Molecular Characterization of Fungi Producing Aflatoxin in *Vigna subterranean* (Bambara Groundnut) sold in Three Selected Markets in Enugu Metropolis

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Being a Thesis Submitted to the Department of Biological Science, Faculty of Natural and Applied Sciences, Lead City University, Ibadan, Nigeria

In Partial Fulfillment of the Requirements for the Award of Master Degree (M.Sc) in General Microbiology

2022

Certification

This is to certify that Victoria Chiyaka, Iwu with Matric No. LCU/PG/001001 carried out this research work titled ''Molecular Characterization of Fungi Producing Aflatoxin in *Vigna subterranean* (Bambara Groundnut) sold in Three Selected Markets in Enugu Metropolis." in the Department of Biological Sciences, Faculty of Natural and Applied Sciences, Lead City University, Ibadan, Oyo state, for the award of Master Degree (M.Sc.) in General Microbiology and that this has not been previously submitted.

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Dedication

This research work is dedicated to everyone facing health challenges as a result of the consumption of food contaminated with aflatoxin.

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Although the institutions and people named above helped with this research project, I alone am responsible for any inaccuracies that are discovered.

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Abstract

Humans require nutritious food to maintain a healthy diet. Toxic strains of *Aspergillus* species produces aflatoxins, which are carcinogenic in nature. Bambara groundnut (BG), *Vigna subterranea* is an underutilized crop used for a variety of delicacies. Consumption of food contaminated with aflatoxin (> 20 ppb) has been linked to death. The aim of this study is to identify the aflatoxin producing fungi in BG using Molecular method. Samples were purchased at five points from three selected markets: Abakpa; New main and Ogbete markets in Enugu Metropolis and transported in sterile polythene bags to the laboratory. Direct plating on Saboraud Dextrose Agar (SDA) with chloramphenicol to suppress bacterial growth was used to isolate fungus from BG samples. The plates were kept at room temperature for 48-72 hours before being examined. Repeated sub-culturing was used to create pure fungus cultures. Aflatoxin quantification of the BG samples was carried out, after which aflatoxigenic analysis of the isolates was conducted using molecular techniques. A total of seventy-seven fungi were isolated. The percentage frequency of occurrence includes *Aspergillus flavus* (32%), *Culvulanaria* (30%), *Aspergillus niger* (12%), *Aspergillus fumigatus* (6%), *Penicillum spp.* (5%), *Colletotrichum spp*. (4%), *Rhizopus stolonifer* (3%), *Aspergillus orchaceous* (3%) and *Trichotothecium spp*. (3%), *Alternaria macrospora* and *Scolecosporae* (1% each). The aflatoxin analysis showed that the samples from the three markets were contaminated with aflatoxin above the permissible dosage of 20 ppb, with the least been 69 ppb (Abakpa) and the highest been 80 ppb (Ogbete). 25 isolates were sequenced from which 14 were identified as *Aspergillus flavus* and screened for aflatoxigencity. All 14 isolates were positive for aflatoxin regulatory gene. The study showed that the BG samples were contaminated with aflatoxin which made them unsafe for human consumption, hence there is need to enhance the knowledge of risk associated with the consumption of BG.

Keywords: Bambara groundnut, aflatoxin, PCR, *Aspergillus,* BLAST

Word Count: 300

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List of Acronyms

Abbreviation	Meaning
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immunodeficiency Syndrome
DNA	Deoxyribonucleic Acid
BG	Bambara Groundnut
CDC	Centers for Disease Control and Prevention
CDC	The center for Disease Control
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
FAO	Food and Agriculture Organization
IARC	International Agency for Research on Cancer
EU	European Union
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
RNA	Ribonucleic acid
tRNA	Transfer Ribonucleic acid
ZEN	Zearalenone
DON	Deoxynivalenol
UV	*Ultraviolet*
DAP	Days After Planting
MPa	Megapascal
µCT	X-ray micro-Computed Tomography
IITA	International Institute of Tropical Agriculture's
GRC	Genetic Resources Center
SSA	Sub-Saharan Africa
GEI	Genotype x Environment Interactions
QTL	A quantitative Trait Locus
EC	European Commission
LAB	Lactic Acid Bacteria

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AF	Aflatoxin
mitDNA	Mitochondrial DNA
ATP	Adenosine Triphosphate
FAD	Flavin Adenine Dinucleotide
NAD	Nicotinamide Adenine Dinucleotide
ADP	Adenosine Diphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
pRb	Percutaneous Renal biopsy
NF-Kb	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
CDK	Cyclin-Dependent Kinase
CKI	Casein Kinase 1
HCC	Hepatocellular Carcinoma
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
rRNA	Ribosomal Ribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
DNase	Deoxyribonuclease
RNase	Ribonuclease
PFGE	Pulsed Field Gel Electrophoresis
PAGE	*Polyacrylamide Gel Electrophoresis*
PCR	Polymerase Chain Reaction
dNTPs	Deoxynucleotide Triphosphates
pH	Potential of Hydrogen
DAF	DNA Amplification Fingerprinting
RAPD	Random Amplified Polymorphic DNA
REA	Restriction Endonuclease Analysis
RFLP	Restriction Fragment Length Polymorphism
WGS	*Whole Genome Sequencing*
AFLPs	Amplified Fragment Length Polymorphisms

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NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
GTR	The General Time-Reversible
SNP	Single Nucleotide Polymorphism
GUI	Graphical User Interface
MEGA	Molecular Evolutionary Genetics Analysis
iTOL	Interactive Tree of Life
USAID	United States Agency for International Development
PDA	Potato Dextrose Agar
ELISA	Enzyme-Linked Immunosorbent Assay
ppb	parts per billion.

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Chapter One

Introduction

1.1 Background to the Study

The use of agricultural food commodities has a number of downsides that have an impact on global food security[1]. Humans require nourishing food that contains necessary nutrients in order to maintain a healthy diet. Proteins are required for structure (growth, tissue repair), while lipids and carbohydrates are required for energy[2,3]. Cereals and legumes such as cowpea and Bambara groundnut, which are consumed throughout Africa, are high in these components. Foods (cereals, tubers, legumes, vegetables, etc.) can be contaminated by a variety of contaminants, including mycotoxins which are secondary metabolites capable of causing sickness and death in humans and animals[4]. Toxigenic molds of the genera *Aspergillus* (A), *Penicillium* (P), and *Fusarium* (F) produces mycotoxins[3].

