as well as in Thin Layer Chromatography. Furthermore, there are combinations of the methods above with pre-process techniques, which can detect the concentration of aflatoxin in a solution in a better way. For example, immunoaffinity column sample clean-up followed by a normal or reverse phase of HPLC separation with fluorometric detection is mostly used for quantitative determination of AFM1 due to the characteristics of specificity, high sensitivity and simplicity of operation (Muscarell *et al.,* 2007).

2.15.2 Immunoassay

Immunochemical detection for aflatoxins is based on antibody-antigen reactions (Ab-Ag) (Lee *et al.,* 2004). Since different kinds of aflatoxin molecules can be considered as antigens, it is possible to detect them by developing antibodies against the compounds. Most of the immunological methods are based on enzyme-linked immunosorbent assays (ELISA), which have good sensitivity, speed and simplicity. In addition, some lateral flow immunoassays (LFIAs) also are applied for the qualitative and semi-quantitative detection of aflatoxin in food, feed and milk (Ho *et al.,* 2002; Anfossi *et al.,* 2011; Salter *et al.,* 2006). Even though several reports have been published on the immunochemical determination of aflatoxin in food, only a few validation studies are available to show that the results comply with certain regulations because of the requirement for expensive instrumentation.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 SAMPLE COLLECTION AND PREPARATION

Raw samples of groundnut, maize, beans and rice were collected from different sales points in Kumasi Central Market-Ghana. Batches were collected from the same sales points in an interval of two weeks (the maximum time limit for the sales' consignment to be completely sold). Sampling was done according to the sampling protocol for Official Control of Mycotoxins in Food (EU, 2003) to give a representative sample which was then put in sealed bags and transported to the Biotechnology Laboratory of the Department of Biochemistry and Biotechnology. The entire primary samples were ground to powder by milling and homogenized. Thereafter subsamples were made into different portions for HPLC, microbiological culture and molecular analysis, Samples were stored at -20ºC until analyzed.

Table 4: Sample collection and size

Food crops	No. of Batches	No. of samples per batch	No. replicate	Sample size (kg)	Total No. of samples
Groundnut	3	1	3	5	3
Maize	3	1	3	5	3
Beans	3	1	3	5	3
rice	3	1	3	5	3

3.2. MATERIALS, MEDIA AND REAGENTS

3.2.1 Materials for HPLC

Methanol (analab), methanol (HPLC grade), acetonitrile (HPLC grade), sodium Chloride, filter paper (whatman no.1), microfilter (0.45 nm), distilled water, chemical balance, blender jar, pipette, a whole HPLC set up, standards of aflatoxins (AFS), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) glass syringe.

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3.2.2 Materials for microbial culture

Potato dextrose agar (PDA), Malt extract agar (MEA) were acquired from Oxiod Ltd., Basingstoke, England while Sabouraud dextrose agar (SDA*), Dichloran Rose-Bengal Chloramphenicol* (DRBC) agar were obtained from Mast Group Ltd., Mersyside, U. K. Peptone water, chloramphenicol were from Sigma-Aldrich Co. MO, USA. sterile Petri dishes, sterile wash bottles, waterbath, glass rod, autoclave, incubator, measuring cylinder, inoculating loop, pipette tip, pipette filler, beakers, microscope, laboratory weighing balance, spatula, filter paper, test tubes, test tubes rack, water bath, beakers, colony counter, Bunsen burner, microscope, Medium preparation (Appendix II).

3.2.3 Materials for molecular analysis

Fungal DNA extraction kits, (NH_4OAc, SDS, NaCl, protease). Primers, Taqpol, dNTPs, Luna Taq. were all purchased from Biolegio Molecular Biology Laboratory, Wageningen, The Netherlands), PCR machine, molecular biology grade water, pipettes, centrifuge, vortex-genie, molecular grade water (nuclease free), analytical balance, EDTA, liquid nitrogen, incubator, phenol, chloroform, RNase, ethanol, ice, laminar flow hood, Tris-HCl buffer, bromophenol blue, microfuge tubes, trays, mortar and pestle, gel electrophoresis complete setup, current supply, ethidium Bromide.

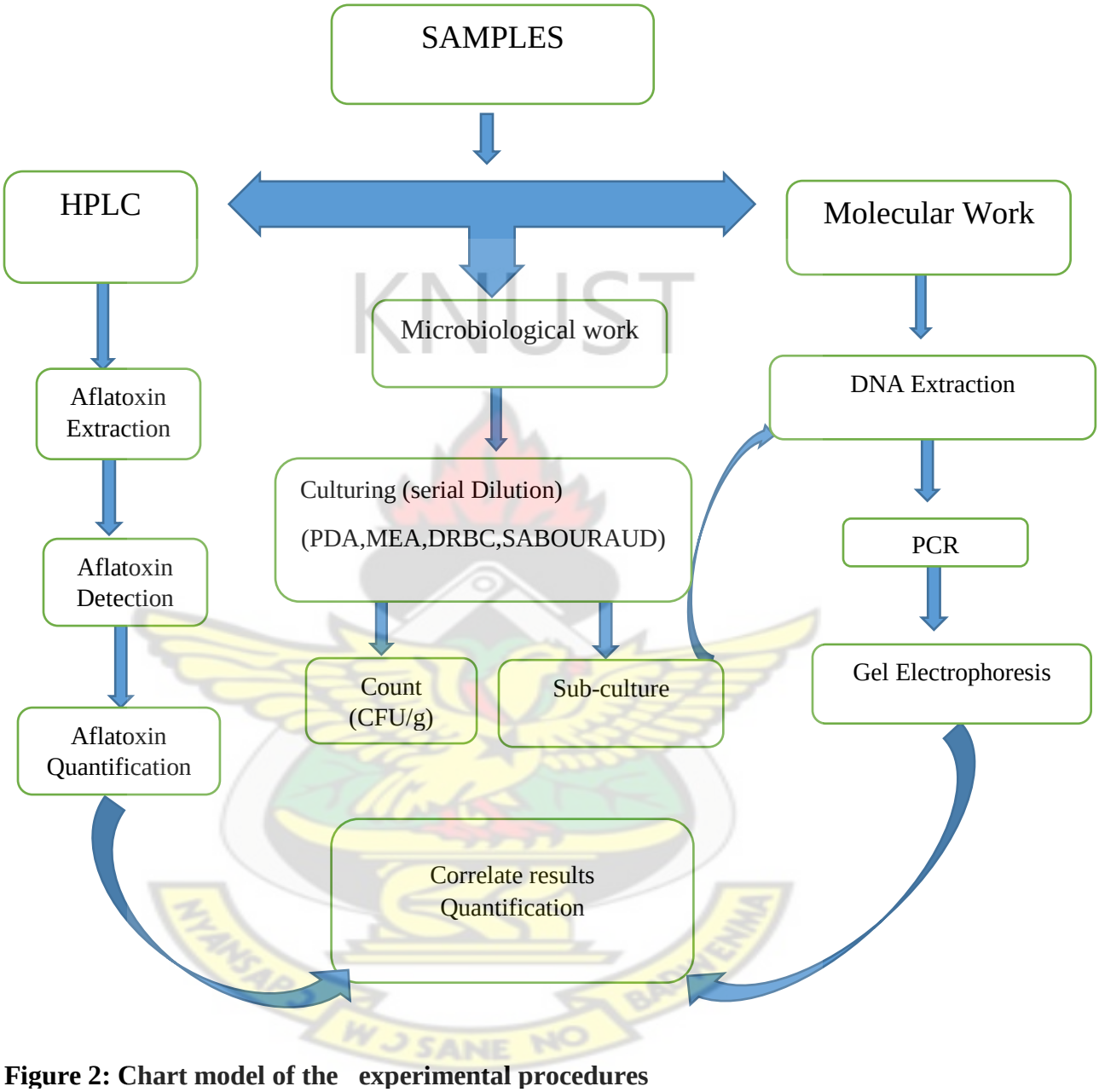

Figure 2: Chart model of the experimental procedures

3.3 AFLATOXIN HPLC PROCEDURE

3.3.1 Extraction of aflatoxin

Each sample groundnut, maize, beans and rice was ran separately, with 25 g of each ground sample and 5 g of Sodium Chlorine (NaCl) weighed and poured in a blender jar. 125 ml of 70% methanol 30% water (v/v) was added to the jar blender and blended at high speed for 2 minutes. The content was filtered with a Whatman No1 filter paper into a clean vessel. The purpose of this step is to dissolve the analyte quantitatively in the solvent.