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Toxic strains of *Aspergillus* species produce aflatoxins, which are carcinogenic secondary metabolites found in agricultural foods. Aflatoxins which are difuranocoumarin molecules produced through the polyketide pathway are highly stable and they can resist standard food processing[5]. Aflatoxins have been found in barley, wheat, sorghum pear millet, oilseeds, tree nuts, milk, and butter, among other foods. In both animals and humans, aflatoxins have been related to carcinogenicity, mutagenicity, teratogenicity, and immunological suppression, they are the most dangerous of all mycotoxins, inflicting damage to the human body[6]. Aflatoxins can also aggravate kwashiorkor and stunted growth in children**Error! Bookmark not defined.**. They can induce stunting in children by suppressing cell-mediated immunity[5]. A number of studies have found that aflatoxins hasten the progression of HIV infection to AIDS[7].

Aflatoxins can attach to cell DNA and alter protein synthesis, as well as contribute to thymic aplasia, which leads to a reduction in cell immunity[8].

Bambara groundnut (BG), *Vigna subterranea* is an underutilized, under-researched indigenous orphan crop produced across Sub-Saharan Africa with tremendous potential. It's drought-resistant and nutrient-dense. *Vigna subterranea* can be cultivated without the use of fertilizers, which are sometimes expensive and difficult to get[9]. In Nigeria, it is an essential leguminous crop, believed to have originated in Nigeria, particularly between Yola and Jos. It is varyingly referred to as Okpa in Igbo region of Nigeria; Kwaruru or Gurjiyain the Hausa region of Nigeria; and Epa-Roro in the Yoruba region of Nigeria[10]. Bambara groundnut is gaining popularity in a variety of food applications thanks to its favorable nutritional profile, particularly its high protein content. It is used as a supplement in cereal-based diets in numerous parts of Africa, particularly in weaning food for newborns and young children. Bambara groundnut, which grows near or under the soil, acts as inoculum of harmful fungi[1].

The most prevalent kind of BG product consumed in the West African sub-region is processed and cooked BG flour, also known as "okpa" in Nigeria's south east. It is consumed by millions of individuals of all ethnicities, religions, and socioeconomic classes in Nigeria, making it one of the most common meals for both the rich and the poor. In general, "okpa" is enjoyed by almost every Nigerian, and as a result, it enjoys widespread popularity among people of various ages, genders, and socioeconomic position. The patronage of "Okpa" is particularly prominent among travelers, students, and families[11].

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1.2 Statement of the Problem

Access to sufficient food for an active and healthy life defines food security, which is a critical factor for economic growth and development. Food insecurity ranks among developmental challenges[9]. Bambara groundnut is used to make different kinds of food delicacies cherished and consumed by a large number of people in Nigeria. However, seed quality, processing and handling methods have not been standardized resulting in products with varying quality and safety levels due largely to contamination by mycotoxins[11]. Aflatoxins have been linked with carcinogenicity, mutagenicity, teratogenicity, and immune suppression in animals and humans[12]. Previous research shows that they are the most hazardous of all mycotoxins, causing damage to the liver[6]. Aflatoxins also influence aggravation of kwashiorkor and impaired childhood growth, they can depress cell-mediated immunity and cause stunting in children[5,13]. Evidence from studies indicates that aflatoxins increase the rate of progression from HIV infection to AIDS[7]. Aflatoxins can bind with the DNA of cells and affect protein synthesis, and also contribute to thymic aplasia resulting in reduced cell immunity[8]. Consumption of food heavily contaminated with aflatoxin (> 20 ppb) has been linked to death[14]. The detection of aflatoxigenic *A. flavus* and aflatoxins in Bambara groundnut flour at levels above the maximum tolerable limits raises health concerns on its utilization in food applications, and in supplementary feeding for infants and young children[1].

1.3 Justification of the study.

Bambara groundnut is used to make different kinds of food delicacies cherished and consumed by a large number of people in Nigeria. Sadly, very little findings have been reported on fungal contamination of bambara groundnut and its safety for consumption. However, this molecular method has justified the presence of aflatoxin producing fungi

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in Bambara groundnut sold at Enugu State Metropolis. The fungal isolates produced aflatoxins beyond the maximum containment level for the toxins, hence not good for human health. The findings from this research will help to educate the public on the negative implications of aflatoxins synthesized by these pathogens so as prevent an epidemic outbreak in Enugu Metroplolis.

1.4 Aim and Objectives of the Study

The aim of the study is to identify the Aflatoxin producing fungi in Bambara groundnut using Molecular method.

The specific objectives of this study are:

1. Isolate the fungi associated with the Bambara groundnut purchased.
2. Identify and characterize the isolates using mycological method
3. Identify the Aflatoxin producing fungal spp using molecular method
4. Determine the Aflatoxin concentrations in the Bambara groundnut purchased

1.5 Significance of the Study

Due to food insecurity, poverty and lack of awareness of food contamination. Many people are exposed to food poisoning resulting from mycotoxins ingestion. Good hygiene in food handling and basic hygiene practices are often neglected by the populace. These to a greater extent have posed serious threat to human health. Despite the numerous contribution of Bambara groundnut to human diet and its economic importance, the plant has received little scientific attention. Hence, there is need to characterize the aflatoxigenic fungi present in it using molecular techniques thereby creating awareness of possible contamination by the fungi.

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1.6 Scope of the Study

Bambara groundnut was purchased from three markets in Enugu Metropolis, mycological examination of the Bambara groundnut was done followed by quantification of the Aflatoxin load of the Bambara ground. Finally, molecular identification of the Aflatoxin producing fungi isolated from the Bambara groundnut was carried out.

1.7 Limitation of the study

Few challenges encountered during the course of this research work includes non-availability of Bambara groundnut samples in Oyo state metrtopolis, which lead to the change in the study area, hence the study area was changed to Enugu metropolis and this involved travelling to the South Eastern part of the Nigeria for sample collection. The delay in the delivery of commercial kits for the Molecular Characteriziation and Aflatoxin assay from USA affected the time stipulated for the research work; hence, the completion of the research work was beyond the expected period.

1.8 Operational Definition of Terms

i. **Molecular Characterization**: The term molecular cgaracterization is this research work is a method used to determine the genetic characteristics of the fungi.

ii. **Parts per Billion (ppb)**: The term ppb is a ratio used to measure the concentrations of a contaminant per million unit of mass. It is also referre to as microgram per litre (μg/L)

iii. **Mycological Method:** This term refers to methods used in the study of fungi

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Chapter Two

Literature Review

2.1 Aflatoxin

Majority of agricultural produce are vulnerable to mycotoxins produced by some group of fungi. Among these are aflatoxins that cause numerous health challenges in humans, poultry birds and livestock. According to a report by International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), attention was drawn to aflatoxin contamination worldwide when peanut meal from Brazil fed to poultry killed thousands of birds in the United Kingdom. After proper investigation, it was discovered that the meal fed to the poultry birds was highly contaminated with aflatoxin[1].

Aflatoxin is a poisonous type of mycotoxins produced by mould fungi strains (*Aspergillus flavus*, *Aspergillus parasiticus*and *Aspergillus niger*) due to poor management of agricultural crops. Aflatoxin was dicovered in the 1960 but regulatory limits were not enforced until the 1980s. Aflatoxin which is carcinogenic in nature can cause sickness that can lead to several types of cancer in humans if consumed in sufficient quantities. Aflatoxin is well known to increase cases of hepatitis viruses' B and C, weakens the immune system and causes of retarded growth in children especially in the Africa region. Aflatoxin also affects animals and livestock, causing feed refusal, loss of weight, reduced egg production and milk contamination. According to Food and Agriculture Organization (FAO), 25% of crops globally are affected by aflatoxins and these toxins affects cereals and millets, oilseeds, spices, tree nuts, pulses figs, meats, dairy products and fruit juices. The center for Disease Control (CDC) has estimated that more than 4.5 billion people in developing countries are chronically exposed to aflatoxins in their diets[1].

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2.1.2 Types of Aflatoxins

Currently, eighteen types of aflatoxins have been identified but only six; AFB1, AFB2, AFG1, AFG2, AM1 and AM2 are common to agricultural produce[2]. The letter B shows that the aflatoxin type has blue fluorescence to ultraviolet light, while letter G shows the yellow-green flourescence. Aflatoxins exist between the temperature of 25˚C and 35˚C[3].

Aflatoxins have been classified by the International Agency for Research on Cancer (IARC) as a group 1 carcinogen.AFB1 was classified as a principal carcinogen by IARC. Aflatoxins M1 and M2 are hydoxylated products of the AFB1 and AFB2 respectively which are found in milk and dairy products. There are other derivatives of aflatoxins that have been identified as products of biotransformation of major metabolites in mammals. These are B2A, G2A, Q1 and P1. However, GM1, GM2, GM2A, B3, M2A and parasitol have been reported as other types of aflatoxins[2].

Aflatoxin B1 has been identified as the strongest hepatocarcinogenic agent known and most potent naturally formed carcinogen. This type of aflatoxin is the only mycotoxin regulated by the European Union (EU) with limits of 5ug/kg for livestock feeds and 20ug/kg for raw materials and other finished feeds[2]. Health hazard caused by aflatoxin B1 is dependent on the amount of dose consumed. At high doses, aflatoxin B1 can be lethal and cause a lot of damage to internal organs such as liver and kidney. The maximum amount of AFB1 in cereals, peanuts and dried fruit for human consumption in foods are 4ug/kg for total aflatoxins (AFB1, AFG1, AFB2, & AFG2) and 2ug/kg for AFB1[4].

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2.1.3 How Crops are contaminated with Aflatoxins

Crops are extremely susceptible to *Aspergillus* infection when exposed to high-humid environment for a prolonged period of time and also stressful conditions such as drought, a condition that lowers the barrier to entry. Fungi contamination can occur at any point of the crop value chain. There are three stages involved in the crop value chain and *Aspergillus* contamination can occur at any of these stages: a). preharvest entry/during plant growth b) entry of fungus during harvest and postharvest entry of fungus.

a). Preharvest Contamination

Aflatoxin contamination that happens in the field during plant growth is called preharvest contamination. Farming practices influence preharvest contamination of crops and makes them susceptible to infections. The following are the farming practices that influence the preharvest contamination of crops:

Repeated cultivation of host plants**:** Repeated cultivation of the same crop or susceptible crop species on the same plot of land promotes *A. flavus* population growth, eventually leading to preharvest contamination of crops in the field[6].

Late planting: Late-planted crops are more susceptible to end-of-season dryness and insect pest attacks, particularly termites. The fungus can easily enter pods that have been injured by insects[6].

Drought: Drought causes pods to fracture, allowing *Aspergillus* to enter and flourish.

Termite infestation and poor field hygiene: Using fields with a record of termite infestation creates an environment conducive to termite pod damage and ultimately

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fungus penetration. Termite and pest damage to growing pods is common in unweeded fields, increasing the risk of Aspergillus infection[6].

Plant density and poor water management: A low plant population and minimal ground cover causes soil erosion, loss of soil moisture, and degradation of soil structure, all of which can exacerbate drought impacts. These factors encourage the growth of *A. flavus* on the land[6].

b). Contamination During Harvest

The degree to which a crop is susceptible to infection is determined by how it is treated during harvest. The following are some of the factors that encourage fungal infection during harvesting:

Poor harvesting techniques**:** Groundnuts are commonly picked using hand hoes, which can easily break the nuts and provide easy entrance sites for the fungus. If dirt adheres to the pods of groundnuts and Bambara nuts, they might become infected with *Aspergillus* from the soil. Crops such as Maize, sorghum, millet, and sunflower, which are often picked and dried on the ground, are susceptible to fungus existing on the ground[6].

Premature harvesting: Immature crops have a high moisture content, which encourages fungal infection. Harvesting immature nuts raises the risk of fungus infection[6].

c). Postharvest Contamination

Premature harvesting: Premature harvesting increases fungal infection because immature crops have high moisture content. Immature nuts are more susceptible to fungal infestation when harvested[6].

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Improper drying: When grains are dried on rooftops or on the floor, they are exposed to moisture, which encourages fungal development[6].

Improper shelling: Methods such as spraying water on pods to soften the shells and increase the weight of the nuts to increase market value result in *Aspergillus flavus* infection and aflatoxin contamination. Threshing groundnuts in a sack is another bad practice[6].

Poor curing techniques: Extreme drying of nuts causes the pod and seed coat to fracture, exposing the nuts to aflatoxin contamination[6].

Poor stripping: Stripping groundnuts with dust transports the fungus into storage, creating an environment that is favorable to fungal infection and aflatoxin contamination.

Sorting: Poor grading, particularly of damaged nuts, is a cause of contamination before storage. Before storing, wounded, broken, shriveled, and cracked kernels must be removed from healthy kernels[6].

Poor storage conditions: Fungus development is aided by storing groundnuts with a high moisture content and poor storage (at the household level, market/shops) that exposes grains to winter rains, high humidity at night, and poor air circulation, all of which contribute to high temperatures[6].

Use of airtight containers: The use of non-porous nylon bags and other airtight storage materials reduces insect pest assault and, as a result, fungal infection.

Poor transportation: When grains are transported in cars with open roofs, they are exposed to unexpected rain and moisture, which can contribute to fungal growth[6].