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3.3.2 Extract dilution

A 15 ml of the filtrate extract was collected with clean pipette and transferred into a clean vessel and diluted with 30 ml of distilled water and well mixed. The dilute extract was filtered through a glass microfiber filter.

3.3.3 AflaTest affinity chromatography

A 15ml of the diluted filtered extract (equivalent to 1g of sample) was loaded into a syringe and was passed through the AflaTest column. The AflaTest column was washed using 10 ml of distilled water. The column was rewashed one more time with equal amount of distilled water. The aflatoxins from the AlfaTest column was eluted with 1.0 ml HPLC grade methanol and was collected in a glass cuvette or vial.

The aflatoxin analysis was conducted with a detector of wavelength 360 nm excitation and 440 nm emissions. The mobile phase pumped at a rate of 1 ml/min consisted of water: methanol (60:40) with addition of 120 mg of potassium bromide and 350 µl of nitric acid. The injection volume of both the analyte and the standard was 100 µl. The column oven temperature was 40^0C and the lower detention limit was 0.5 ppb while the limit of quantification was 1.0 ppb.

3.4 MICROBIAL CULTURE PROCEDURE

A microbiological analytical culture procedure was carried out under aseptic conditions. A 10 ml of peptone water was pipetted into a sterile test tube 1, and 9 ml into another test tube 2, and tube 3 i.e. (dilution factor at 10^{-3}). Then 1g of each of the samples (groundnut, maize, beans and rice) were weighed into the respective labelled test tube 1 and shaken vigorously to dissolve the sample. Thereafter, 1ml of the mixture was serially diluted in test tube 2 and test tube 3. Finally 1 ml from each dilution factor was cultured by spread plate technique (Appendix II*)* on the various agars (Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), Sabouraud Dextrose Agar (SDA), DRBC agar) containing 100 mg/l of chloramphenicol and 50mg/l chlortetracycline hydrochloride to suppress the growth of bacteria both incubated at room temperature for 5-7 days. Fungal growth was observed within the 5-7 days in plate colonies.

3.4.1 Isolation and identification

After the incubation period, colonies were sub-cultured to the appropriate media and further incubated to attain pure cultures of each fungal species.

After obtaining colonies of pure isolates, they were identified by observing them phenotypically and verified microscopically by examining colony colour, size, appearance and cell morphology (Hogg, 2005). The colonies were further counted using a colony counter. Samples were calculated in colony forming units per gram of sample (CFU/g) as

$$\text{CFU/g} = \frac{\text{number of colonies x reciprocal of the dilution factor}}{\text{Plating volume (1 ml)}}$$

3.5 MOLECULAR WORK PROCEDURE

3.5.1 Fungi DNA extraction

Isolates of the various fungus strains from the grain samples were sub-cultured to pure isolates and incubated for 6 days at room temperature. Fungal hyphae collected were centrifuged and washed with water once to rinse out the spores and dried under a laminar hood. About 100 mg portion of mycelia from the pure fungal strain were freeze-dried with liquid nitrogen and ground with a mortar and pestle. A 900 µl of 2% CTAB pre-warmed extraction buffer was added, mixed and incubated at 65°C for one hour. Again 900 µl phenol: chloroform (1:1) was added. The mixture was homogenized by vortexing for 30 sec and phases were separated by centrifugation at 10,000 rpm for 10 mins. A 500 µl of the supernatant was pipetted in a fresh 2 ml centrifuge tube and 2 µl RNase (5µg/ml) was added and incubated at 37° C for 30 mins. An exact 750 µl of chloroform was then added with quick vortexing (5 s) and then again centrifuged at 10,000 rpm for 10 mins and the aqueous collected into a sterile microtube. A 250 µl of 7.5M NH_4OAc and 1 ml of ethanol (99%) was added to the aqueous and the mixture kept on ice for over an hour. The tube was then finally centrifuged at 12,000 rpm for 15 mins, and all the liquid in it was discarded and another quick centrifuge of 5 s and remaining liquid Pipetted out. Tubes were then dried in the laminar flow hood for 45 mins. The dried pellets were dissolved in 45 µl of Tris-HCl EDTA buffer at pH 8.0.

3.5.2 PCR amplification

Primers were constituted with molecular biology grade water to their respective volumes as directed by the manufacturer (Biolegio), to give a stock concentration of 100 µM, and centrifuged for about 5 s. To prepare the working concentration (10 µM), 10 µl was pipetted from both forward and reverse primer stock and dispensed in a new microfuge tube and diluted

with 180 µl molecular biology grade water and vortexed . A 8.5 µl of the master mix (Table 6) was dispensed into a 200 µl PCR microfuge tube and 1.0 µl of DNA added and spun for 30 sec. The tubes were placed in the thermal cycler and run with following cycling conditions; initial denaturation at 95 °C for 5 min for 1 cycle; followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 50-55 °C for 30 s, elongation at 72 °C for 1 mins, and final extension step at 72 °C for 10 mins. Five (5) microliters of the PCR products were loaded on 2% agarose gel and visualized with UV.

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Table 5: Primers used in the study arranged based on detection specificity

No.	Primer name	Sequence 5'-3'	Product size (base pair bp)	Annealing T/ ^{0}C	References
	Universal fungi primers				
1	0817F 1196R	**F:**TTAGCATGGAATAATRRAATAGGA **R:**TCTGGACCTGGTGAGTTTCC	422	55	Borneman *et al*., (2000)
2	P1 P2	**F:** ATTGGAGGGCAAGTCTGGTG **R:** CCGATCCCTAGTCGGCATAG	482-503	60	Einsele *et al*., (1997)
3	U1 U2	**F:** GTGAAATTGTTGAAAGGGAA **R:** GACTCCTTGGTCCGTGTT	260	50	Sandhu *et al*., (1995)
4	FF2 FR1	**F:** GGTTCTATTTTGTTGGTTTCTA **R**: CTCTCAATCTGTCAATCCTTATT	425	55	Zhou *et al*., (2000)
5	ITS5 IST2	**F:** GGAAGTAAAAGTCGTAACAAGG **R:** GCTGCGTTCTTCATCGATGC	315	53	White *et al.*, (1990)
Aflatoxin producing fungi primers					
6	Nor1 Nor2	**F:** ACCGCTACGCCGGCACTCTCGGCAC **R:** GTTGGCCGCCAGCTTCGACACTCCG	400	63	Geisen *et al.*, (1996)
7	Ver1 Ver2	**F:** GCCGCAGGCCGCGGAGAAAGTGGT **R:** GGGGATATACTCCCGCGACACAGGC	537	60	Geisen *et al.*, (1996)
8	Omt1 Omt2	**F:** GTGGACGGACCTAGTCCGACATCAC **R:**GTCGGCGCCACGCACTGGGTTGGGG	797	60	Geisen *et al.*, (1996)
9	VER-496 VER-1391	**F:** TGTCGGATAATCACCGTTTAGATGGC **R:** CGAAAAGCGCCACCATCCACCCCAATG	895	62	Shapira *et al.*, (1996)
10	APA-450 APA-1482	**F:** TATCTCCCCCCGGGCATCTCCCGG **R**: CCGTCAGACAGCCACTGGACACGG	1032	55	Shapira *et al.*, (1996)
11	OMT- 208 OMT-1232	**F:** CGCCCCAGTGAGACCCTTCCTAG **R:** CGC-CCC-AGT-GAG-ACC-CTT-CCT-CG	1024	55	Shapira *et al.*, (1996)
12	AflR620 AflR1249	**F:** CGCGCTCCCAGTCCCCTTGATT **R:** CTTGTTCCCCGAGATGACCA	630	55	Sweeney *et al.*, (2000)
13	Ord1508 Ord2226	**F:** TTAAGGCAGGGGAATACAAG **R:** GACGCCCAAAGCCGAACACAAA	598	60	Sweeney *et al.*, (2000)