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2.1.4 Control of Aflatoxin Contamination in Crops

Aflatoxin contamination and fungal development in crops are caused by a variety of reasons, some of which are environmental and others, which are related crop management. Farmers have very little influence over environmental conditions, but they may enhance crop production techniques to decrease fungal infection, growth, and aflatoxin formation[6].

To control aflatoxin contamination in the three stages of crops value chain, several factors should be considered[6].

Control of Aflatoxin Contamination of Crops during Preharvest

These techniques are good towards reducing fungal preharvest infection. They attempt to provide the greatest possible growing environment for crops in order to minimize insect infestation, drought, and fungal infection[6].

Early planting: Early planting allows plants to avoid the end-of-season dryness, which can cause pods to fracture and allow *A. flavus* to penetrate[6].

Maintaining field hygiene: Weeding on a regular basis helps to preserve soil moisture, which is necessary for optimal plant growth, as well as avoid dry conditions, which can cause growing pods to split. Termite management is also essential to avoid harm to growing pods, particularly as the crop matures[6].

Harvesting of water in the field: Drought condition when the crop is developing in the field is a prerequisite for fungal infection and subsequent aflatoxin contamination. Farmers must ensure that moisture is retained in their fields. Tied ridges (box ridges) can enhance water penetration into the soils, lowering the risk of A. flavus infection in the growing crop. Early in the cropping season, box ridges should be installed to catch

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adequate precipitation and prevent the impacts of end-of-season dryness. Mulching also aids in the retention of moisture in the field[6].

Soil amendments: Application of lime to the crop promotes the formation of hard shells (pod resistance). The first line of defense against insect and fungal attack is strong hard shells[6].

Control of Aflatoxin Contamination of Crops during Harvesting

These are management methods that limit or eliminate grain exposure to fungus, therefore minimizing or avoiding contamination of pods and grain[6].

Harvesting at the right stage: Premature kernels contain a high moisture content, which promotes fungal infection and development, as well as aflatoxin contamination. As a result, harvesting at the proper time when the crop is ripe reduces the crop's exposure to high heat, sudden rain, or drought, all of which might influence infection[6].

Avoiding pod injuries: When using hand hoes, take caution to avoid damaging the pods. To prevent damaging pods or kernels, proper harvesting requires that the hoe is focused on the plant's rooting zone[6].

Removal of soil: To avoid bringing the fungus into shops and processing facilities, it is critical to remove dust clinging to the pods during harvesting[6].

Control of Aflatoxin Contamination of Crops during Postharvest

Another important source of contamination is postharvest crop processing, which, if properly handled, will reduce fungal infection and aflatoxin contamination. There are two levels to which management techniques may be adopted[6].

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At the Household Level

Proper drying: Drying on a roof or in the soil absorbs moisture and promotes fungal development, which results in aflatoxin contamination. The use of Mandela Corks (ventilated stacking) is one of the best ways for curing groundnuts, and it is especially liked since it reduces the amount of time groundnuts are exposed to the sun directly. Mandela Corks should be placed on an elevated platform with a hollow gap in the centre for air circulation[6].

Proper shelling: sprinkling water on the pods to shell the groundnut leads to fungal growth and aflatoxin formation. As a result, it's best to prevent soaking pods when shelling. Mechanical shellers can also help you save money on labor[6].

Grading and sorting: Fungi are easily attracted to kernels with broken or damaged pods or seed coats. The amount of diseased product in the lot is reduced by removing such damaged, discolored, undersized, and shrivelled pods[6].

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Figure 2.1: Mandela Corks Support Slow Curing of Harvested Groundnuts. Source: ICRISAT, 2016

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Proper storage: Insects and moisture can introduce fungus into storage lots, resulting in aflatoxin contamination. Grains should be kept in a dry, safe location where insects and moisture cannot get in[6].

At the Processors' Level

Sorting before shelling: Aflatoxin contamination is more likely in loose shelled kernels, shriveled, broken, or discolored pods than in big, mature, and well-filled pods. Sorting alone can help to keep contamination under control and below acceptable limits[6].

Grading after shelling: After shelling, all kernels must be sorted into different grade groups depending on size, and it is preferable to use electronic color sorters to remove discolored, damaged, and shrivelled kernels[6].

Avoid using grade-outs: Grade-outs often contain greater levels of pollutants and should not be consumed or fed to animals. Consuming grade-outs with high aflatoxin levels exposes humans and animals to dangerously high quantities of aflatoxin, which can cause mortality[6].

2.2 Other Mycotoxins

2.2.1 Ochratoxin A

Ochratoxins: Ochratoxins are a group of mycotoxins produced by as secondary metabolites by a group of fungi primarily *Aspergillus ochraceus* and *Penicillium verrucosum*[6]. Other isolates such as *Aspergillus carbonarius*, *Aspergillus niger*, *Neopetromyces* spp. and *petromyces* spp. produce ochratoxins[7]. Ochratoxins can have a chlorine atom present in their structure and can naturally be found in various

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agricultural produce such as cerels, coffee, beans, cocoa, and nuts. Common types in this group are OTA, OTB, OTC, methyl ester of OTA and ethyl ester of OTB. Ochratoxin A (OTA) is the most common and is stable when exposed to heat and affects protein, DNA and RNA synthesis in the body[8]. Ochratoxin A has shown to be carcinogenic, tetretogenic, immunotoxic, and neurotoxic properties. It has been recognised as a cause of nephropathy in humans[3].The toxicity of OTA is exhibited through various mechanisms. An example is the phynlalanine-tRNA-catalysing reaction which inhibits synthesis of protein by competing with phenylalanine and the DNA adducts formed interfere with DNA repair system and control system of the cell cycle thereby initiating carcinogenesis. Ochratoxin A contaminates various crops and feeds[7]. European Union regulatory limits for OTA differ according to food type. A limit of 5ug/kg is regulated in unprocessed cereals and 3ug/kg in cereal-based processed foods. A study conducted in Portugal shows that OTA was detected in 59% of 601 samples of cereals and cereal based products analyzed, at concentrations of 0.02 to 7.97ug/kg with four of the samples having values above the accepted limits[2].

2.2.2 Fusarium Toxins

Fusarium toxins are the dominant contaminant in temperate regions and corn is the most affected crop. It can also be found in grains and malt brewing[3]. Fusarium toxins can be grouped as follows:

Estrogenic Toxins: Estrogenic toxins which contains lactone structure such as zearalenone and zearalenol can have the lactone ring-OH group formed at such a position that it can interact with the estrogen receptor[3].