F; Forward primer, R; Reverse primer

Table 6: Preparation of PCR reactants

Master mix	Reagent Used	Volume(uL)	Final conc.
	10X buffer	5	1X
	10uM Primers (F and R)	2	10µM
	10mM dNTPs	0.5	100µM each
	Taqpol	1.0	2U
	DNA template	4.0	-
	H_2O	37.5	-
	Total volume	50	-

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3.6 STATISTICAL ANALYSIS

All the results generated from the experiments were analyzed using GraphPad Prism (V.5) one-way and two-way ANOVA and Bonserroni at 5% probability and 95% confidence interval.

CHAPTER FOUR
4.0 RESULTS

4.1 LEVELS OF AFLATOXIN IN FOOD SAMPLES

Although the focus was on AFB1, during assessment of aflatoxin contamination of four food was as from three sales points (SP) in Kumasi, the results showed presence of all the four (4) types of aflatoxins (AFB1, AFB2, AFG1 and AFG2) in the crops Table 7: Out of the twelve (12) crops only two groundnut samples (P1, P3,) and two maize samples (C1 and C2) recorded significant amounts of aflatoxin, ranging from 2.20 to 31.11ppb. One beans sample (N2) and rice sample (R3) showed aflatoxin contamination (0.0006 and 0.0001 ppb respectively), but too low to pose health effect. However, the rest of the samples from all the crops tested negative for AFB1.

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Table 7: Aflatoxin contamination levels detected by HPLC.

Crops		Parts per billion (ppb)				
		AFB1	AFB2	AFG1	AFG2	Total AF
Groundnut	P1	**2.20**	0.20	0.07	0.34	2.81
	P2	-	-	0.021	23.87	23.89
	P3	**31.11**	2.50	14.80	0.39	48.80
Maize	C1	**8.79**	0.43	0.21	-	9.43
	C2	**3.15**	0.13	0.60	0.02	3.91
	C3	-	-	-	0.0021	0.0021
Beans	N1	-	-	-	-	-
	N2	**0.0006**	0.0003	0.0008	0.0013	0.003
	N3	-	-	-	0.0003	0.0003
Rice	R1	-	-	0.03	0.08	0.11
	R2	-	-	-	-	-
	R3	**0.0001**	0.0002	-	0.0018	0.0021

P=groundnut; C= maize; N= beans R= rice; (P1, C2, N1...) = crop with batch number (- = negative), AF=aflatoxin (B1, B2 =Blue and G1, G2 = Green).

No significant difference between SP1 and SP2 in terms of groundnut and maize (P>0.05). There is however, a great significant difference between sales point 1 and sales point 3 with respect to groundnut again (P<0.001). Figure 3.

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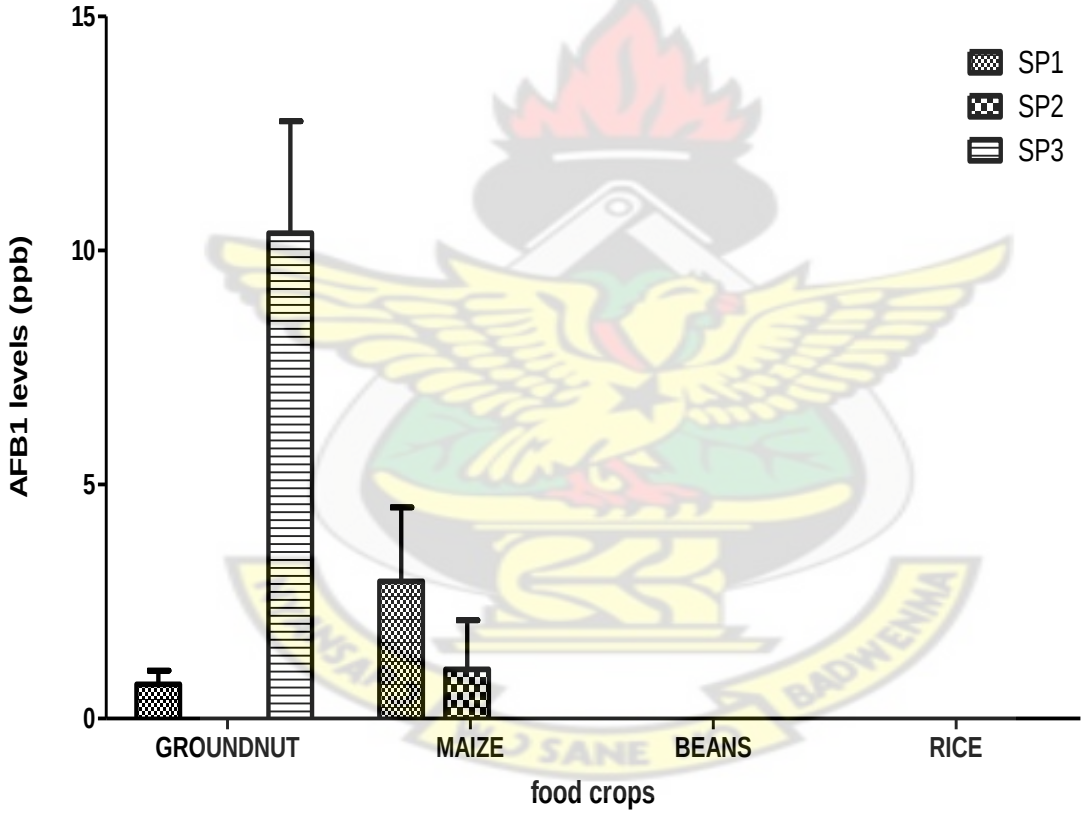

Figure 3: Aflatoxin levels within food at various sales points (SP)

Aflatoxin results obtained for the grains samples from sales point 1 showed no significant difference between maize and beans, maize and rice, and between beans and rice within the other sales points Figure 4. (i.e. P>0.05). The analysis however, revealed a great significant difference between groundnut and all the rest of the crops (Maize, beans and rice) samples from Sales Point 3, SP3 (i.e. <0.001)