Zearalenone: As a secondary metabolite, *Fusarium* species, particularly *F. graminearum* and *F. culmorum*, generate zearalenone (ZEN)[8]. Zearalenone has been

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found in a variety of cereals and animal feeds all over the world. It's harmful to the liver, hematotoxic, genotoxic, and immune system. It has the ability to connect to estrogen receptors and has a substantial impact on the reproductive system, as well as the potential to induce serious reproductive problems[2]. It has been related to the early onset of puberty in young children and has been suggested as a possible promoter of human breast carcinogenesis[7]. Zearalenone exposure in pigs can result in vaginal and/or rectal prolapse, vulvovaginitis, abortion, and infertility[9]. To regulate the presence of this fungal toxin in foods and feeds and reduce its harmful effects on human and animal health, regular monitoring is required[2].

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Non-Estrogenic Toxins: Deoxynivalenol, nivalenol, T-2, and HT-2, as well as diacetoxyscirpenol, are non-estrogenic toxins known as trichothecenes. The most significant and prevalent toxin among the trichothecenes is deoxynivalenol (also known as "vomitoxin"), which is extremely stable in technological treatments. T-2 toxin is generated in cold environments[3].

Deoxynivalenol: Deoxynivalenol (DON) is a trichothecene found in grains such as rye, barley, and wheat[9]. It is mostly produced by *Fusarium. graminearum* and inhibits RNA, DNA, and protein production. It causes genotoxicity, cytotoxicity, teratogenicity, and foetal skeletal abnormalities in experimental animals. Animals ingesting DON24 contaminated foods at higher levels have vomiting, diarrhoea, and feed refusal, resulting in significant weight loss, hematological system damage, and immunological dysregulation[9]. In low dosages, it induces sickness and feed rejection in pigs and other animals[9]. Although deoxynivalenol's concentration can be decreased by boiling in water, it is extremely heat resistant[7].

2.2.3 Fumonisins

Fumonisins are nephrotoxic and hepatotoxic mycotoxins that have been found to have significant cancer-inducing characteristics. Fumonisins are a kind of fumonisin that causes neural tube abnormalities in children. Oesophageal cancer has been related to experimental animal species and their prevalence in Maize grains in the Transkei area of Southern Africa, China, and North-Eastern Italy[7]. According to the International Agency for Research on Cancer, fumonisin has immunosuppressive properties and has been classed as a category 2B carcinogen (possibly in humans)[10]. Fumonisins are a kind of fumonisin produced by Fusarium molds[3]. Mycotoxins belonging to the fumonisin group include B1 (FB1, FB2, FB3, and FB4), A1, A2, A3, AK1, C1, C3, C4, P1, P2, P3, PH1a, and PH1b.The fumonisin B1 produced by *Fusarium verticilloides* and *Fusarium proliferatum* is the most significant. These fungi are common Maize pollutants, and their poisons have been linked to esophageal and liver cancer, neurological abnormalities, and poisoning[3].

The most widely researched fumonisin is B1, a diester of propane 1,2,3-tricarballylic acid and 2-amino-12, 16 dimethyl-3,5,10,14,15-pentahydroxycosane. Some *Fusarium* species generate fumonisins[7]. *Fusarium proliferatum*, *Fusarium verticillioides* (formerly classed as *Fusarium moniliforme*), and *Fusarium nygamai* are the main manufacturers of fumonisins[8]. *F. polyphialidicum*, *F. anthophilum*, *F. dlamini*, *F. napiforme*, *F. subglutinans*, and *F. oxysporum*are among the other producers. Fumonisins, particularly FB2, have been reported to be produced by *Aspergillus niger*[7,11]. Beer, rice, corn, herbal tea, bovine milk, medicinal herbs, figs, and other agricultural commodities have all been discovered to contain fumonisins[7]. FB1 was found in corn meal, Maize flour, popcorn, cornflakes, and polenta[12]. Human daily fumonisin consumption should be kept below 1µg/kg body weight per day, according to early evaluation results[11].

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2.2.4 Patulin

Patulin was discovered as an antibacterial ingredient in the plant P. patulum, which is now known as *P. griseofulvum*[2]. Patulin is a mycotoxin produced by several *Penicillium*, *Aspergillus*, and *Byssochylamys* species[13,14]. *A. clavatus*, *A. giganteus*, and *A. terreus* are the species that produce patulin. Its toxicity to animals and plants was found in the 1960s, leading to its reclassification as a recognized mycotoxin. It was originally used to treat common colds and skin diseases[11]. Apple, pear, cherry, grapes, cereal grains, silage, and other fruits are affected by *Penicillium expansum*, a natural and efficient producer of patulin. Apple and apple products have the highest prevalence[3,13,14].

2.2.5 Citrinin

Citrinin is a mycotoxin produced by *Aspergillus*, *Penicillium*, and *Monascus* species. Despite being classified as antibiotics, antifungal and bacteriostatic, they were later cited for swine neuropathy in numerous European nations. They've been found in a variety of foods, including cereals, fruits, cheeses, acorns, nuts, carrots, tomatoes, and meat. Citrinin possesses mutagenic and nephrotoxic properties[3].

2.2.6 Sterigmatocystin

A. versicolor produces this mycotoxin, which is a precursor to AFB1, AFG2, and AFG1. sterigmatocystin is in relation to cancers of the stomach, liver, and esophagus. Cereals, coffee, ham, pepper, and cheese have all been shown to contain them[3].

2.2.7 Ergot Alkaloids

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The clavines and lysergic acid alkaloids are ergot alkaloids that are indole alkaloids produced from a tetracyclic ergoline ring structure[2]. The fundamental structure of clavines is ergoline, whereas lysergic acid alkaloids such as ergotamine and ergine make up the lysergic acid alkaloids[2,15]. These compounds are produced in the sclerotia of *Claviceps* species that cause infections in grass plants, most notably infecting rye grains, wheat, barley, millet, and oats[3]. 2014 Ergotism, also known as St. Anthony's fire, is caused by eating cereals contaminated with ergot sclerotia[2]. Ergotism can be gangrenous, affecting blood flow to the body's extremities, or convulsive, affecting the central nervous system[15]. Ergotism causes miscarriage, convulsions, gangrene, lactation suppression, hypersensitivity, and ataxia in sensitive animals such as cattle, ovine species, pigs, and birds[2].

Human ergotism cases were documented in Europe throughout the middle Ages as a result of bread produced from tainted grain[2, 15]. Because contemporary techniques of grain washing and processing in mills leave relatively low amounts of the toxin, human ergotism is less frequent nowadays. Heat labile ergot alkaloids are also eliminated during bread manufacturing.