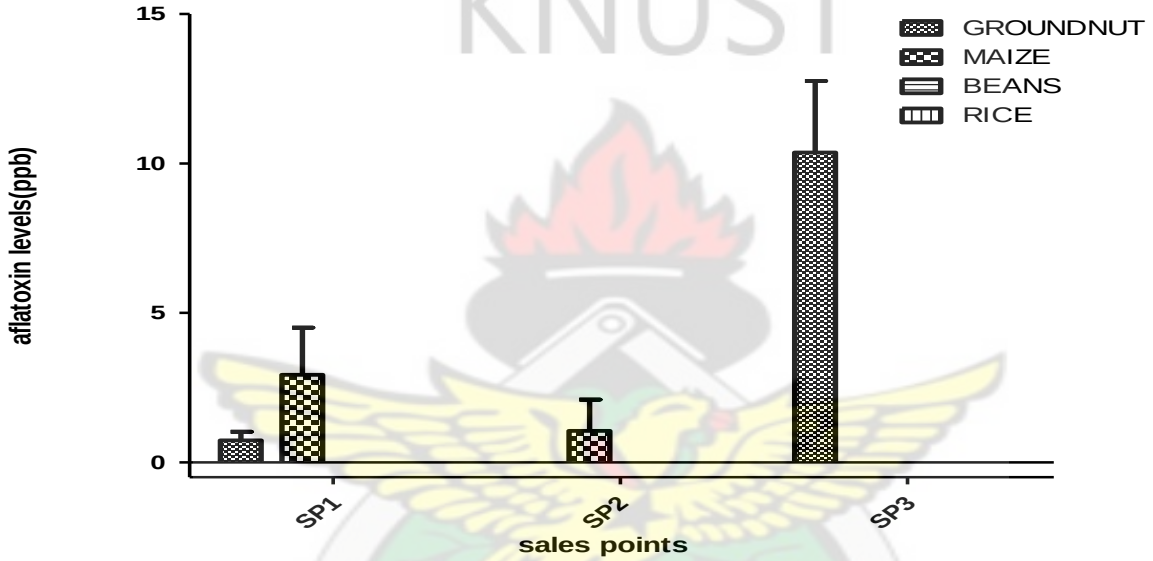

Figure 4: Measured of aflatoxin levels in crop from Sales Point

4.2 MICROBIAL CULTURE RESULTS

The commonest fungi genera found in the food samples were *Aspergillus* and *Penicillium.* Table 8 shows the types of fungi identified for each crop sample. From the study, *A. flavus and A. paraciticus* were the predominant species in the *Aspergillus* genera. In general, eleven (11) different moulds were found to be present in the groundnut samples (P), 10 from maize (C), 8 isolates from the bean samples (N), whilst rice had the least amount of fungal species with 5 isolates.

Table 8: Moulds Identified on the various samples

Crops	Samples	Fungi identified
Groundnut (P)	P1	*A. flavus, A. niger, P. citrinum, Moniliella*
	P2	*A. flavus, A. terreus, Emericella nidulans, P. expansum*
	P3	*A. flavus, A. paracitius, P. italicum, Absidia corymbifera, Aureobasidium,*
Maize (C)	C1	*A. paracitius, A. tamarii, P. expansum, Mucor hiemalis*
	C2	*A. paracitius, Emericella nidulans, Rhizoctoinia solani*
	C3	*A. flavus, A. terreus, P. citrinum, A. versicolor*
Beans (N)	N1	*A. niger, P. expansum, Fusarium*
	N2	*P. citrinum, Eurotium amstelodami*
	N3	*Mucor hiemalis, Cladosporium, Botrytis*
Rice (R)	R1	*P. italicum, Cladosporium*
	R2	*Moniliella, Aureobasidium*
	R3	*Eurotium amstelodami*

P=Groundnut; C=Maize; N=Beans; R= Rice. ; (P1, C2, N3 ...) =sample with batch number

Table 9 shows contamination levels of microbes on the samples analyzed (cfu/g). The Groundnut (P) samples recorded the highest level of microbial contaminants ranging from $3.8 \times 10^5 \pm 3.74$ cfu/g to $4.3 \times 10^6 \pm 0.49$ cfu/g compared to Rice (R) which had the lowest microbial loads ranging from $2.1 \times 10^3 \pm 0.04$ cfu/g to $4.3 \times 10^3 \pm 4.92$ cfu/g. Maize (C) had microbial loads ranging from $4.6 \times 10^4 \pm 3.09$ cfu/g to $3.4 \times 10^6 \pm 0.29$ cfu/g whilst the microbial loads recorded for Beans (N) ranged from $2.9 \times 10^4 \pm 0.24$ cfu/g to $5.5 \times 10^4 \pm 0.24$ cfu/g.

Table 9: Microbial Count (CFU/g ± SD) of the samples

Crops	Samples	Contamination level (CFU/ g)	Standard deviation (SD)
Groundnut	P1	3.8×10^5	3.74
	P2	5.3×10^5	0.16
	P3	4.3×10^6	0.49
Maize	C1	3.4×10^6	0.29
	C2	3.4×10^5	0.44
	C3	4.6×10^4	3.09
Beans	N1	5.5×10^4	0.24
	N2	3.2×10^4	2.44
	N3	2.9×10^4	0.24
Rice	R1	4.3×10^3	4.92
	R2	2.7×10^3	0.17
	R3	2.1×10^3	0.04

P=Groundnut; C=Maize; N=Beans; R= Rice; (P1, C2, N3 ...) =sample with batch number

CFU/g: Colony forming unit per gram, Dilution factor 10^{-3}

The relationship of the type of mould with the type of crop used was also analyzed (Figure 5). It was observed that, the *Aspergillus* and *Penicillium species* such as *A. flavus, A. paracitius, A. terreus, P. citrinum P. expansum,* and *Emericella nidulans* dominated in all the three groundnut and three maize samples. *Absidia corymbifera* was found in the groundnut samples only. *A. tamari, A. versicolor, Rhizoctoinia solani* were also found only in the maize. *Aureobasidium, P. italicum* and *Moniliella* were observed in both the groundnut and rice samples. Furthermore, *A. niger* was observed in both groundnut and the beans samples. *P. citrinum, P. expansum* were observed in the bean samples**.** *Mucor hiemalis* was found in both beans and maize. *Fusarium* and

Botrytis fungi were observed in the beans only. Finally, *Eurotium amstelodami* and *Cladosporium* were found in both the beans and the rice.

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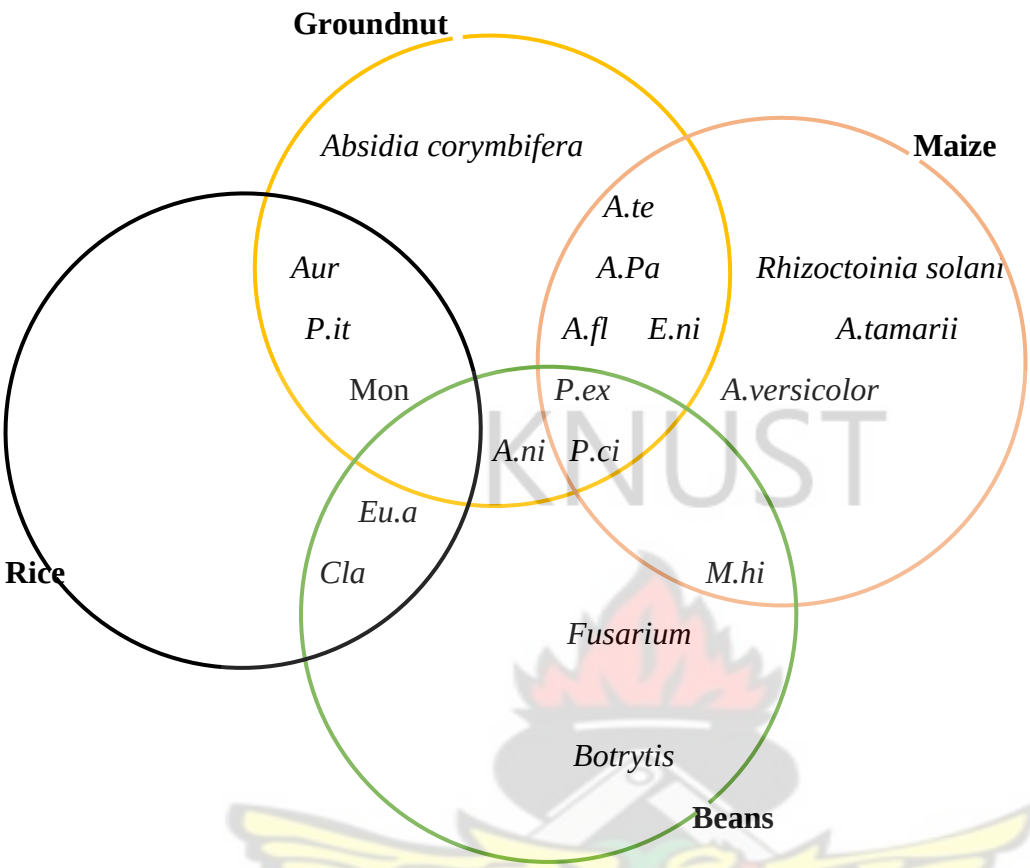

Figure 5: Isolated fungi and relationship with the type of crop sample used

Key: ***A.fl=Aspergillus flavus; A.Pa= Aspergillus parasiticus; A.ni= Aspergillus niger; A.ta= Aspergillus tamari; A.te= Aspergillus terreus; P.ex=Penicillium expansum; P.ci=Penicillium citrinum; P.ita= Penicillium italicum; mon=Moniliella; E. ni=Emericella nidulans; Aur=Aureobasidium; Cla=Cladosporium; M.hi=Mucor hiemalis***

4.3 MOLECULAR ANALYSIS OF AFLATOXIN-PRODUCING FUNGI

Table 10: Fungi identified and arranged for PCR

1	*A. flavus*	11	*Eurotium amstelodami*
2	*Mucor hiemalis*	12	*P. citrinum*
3	*Absidia corymbifera*	13	*A. niger*
4	*Emericella nidulans*	14	*A. parasiticus*
5	*Rhizoctoinia solani*	15	*A. terreus*
6	*P. expansum*	16	*A. versicolor*
7	*Fusarium*	17	*P. italicum*
8	*Aureobasidium*	18	*A. tamarii*
9	*Botrytis*	19	*Cladosporium*
10	*Moniliella*		

Various DNA markers of fungal strains were amplified using both universal and specific primers and were separated on agarose gel (Appendix lll).

Figure 6: Amplification of universal primer set (FF2 & FR1) on the fungal isolates 1-19

M –kb plus ladder. **(1-19 fungal isolates arranged accordingly as represented in Table 10.**

Bands representing PCR products were scored e.g. lanes 2,5,6,10,16, and 18 in Figure 6 were negative with respect to 400bp locus whilst lanes 1,3,4,7,8,9,11,12,13,14,15, 17 and 19 were positive.

Table 11 shows the results of both universal and specific primers for aflatoxin producing fungi on the 19 fungi isolates shown in Table 10 *A. flavus, A. paracitius, A. terreus, P. citrinum P. expansum Emericella nidulans and Absidia corymbifera* among others. It displays how efficiently primers can discriminate between aflatoxin producing fungi and those that cannot in this study. The positive (+) and negative (-) sign in the table represents presence or absence respectively of the ability of the fungi to produce aflatoxin.

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Table 11: Loci scores for various primer pairs on different samples

	Primers(F&R)	**fungal isolates 19-1 arrange accordingly as represented in Table 10**																		
No.		19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
1	**F:** 0817F **R:** 1196R	+	+	+	+	-	+	+	-	+	+	+	+	-	+	+	+	-	-	+
2	**F:** P1 **R:** P2	+	-	-	-	-	-	+	+	+	+	+	-	-	+	+	-	+	-	+
3	**F**: U1 **R**: U2	-	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+
4	**F**: FF2 **R**: FR1	+	-	+	-	+	+	+	+	+	-	+	+	+	+	-	-	+	+	+
5	**F**: ITS5 **R**: IST2	-	+	+	-	+	+	+	+	-	-	+	-	-	-	+	+	+	+	+
6	**F:** nor1 **R:** nor2	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	**F:** Ver1 **R:**Ver2	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	+
8	**F:** Omt1 **R:** Omt2	-	+	-	+	-	+	-	+	-	-	-	+	-	-	-	-	-	-	-
9	**F:** VER-496 **R:** VER-1391	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
10	**F:** APA-450 **R**: APA-1482	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	**F:** OMT-208 **R:** OMT-1232	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-
12	**F:** AflR620 **R:** AflR1249	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-
13	**F:** Ord1508 **R:** Ord2226	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-

- =absents, + = present, F= forward, R= reverse

CHAPTER FIVE
5.0 DISCUSSION

This study was aimed at the determination of contamination levels, at which consumers might undoubtly encounter an increased risk of excessive amounts of aflatoxin in agricultural commodities. Due to consumer relevant health concerns related to aflatoxin, aflatoxin contaminated food and food raw materials have repeatedly been rejected by law enforcement authorities, which results in high economic losses both for producers and traders (Robens and Cardwell, 2005).

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Different grains from Kumasi Central market were analyzed for the concentration of total aflatoxins by HPLC and the fungal contamination by microbiological analysis. In addition to identifying pure fungal cultures, another goal of the current research was detection of the presence of aflatogenic fungi using PCR assays parallel in the studies. The results from the study showed significant contaminations from groundnut and maize while beans and rice recorded low level. The microbial counts also recorded a variety of fungi with a range of high cfu which are later discussed. Similarly, highly specific primers were employed to identify aflatoxin potentially producers.

The insignificant difference between Sales Point (SP) 1 and Sales Point (SP) 2 in terms of aflatoxin levels Figure 3 could be based on the hygienic conditions of the two marketing points. The sales women were continuously sorting out damaged grains when visited during the research. The grains at these SPs were exposed to the heat of the sun while being sold, a process known to inhibit aflatoxin (Wu *et al.*, 2010). This could be the reason why low aflatoxin level was recorded. Magan *et al.*, (2007) reported that proper heat drying can effectively limit the spread of harmful fungi that produce different mycotoxins especially aflatoxin. Different storage

conditions could also be account for why SP3 differ greatly in its aflatoxin level for groundnut as compared to the rest of the two Sales Points. The great difference shown by SP3 is further clarified in groundnut sample P3 where groundnut samples registered the highest aflatoxin level. Bad agronomical practices in the field such as continuous cropping, breaking shell during harvesting could also account for the contamination as stated by Hell *et al.,* (2000).

Olsen *et al.,* (2008) reported from microbiological study of a typical aflatoxin producers in a variety of commodities that, there was a considerable risk of exceeding the European legislative limit for aflatoxin (4 μg/kg) when the level of the aflatoxin-producers contamination exceeds 100 CFU/g of commodity. Moreover, the probability of excess total aflatoxin levels exceeding the European legislative limit of 4 μg/kg increased rapidly from approximately 30% to above 80% when the mould levels increased from 100 to 1000 CFU/g, respectively (Johnsson *et al.,* 2008).