2.2.8 Masked mycotoxins

The natural poisons in food contaminated with mycotoxins may have structurally similar chemicals produced by plant metabolism or food processing that co-exist with them. These are referred to as Masked or conjugated mycotoxins[2, 16]. Plant enzymes, particularly those engaged in detoxification, act as catalysts for chemical reactions that produce masked mycotoxins[17]. They are undetectable using regular analytical techniques because their molecules display variations in physicochemical characteristics that affect their chromatographic behavior[17]. These masked mycotoxins

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are hydrolyzed in the digestive tracts of animals to their respective precursors, which may or may not have comparable harmful effects as their parent compounds[16]. Masked mycotoxins are divided into two types: extractable conjugated mycotoxins that can be detected using appropriate analytical methods, and bound (non-extractable) mycotoxins that are not directly accessible and must be liberated from the matrix using chemical or enzymatic treatment before being analyzed. Deoxynivalenol, nivalenol, fusarenon-X, T-2 toxin, HT-2 toxin, zearalenone, ochratoxin A, destruxins, and fusaric acid have all been reported as plant metabolites[17]. Naturally contaminated grains such as wheat, barley, and Maize have been shown to include zearalenone-14—D-glucopyranoside (Z14G) and deoxynivalenol-3—D-glucopyranoside (D3G). Infected veggies have been shown to contain fusaric acid methylamide. Raw Maize and cereal-derived foods have been found to contain bound fumonisin[17].

Although data on the recovery of masked mycotoxins in Bambara groundnut is limited, the discovery of masked mycotoxins in Maize raises concerns about its use in Maize-Bambara composite flour. Because masked mycotoxins are produced during plant metabolism or food processing and may or may not have the same harmful effects as their parent compounds, more research into identifying masked mycotoxins in Maize-Bambara composite flour could be critical in reducing mycotoxin contamination in seeds[2].

2.3 Bambara Groundnut

The Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is an indigenous African crop that is widely grown in other parts of the world.[2,18]. Its origins may be traced to West Africa's Sahelian area, and the name appears to be originated from the Bambara tribe, which today resides mostly in Mali[19]. The Bambara plant, like groundnut (*Arachis*

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hypogea) and cowpea (*Vigna unguiculata*), belongs to the *Leguminosae* family and produces edible seeds[19].

Figure 2.2: Bambara Groundnut
Source: Future Food, 2021

Bambara groundnut (*Vigna subterranea*) is an annual, creeping legume with glabrous, trifoliate leaves that, like the peanut, ripens its pods underground (also called groundnut). It possesses a taproot that is encircled by many lateral roots that contain nitrogen-fixing nodules[20]. Flowers appear in the form of papilionaceous racemes. Fruits are two or three seeded subterranean pods that are 1.5 to 3cm long, wrinkled, and indehiscent at first. When mature and dried, pods dehisce[21].

When dried, the seeds are spherical, smooth, and very firm. The mature and dried seed is firm, smooth, and spherical, with a diameter of up to 1.5cm. The seeds come in a variety of colors, including cream/white, dark brown, red, black, and speckled, spotted, or patterned with a mix of these colors[21].

Despite its importance in many Africans' daily diets, Bambara groundnut growth has been low, and it is considered as a famine culture crop in semi-arid Africa, particularly among farmers[18]. It is said to be largely farmed by rural farming women, with approximately 10-40% of the crop sold and the remainder consumed domestically[18]. Annual output is over 330, 000 tons worldwide, with Africa accounting for half of the total and Nigeria leading the way[2,18].

Bambara groundnut is grown for its edible seed, which provides a significant source of nutritional protein and calories. For many Africans, it is one of the five most significant protein sources[19]. The proper usage of Bambara groundnut is hampered by a number of

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reasons. It is difficult to dehull and grind, is difficult to prepare, has a strong beany flavor, and contains antinutrients[22].

Crop improvement and large-scale cultivation have been ignored due to a lack of research. Despite these difficulties, most farmers in Swaziland believe Bambara groundnut to be a lucrative crop[18]. This is due to its several advantages over other legumes, including drought and pest tolerance, long storage life, and capacity to yield relatively well on poor soils[19]. Bambara groundnut produces better yields in conditions that are too dry for other crops including groundnut, Maize, and sorghum[2].

Bambara groundnut does not require fertilizers or chemicals to grow[23]. Because of the thick seed coat, the nut may withstand weevil assault and be stored for long periods of time without losing quality[2]. These features have reignited interest in Bambara groundnut growing in the dry savannah zone[19]. It also has a role in international trade, since it is exported from Ghana, Burkina Faso, Cameroon, Zimbabwe, and Madagascar to African markets in Western Europe and North America[23].

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2.3.1 Common Names of Bambara Groundnut

Bambara groundnut is known by different names in Africa and other continents where it is being cultivated. In different regions of the world, BG is known by a variety of distinct names. The nut is known by several ethnic groups in Nigeria by different names. The nut is known as ‘okpa' in the Igbo language, which is spoken by the majority of Nigerians in the south-east sub-region. BG is referred to as ‘gurjiya' or ‘kwaruru' in the Hausa language, which is spoken by the majority of people in the north. In the Goemai language, spoken by the Plateau people in the northern Nigeria, the nut is known as ‘kwam’; while Kanuri people primarily located in Borno State named it ‘ngamgala’.Bambara Groundnut is commonly known as "epa-kuta or Epa ororo"

among Yoruba people in the south-west of Nigeria[2, 21]. Other popular names for Bambara groundnut are Bambara bean, congo goober, earth pea, stone groundnut, ground bean, or hog-peanut[2].

Bambara Groundnut is also known as jugo beans in South Africa, ‘ntoyo cibemba', ‘katoyo', or ‘mbwiila' (chitonga) in the Republic of Zambia, ‘njugumawe' in Swahili, ‘tindluwa' in Shangaan, and ‘voanjobory' in Malagasy, the present-day Madagascar. The nut is called 'nyimo' in the Shona language of Zimbabwe, and 'indlubu' in the Ndebele language. The nut is known as ‘akwei' among the inhabitants of Greater Accra, Ghana, and azi nogui, which translates as round peanut in the Ghanaian Ewe language. The nut is known in France as pois Bambara or voandzou, and in English as stone groundnut[21].

Because the nut is extensively produced in Bogor, West Java, throughout Asia, notably in Indonesia, BG is known as ‘kacang Bogor,' which literally means Bogor peanut. There are several more local names for Bambara Groundnut, some of them are related on the place where the nut was produced[21].

2.3.2 Scientific Classification of Bambara Groundnut

The edible leguminous vegetable crop Bambara groundnut belongs to the kingdom *Plantae*, order *Fabales*, family *Fabaceae*, genus *Vigna*, and species *Vigna subterranea*. Although *Vigna subterranea* is the scientific name for Bambara groundnut, it is also known by the names *Arachis Africana*, *Burm. F.*, *Glycine subterranea* L., *Voandzeia subterranea* (L.) *Thouars*, and *Voandzeia subterranea* (L.) DC[21].