Fungi are part of the normal flora of food products; however, some produce toxic metabolites such as mycotoxins. Mycotoxins are harmful to human health also degrades food taste and flavor, with consequence economic losses (Wu *et al.,* 2010). It is worth mentioning first of all that, the outcomes acquired by cultivation and identification of microorganisms are influence by many factors such as chemical composition of the suspending solution, growth medium, cultivation conditions and presence of other species as well as dilution factor.

These contaminations of crops may occur during the agronomical stages and as far as the processing, packaging and storage stages (Navarro *et al.,* 2010). Fungi especially the *Aspergillus* spices are ubiquitous, they grow on virtually any organic substrates, living or dead and is astonishing on how they adapted to adverse environments or areas subjected to periodic droughts. The adaptation Of *A. flavus* for growth enable it to outcompete neighbouring species and other commonly occurring micro flora such as bacteria under drought and stress conditions.

Shapira *et al.,* (1996) revealed that concentrations of aflatoxins can be correlated with the level of CFU/g of aflatoxigenic species detected on naturally contaminated samples. Verification of fungal contamination levels in agricultural produce is therefore essential since previous reports have also established the correlation between levels of aflatoxigenic fungi and aflatoxin concentrations at a level exceeding legal limits (Lund *et al.*, 2003).

A. flavus is the most common species associated with aflatoxin contamination of agricultural crops (Cotty, 1997). Generally, from the results of the findings, *A. flavus and A. paraciticus* were presents and dominant in all the groundnut and maize sample but absent from the rice and beans sample. This finding is in agreement with research by Samuel *et al.*, (2013) who stated that the two fungi (*A. flavus* and *A. paracitius*) are commonly found in maize, cotton, tress, groundnuts. Chen *et al.*, (2013) also stated that they seldomly found on rice. There was no sign of presence of *A. flavus and A. paraciticus* on all the bean samples. Only the black spored *Aspergillus* species *A. niger* appeared in one of the bean samples. Though, *A. niger* is not known to be pathogenic, however, in very rare cases it could be an opportunistic microorganism capable of colonizing and causing ear, nose and lungs infection on people with acute illness and immuno-compromised individuals (Schuster *et al*., 2002).

A. tamarii was part of the isolates identified, alongside *A. paraciticus* in the maize sample (C1), *as* shown in Table 8, *A. tamarii* constitutes 26% of the total fungi in this particular sample. The total CFU/g of fungi in this sample was 3.4×10^6 which is higher than the acceptable limits of $<10^5$ CFU/g (Abadias *et al*., 2008). The total level of aflatoxin contamination in the maize sample was 8.72 ppb. Despite *A. Parasiticus* being a known aflatoxin producer, it showed negative to all the aflatoxin specific primers except Ver1/Ver2. Some of the molecular markers

U1/U2 and ITS5/ ITS2 has shown some positivity in *A. tamarii* which shows that the fungi is capable of producing aflatoxin. This is in concordance with (Dorner, 1983) who reported that *A. tamarii* has been found for the first time to produce aflatoxin.

A. versicolor is a common indoor fungus in damp houses, carpet dust among many others. It is capable of producing Sterigmatocystin which is a precursor of aflatoxin biosynthesis (Yu *et al.*, 2004). *A. versicolor* is identified to be among the major producer of the hepatotoxic and carcinogenic mycotoxin sterigmatocystin (Barnes *et al.*, 1994). Scudamore *et al.*, (1996) states that, however, the acute and chronic toxicities effect of sterigmatocystin from *A. versicolor* are significantly lower. *A. versicolor* represents about 16% of the total fungi isolated in this experiment scoring 4.6 x10^4 CFU/g with *A. flavus, A. terreus, and Penicillium citrinum* in the maize (C3) sample Table 9. Even though the CFU/g is high enough with the presence of *A. flavus*, no aflatoxin contamination was recorded by the HPLC. Four universal primers 0817F/1196R, U1/U2, FF2/FR1 and ITS/IST2 and one specific aflatoxin producing fungi primer nor1/nor2 was able to show positive bands from *A. versicolor*. This may means that *A. versicolor* has the gene to produce aflatoxin, however, it could not produce detectable aflatoxin. It can be concluded that the presence of aflatoxin producing fungi in the seeds or grains does not necessarily mean there will be aflatoxin contamination. This point seems to be supported by Cole *et al.*, (1983) who established poor correlations between infection percentages and aflatoxin contamination.

The Dorner *et al.*, (2003) also suggested that infection of groundnuts by *A. flavus, A. parasiticus* and other mycotoxins-causing fungi was not a prerequisite of aflatoxin contamination. It is very possible to detect significant aflatoxins level in biochemically clean seeds while an infested

looking seed may not contain any amount of aflatoxin. It is also difficult to use percentage pathogenic infections as a guide to aflatoxin build-up since even disinfected seeds may still carry the fungal propagules and continue to sporulate in due cause (Dorner *et al.*, 2003).

Plant seeds contained other proteins which may act as inhibitors of fungi infection and growth during storage and germination (Guo *et al*., 1999). When these proteins are concentrated in the seed, it may prevent fungal invasion and reproduction and also exhibit bioactivity against growth of *A. flavus*, and other mycotoxin-causing fungi. In the current study, some of the samples Table 7 may have recorded no aflatoxin contamination probably due to the interactive effects of the seed inhibiting proteins (phytoalexins) and competition among the fungi.

Penicillium species are very commonly found in the soil, on decaying vegetation and compost or on wood, dried foodstuffs, spices, dry cereals, fresh fruit and vegetables among others. Bancerz *et al.*, (2005) found that this species was one of the best lipase producers among other fungi they studied in the arctic tundra. *Penicillium chrysogenum* has high enzymatic activity and has the ability to produce alpha-amylase. Most of the species produce secondary metabolites. These are often used to create antibiotics such as penicillin and griseofulvin. *P. italicum* causes slimy rot and produces blue-green conidia. These species thrive well in cooler temperatures, which explain why they are usually found on foods left too long in the refrigerator. Several species of the genus *Penicillium* play a central role in the production of cheese and of various meat products, cheeses. Many other *Penicillium nalgiovense* is used to improve the taste of sausages and hams, and to prevent colonization by other molds and bacteria (Marianski *et al*., 2009). *Penicillium* fungi are versatile and opportunistic and many species are one of the most common causes of fungal spoilage in fruits and vegetables and are also post-harvest pathogens (ICMSF, 1998). Some

Penicillium species produce mycotoxins, for example, *P. expansum* produces Patulin. *P. expansum* was positive to three of the universal primers Table 11 and two of the specific aflatoxin producing fungi primers (Ver1/Ver2 and omt1/omt2) indicating that *P. expansum* is capable of producing aflatoxin.

Fusarium species are widely distributed in soil, buried or subterranean and aerial plant parts, plant debris, and other organic substrates (Nakar *et al.,* 2001) and are present in water worldwide as part of water structure biofilms (Elvers *et al.,* 1998). The widespread distribution of *Fusarium* species may be attributed to their ability to grow on a wide range of substrates and their efficient mechanisms for dispersal (Burgess *et al.,* 1981).

Fusarium species are important plant pathogens causing various diseases such as crown rot, head blight, and scab on cereal grains. They are capable of producing mycotoxins such as *Fumonisin* or *Tricchothecenes* responsible for diarrhoeal diseases as clearly spelled out by Nakar *et al*., (2001), and they may occasionally cause infection in animals **(**Ferrer *et al*., 2005**).** In humans, *Fusarium* species cause a broad spectrum of infections to almost exclusively in severely immune-compromised patients.