2.3.3 Plant Morphology of Bambara Groundnut

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Bambara groundnut is a self-pollinated annual plant. With many nitrogen-fixing nodules, the stem and tap root are well-developed. The stem bears trifoliate leaves with long petioles and a green or purple base. Flowers are borne in a raceme on the stem's nodes[18, 19]. The seeds are found in single or many pods that are broad, spherical, and wrinkled. After fertilization, the flower stem bends downwards, forcing the young developing pod into the soil or slightly above the ground to develop and mature, similar to groundnut[19].

The plants are manually taken out of the ground during harvest when the pods have matured for about four months after planting[18]. There are seven different kinds of BG seed cultivated across the continent[2]. Bambara groundnut seeds are smooth and very hard when dried with differences in the seed coat, size, and form. White, cream, dark-brown, red, and black seeds are common, and some seeds are speckled or patterned with various color combinations. Some round or elliptical-shaped seeds have been observed to have different seed shapes[2]. Despite these differences, the nutritional content of the various cultivars does not differ much[18].

The black seed (early maturity, small to medium size kernels, mainly one seeded); the red seed (late maturity, large kernels, good yielder, and prone to rotting onsite); the cream/black eye seed (a large kernel and a good yielder); the cream/brown eye seed (a large moderate kernel and a good yielder); the cream/white no eye seed (very small pods and kernel; mainly produces one seed and yields a small amount); the cream (purple colour predominates, kernels are small and pods are mainly one-seeded)[21]. Cream/white and red or brown seeds are the two most common Bambara Groundnut types produced in Nigeria. Because of their excellent yield and better taste (sweet taste) and flavor when processed as food, less tannin, shorter time to cook, and beautiful color, the cream/white seeds are more sought after or accepted among the

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locals. Red seeds are said to be beneficial in places where iron shortage is a concern, as they contain nearly twice as much iron as cream seeds. For example, large seeds are favored over smaller seeds as snacks; smaller seeds are crushed into flour for use in other delicacies[21].

Red seeds are more popular than cream/white seeds in Zimbabwe, where they command a high price with the Grain Marketing Board. Unlike in Nigeria and Ghana, where cream/white Bambara Groundnut seeds are more sought after and command up to a 10% premium, red seeds are more popular than cream/white seeds in Zimbabwe, where they command a high price with the Grain Marketing Board[21].

2.3.4 Nutritional Value of Bambara Groundnut

The Bambara groundnut is a nutrient-dense grain legume that provides growth and development with important elements. It is commonly used to replace animal protein in households that cannot afford such sources[23]. The seed is a highly nutritious meal for humans and animals, comprising 54.5–69.3% carbohydrate, 17–24.6 percent protein, and 5.3–7.8% fat, and providing 367–414 calories per 100kg. Other popular pulses such as cowpea (*Vigna unguiculata*), lentil, and pigeon pea (*Cajanus cajan*) have a higher gross energy value than Bambara Groundnut seed[24]. The carbohydrate component of BG seed is mostly made up of starch and non-starch polysaccharides, with a smaller percentage of reducing and non-reducing sugars[18]. The nutrients thiamine, riboflavin, niacin, and carotene are also found in the nut, although it is minimal in ascorbic acid[25]. The high protein content of Bambara groundnut can be employed to improve the nutritional profile of a variety of food compositions. In terms of essential amino acid needs for children, Bambara groundnut protein, like most other legumes, lacks sulfur-containing amino acids (cysteine and methionine) and tryptophan[21].

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The mineral content of raw BG includes sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), phosphorus (P), manganese (Mn), and copper (Cu)[25]. Calcium is necessary for blood clotting, whereas iron is said to be critical for the proper functioning of the central nervous system[26].

Anti-nutritional elements such as trypsin inhibitors, oxalate phytates (phytic acid), and tannins (tannic acid), especially condensed tannins in the seed coat, are present in Bambara Groundnut seeds, as they are in most legume seeds. Tannin concentration is proportional to coat color, with cream-colored seeds having fewer tannins than brown or black seeds. Tannins can be harmful to livestock performance, although they can also be useful nutritionally in rare instances (by-pass protein in ruminants, anti-helminthic effect)[21].

Antinutritional factors in legumes have long been recognized to bind to nutritious components, rendering them inaccessible for digestion fully or partially. Anti-herbivory factors can vary from basic protease inhibitors to significant toxins, such as the amino acid -N-oxalyll-,-diaminopropionic acid (ODAP) found in grasspea (*Lathyrus sativus*), which can cause paralysis if ingested as the sole source of food during times of scarcity[27].

Dehulling, soaking, cooking, fermentation, germination, and others might potentially decrease antinutritional components and increase bioavailability[28, 29]. However, considering the antinutritional characteristics found in most minor crops, bioavailability is a key caveat for any promotion of underutilized foods. Further research is needed to guarantee that the nutritional status of individuals is really improved via the usage of nutrient dense minor crops[30].

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The antitryptic activity of Bambara groundnut seeds is higher than that of soybean seeds, and the intensity of effectiveness varies depending on the variety. Trypsin inhibitors are frequently destroyed by heat treatments such as cooking and roasting. Phytates are abundant in Bambara Groundnut seeds and are known to decrease cation availability (especially calcium)[21]. Cooking or roasting, as well as other types of processing (such as soaking, milling, dehulling, germination, and fermentation), were found to be beneficial in lowering the concentration of these anti-nutritional components. However, seed processing does not necessarily result in a higher feeding value[21].

2.3.5 Bambara Groundnut Processing Method and Utilization

Bambara groundnut is used in a variety of ways. Okpa, a steaming gel made from flour, is popular in Nigeria. It's made by combining Bambara groundnut paste with seasonings, wrapping it in banana leaves, and boiling it[19]. The antibacterial characteristics of polyphenols in banana leaves may be responsible for Okpa's ability to stay for several days at room temperature without spoiling[31]. Alternatively, Bambara groundnut flour may be made into a variety of different delicacies, such as ‘utara okpa' (a stiff paste made by stirring Bambara flour in boiling water) which is served with a variety of traditional and regional soups, including ogbono-soup, also known as draw-soup. The Nsukka people of Nigeria's south-east enjoy 'Utara okpa.' The flour can also be made into bean-balls known as 'akara' in Nigeria, or flat cakes known as 'owuna.' Both 'akara' and 'owuna' are fried Bambara Groundnut flour paste items made with palm oil or vegetable oil[32]. The Yorubas and Igbos, particularly the Nsukka people of the Igbos, enjoy ‘owuna' as a delicacy[21].