Fusarium was found only in the beans sample, it is possible that the species found is non-aflatoxin producer as it showed negative to all the specific primers. The same result is manifested on *Botrytis* and it is unknown if this type of mould produces toxins. Eurotium amstelodami produces the same results when ran with the same specific primers as shown Table 11.

Some species of *Moniliella* can cause disease in humans (Guarro *et al.,* 1999) and in cats (McKenzie *et al*., 1984). Three of the specific primers show it can produce aflatoxins as well.

The main reason of this research is to identify which fungi are able to produce aflatoxin using optimize conditions with PCR method. Mayer *et al.,* (2003) suggested that the presence or lack of mRNA could permit direct differentiation between aflatoxin and non aflatoxin producing fungi, however, in this situation, after testing nineteen (19) fungi isolates with five universal primers and eight specific aflatoxin producing primers, as revealed in Table 11 *Rhizoctoinia solani, Botrytis, Eurotium amstelodami, A. terreus, A. versicolor and A. tamarii* showed negative results in all the specific primers. This indicates that all these six are non-aflatoxingenic mould. The rest of the fungi displayed different bands with various primers. Some known aflatoxin producing fungi such as *A. flavus* showed no band with the primer nor1/nor2. This may be as results of mutation on the strain of the fungi as suggested by Geisen (1996) that, the lack of aflatoxin production could also be due to simple mutations including substitution of some bases. Various physiological conditions and nutrient source can also affect aflatoxin biosynthesis pathway.

It is practically possible to isolate a fungi as aflatoxigenic by the PCR yet the fungi would not be able to produce aflatoxin, since in this study specific gene are not targeted to identify particular strains.

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CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1. CONCLUSIONS

The aflatoxin level found in all the 12 crops were within the range of the acceptable limit for consumption and exportation (0-20 ppb) except for the groundnut sample P3 which recorded a relatively high amount of 31.11ppb from the HPLC analysis. The microbial culture analysis revealed groundnut samples with the highest fungi contamination of 3.8×10^5, 5.3×10^5, 4.3×10^6 CFU/g. The goes beyond the permissible limit $<10^5$ CFU/g. The fungal levels were beyond the permissible. Rice samples on the other hand recorded value of low CFU/g 4.3×10^3, 2.7×10^3, and 2.1×10^3.

A consistent correlation could not be established between the aflatoxin contamination levels with the CFU/g recorded by the microbiological cultures per sample. For example, Groundnut sample P2 recorded 5.3×10^5 which is high enough to record aflatoxin level but there was no aflatoxin produced. The indication of a band for a particular fungi cannot be said with certainty that the fungi is a aflatoxin producer.

6.2 RECOMMENDATIONS

i). Sampling should be done in various seasons and increase batch number in subsequent studies.

ii). Molecular markers that should target specific genes to identify particular strains that are responsible for aflatoxin production is also recommended to be employed in a later study.

iii). Baseline survey on agronomical and postharvest practices should be conducted to find out where the main problem of crop contamination occurs. This would help regulatory bodies or agencies to put measures on how to protect and control crops contamination with mycotoxins.

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APPENDICES

APPENDIX I

TOTAL AFLATOXINS LEVELS. (n = number of replicates)

	Aflatoxin B1			
Samples	**n1**	**n2**	**n3**	**Total**
P1	1.2	0.2	0.8	2.2
P2	0	0	0	0
P3	13.45	5.66	12	31.11
C1	0	3.37	5.42	8.79
C2	0	0	3.15	3.15
C3	0	0	0	0
N1	0	0	0	0
N2	0.0006	0	0	0.0006
N3	0	0	0	0
R1	0	0	0	0
R2	0	0	0	0
R3	0	0.0001	0	0.0001
	Aflatoxin B2			
P1	**n1**	**n2**	**n3**	**Total**
P2	0	0.2	0	0.2
P3	0	0	0	0
C1	0	0	2.5	2.5
C2	0.43	0	0	0.43
C3	0.13	0	0	0.13
N1	0	0	0	0
N2	0	0	0.0003	0.0003
N3	0	0	0	0
R1	0	0	0	0
R2	0	0	0	0
R3	0	0	0	0
	0	0.0002	0	0.0002
	Aflatoxin G1			
	n1	**n2**	**n3**	**Total**
P1	0.07	0	0	0.07
P2	0	0.021	0	0.021
P3	3.47	11.33	0	14.8
C1	2.21	0	0	0.21
C2	0	0	0.6	0.6
C3	0	0	0	0
N1	0	0	0	0
N2	0	0.0008	0	0.0008
N3	0	0	0	0

R1	0.03	0	0	0.03
R2	0	0	0	0
R3	0	0	0	0

	Aflatoxin G2			
	n1	**n2**	**n3**	**Total**
P1	0	0.34	0	0.34
P2	0.55	12.32	11	23.87
P3	0.39	0	0	0.39
C1	0	0	0	0
C2	0	0.02	0	0.02
C3	0.0021	0	0	0.0021
N1	0	0	0	0
N2	0.0013	0	0	0.0013
N3	0	0.0003	0	0.0003
R1	0	0	0.08	0.08
R2	0	0	0	0
R3	0	0	0.0018	0.0018

	Grand total
P1	2.81
P2	23.89
P3	48.8
C1	9.43
C2	3.91
C3	0.0021
N1	0
N2	0.003
N3	0.0003
R1	0.11
R2	0
R3	0.0021

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APPENDIX II

Preparation of media

A. Potato dextrose agar (PDA)

PDA is a selective medium purposely for culturing and isolation of fungi. The medium was prepared by dissolving 39 g of Potato dextrose powder with 100 mg of chloramphenicol in 1 liter of distilled water and swirled. The chloramphenicol is an antibiotics that suppresses and inhibits the growth of bacteria. The medium was heated with a waterbath to boil and dissolve completely and sterilized by autoclaving at 121^0C for 15minutes. Pour plate technique was carried out into the petri dishes and allowed to solidify.

B. Malt extract agar (MEA)

MEA is also a selective medium purposely for culturing and isolation of fungi. The medium was prepared by dissolving 50 g of Malt extract powder with 100 mg of chloramphenicol in 1 liter of distilled water and swirled. The chloramphenicol is an antibiotics that suppresses and inhibits the growth of bacteria. The medium was heated with a waterbath to boil and dissolve completely and sterilized by autoclaving at 121^0C for 15minutes. Pour plate technique was carried out into the petri dishes and allowed to solidify.

C. Dichloran Rose-Bengal Chloramphenicol *(*DRBC)

This agar was prepared by dissolving 15.75 g of DRBC powder with 100 mg of chloramphenicol in 500 ml of distilled water and swirled. The chloramphenicol is an antibiotics that suppresses and inhibits the growth of bacteria. The medium was heated with a waterbath to boil and dissolve

completely and sterilized by autoclaving at 121^0C for 15minutes. Pour plate technique was carried out into the petri dishes and allowed to solidify.

D. Sabouraud Dextrose Agar (SDA)

This agar is a selective agar for yeast and mould culturing and isolation. It was prepared by dissolving 62.0 g of Sabouraud powder with 100 mg of chloramphenicol in 1litre of distilled water and swirled. The chloramphenicol is an antibiotics that suppresses and inhibits the growth of bacteria. The medium was heated with a waterbath to boil and dissolve completely and sterilized by autoclaving at 121^0C for 15minutes. Pour plate technique was carried out into the petri dishes and allowed to solidify.

E. Spread plate technique

This technique was conducted by adding to each disposable petri dish 15-20 ml of sterilized agar medium, previously prepared, and melted then allow to solidify. 1 ml of each test sample fluid or its dilution was pipetted into each petri dish aseptically, and kept at room temperature for 5-7days. A large number of colonies were observed and viable counts obtained from the plates were calculated. With not more than 100 colonies per plate.

APPENDIX III

TOTAL PCR RESULTS OF FUNGAL ISOLATE FROM THE SAMPLES 1-19

Primer	loci	Fungi isolates																		
		19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
0817F 1196R	1	-	+	-	+	-	+	-	+	+	+	+	-	+	-	-	+	+	-	-
	2	+	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
P1 P2	1	-	-	-	-	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-
	2	-	-	+	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	+
	3	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
U1 U2	1	+	+	-	+	-	-	+	+	-	+	-	-	-	-	-	+	-	+	-
	2	-	-	-	-	+	+	-	-	+	-	+	-	+	+	-	-	+	-	-
FF2 FR1	1	+	-	-	-	+	+	+	+	-	-	+	+	+	-	-	+	+	-	+
	2	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
ITS5 IST2	1	+	+	+	+	+	-	-	-	+	-	-	+	+	+	-	-	+	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
Nor1 Nor2	1			-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Ver1 Ver2	1	-	-	-	-	-	+	+	-	-		-	-	-	+	-	-	-	-	-
	2	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	
Omt1 Omt2 8	1	-	-	-	-	-	-	-	+	-	-	-	+	-	+	-	+	-	+	-
VER-496	1	-	-	-		-		-		-	-		-	-	-	-	+	-	-	-
VER-1391	2		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
APA-450 APA-1482	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OMT-208 OMT-1232	1	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
AflR620 AflR1249	1	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
Ord1508 Ord2226	1	-	-	-	-	+	-	-	+	-	+	-	-	-	-	+	-	-	-	-

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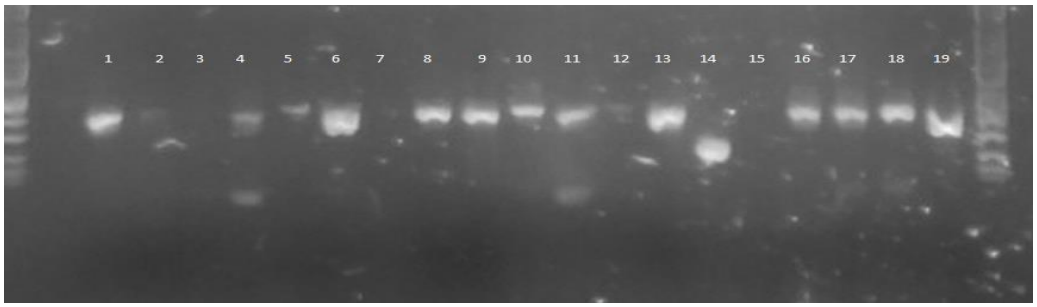
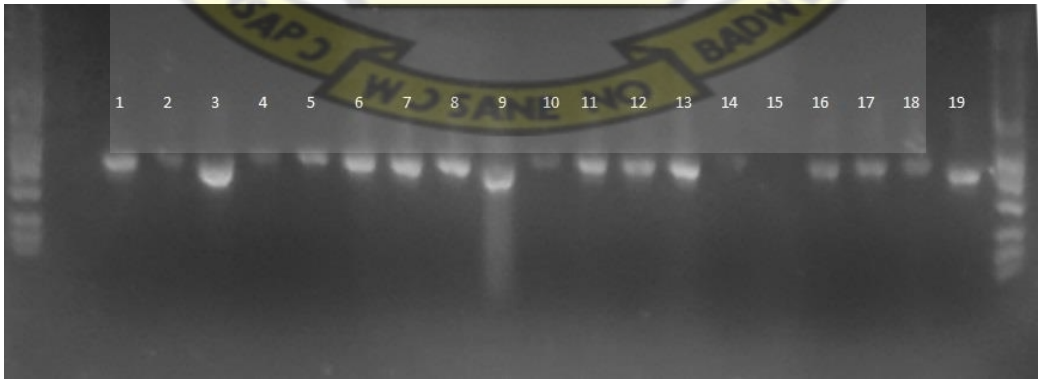


1) 0817F & 1196R

2) P1 & P2

3) U1 & U2



4) FF2 & FR1

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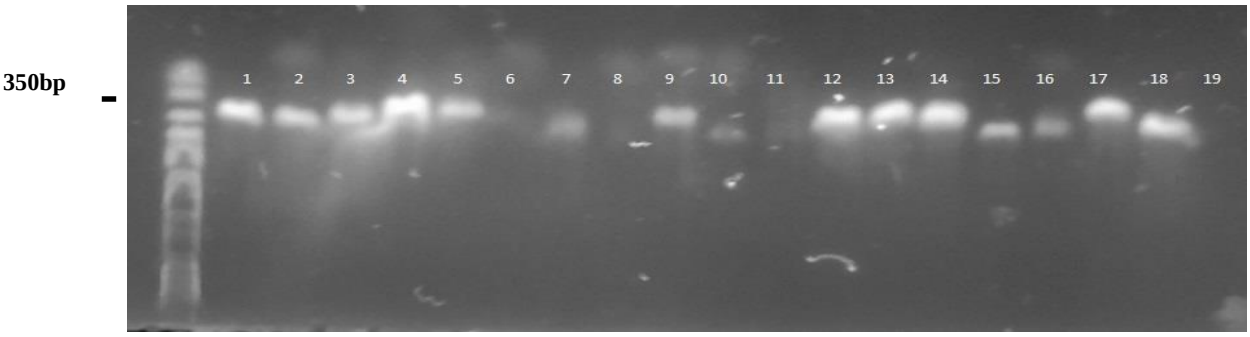

5) ITS5 & IST2

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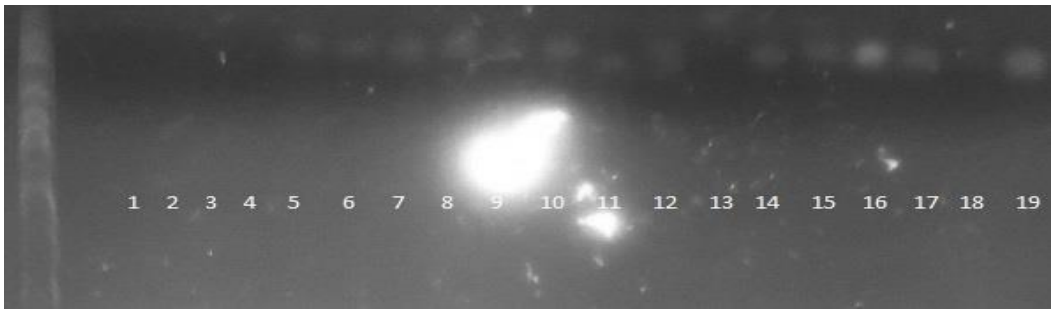
6) Nor1 & Nor2

7) Ver1 & Ver2

8) Omt1 & Omt2

900bp



9) VER-496 & VER-1391

1000bp

10) APA-450 & APA-1482

1000bp

11) OMT- 208 & OMT-1232

600bp

12) AflR620 & AflR1249

KNUST

13) Ord1508 & Ord2226

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