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Figure 2.3: Bambara Groundnut and okpa
Source: Udomoh Eshemokha, 2019

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The seed is fermented to make a condiment similar to dawadawa[33,34]. To obtain oil, certain Congolese communities roast and pound the seed[19]. The seeds can be eaten raw or roasted while still developing. Boiling fresh pods with salt and pepper makes a tasty snack. Immature seeds are also cooked in pods, salted, and eaten with or without Maize seeds in Botswana[19]. Cakes and porridges are made from crushed dry seeds that have been pounded into flour[18]. When the seeds develop, they become hard and must be cooked before being used[23]. Bambara groundnuts are roasted and pulverized into a soup, which can be served with or without seasonings. Cote d'Ivoire produces Bambara groundnut flour, which is used to make bread and cakes in Zambia and Botswana, respectively[19].

In some parts of Malawi, Bambara groundnut is served alongside nsima, a traditional meal prepared from Maize flour and water[23]. Sekome, also known as tihove or tshidzimba in South Africa, is made by combining Bambara groundnut, peanut, or both to a Maize or millet meal, which is then cooked to make stiff dough that is salted and crushed into a ball. It lasts for several days in the refrigerator. In South Africa, the dried beans are also used to make soup by the local white population[18].

Fresh boiled and salted immature beans are consumed in Ghana, while boiled and crushed dry seeds are used to make fried cakes and balls, which are subsequently utilized in stew recipes[18]. Porridge is made with overnight-soaked beans that have been cooked with or without capsicum pepper and salt, and eaten with roasted and grated cassava or mashed, fried, or ripe plantain. Green and dry beans are dehulled and cooked until they are quite soft in Kenya. It's mashed, then boiled and smoothed with coconut liquid. It's served with rice or Maize meal porridge[18].

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Mashed boiling seeds, often combined with cooked sweet potatoes, are a favorite children's meal. In the early 1960s, canned Bambara groundnut in tomato sauce and brine was also claimed to have been made in Ghana[18]. Bambara groundnut may also be used to make animal feed. An investigation was done on fermentation of Bambara Groundnut with *Aspergillus niger*, *Aspergillus flavus*, *Trichoderma sp.*, *Yeast sp.*, and *Penicillium sp*. to enhance protein content and bioavailability as an element in animal feedstuff[2].

The seeds are used to create poultry feed, while the leaves are good for herbivores since they are high in nitrogen and phosphorus. Bambara groundnuts are utilized for therapeutic purposes in various regions of Africa. In Botswana, for example, the seeds are utilized in traditional medicine[18]. Water from cooked Bambara peanuts and Maize is used to cure diarrhoea in Kenya. When crushed and combined with water, the leaves of Bambara groundnut and nyamrithi *(Lantana trifolia* L.) are used as a pesticide on vegetable crops and to spray on animals to avoid ticks[2]. In the treatment of oral illness in cattle, dried leaves pounded with traditional salt are employed[18].

Bambara groundnut has been researched for usage in a variety of foods. To make plain yoghurt, milk from Bambara groundnut and soybeans was fermented with *Lactobacillus delbrueckii* subsp *bulgaricus* and *Streptococcus salivarus* subsp. Thermophilus[35]. Because of its capacity to enhance the protein content and functional characteristics of cereal-based flour, it is recommended as a supplement in the manufacturing of biscuits, bread, and weaning food for newborns. With enhanced functional characteristics, infant supplemental food has been made from sprouted and fermented millet and Bambara groundnut flour[36]. Researchers found that bread prepared from a composite flour of Bambara groundnut and wheat flour had greater crude protein, crude fiber, and ash content[37].

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2.3.6 Bambara Groundnut Post Harvest Processing

Dehulling or Shelling: Indigenous technologies are used in the shelling and dehulling processes. Over drying of pods results in poor shelling output since pods are dried mostly in the sun. To shatter the dried pods, most rural populations use a mortar and pestle. The pods are stacked on the ground and trampled with feet in some communities, while in others they are put inside jute or hessian bags and pounded with sticks on level surfaces. Other techniques include cracking the pods on a flat surface using stone[38].

Winnowing: After the shelling procedure, clean seeds are obtained by winnowing the hull from the seed-hull combination. The seed-hull combination of the shelled material is dropped from a specific height, against the wind. The wind blows away the lighter particles or hull, while the somewhat heavier seeds gather in a container put on the ground below the height[38].

Cooking or Roasting: Dehulled seeds are usually roasted in a roaster at 140°C for 20 minutes. The seeds are roasted and cooled before being processed into flour and stored for further use[38].

Fermentation: Clean entire Bambara groundnut seeds are steeped in water in a 1:3 ratios and left to ferment for 3 days at room temperature. The water is decanted after fermentation, and the seeds are cleaned and dehulled by pressing between the fingers. Cotyledons are cleaned in numerous water rinses before being dried in a convection oven at 65°C for 8 hours. After cooling, the seeds are ground into flour, sealed in plastic bags, and kept until needed[38, 39].

Milling: To make Bambara groundnut flour, dehulled seeds are crushed in a coarse grinder, and the material obtained after grinding is sieved. After that, the flour is sealed in plastic bags and kept until needed[38].

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Storage: The Bambara groundnut is commonly stored both shelled and unshelled. When there is a little quantity of Bambara groundnut, the unshelled pods are usually stored in pots. Granaries hold large quantities of Bambara groundnut. To reduce the infestation of pests and insects, the product is sometimes treated with pesticides and then kept in bags, plastic/metal drums, and other suitable containers[38]. Dried seeds can also be kept in jute/wool bags, rums, tanks, tins, silos, bottles, cans (Bambara groundnut seeds have been canned in Ghana and Zimbabwe), gourds or calabashes, which are often sealed with mud, and clay pots[40]. Some seeds are carried to marketplaces where they are stacked in hundreds or thousands for sale, while others are kept in warehouses, residences, or on the farm for food security and later use[21].

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Figure 2.4: Bambara Groundnut stored in pots
Source: Cape Peninsula University of Technology, 2021

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2.3.7 Bambara Groundnut Product Development

Bambara groundnut contains an ideal combination of high-quality protein and other well-balanced nutrients (carbohydrate, fat, and mineral content), making it suitable for use in a variety of food compositions[41]. The immature seeds of the Bambara groundnut are eaten fresh, cooked, or grilled, and are occasionally combined with groundnut or Maize. The seeds are roasted, cooked, or ground into flour once they have ripened. The seeds or milled flour are then consumed on their own or in conjunction with Maize[42, 43]. In certain regions of Africa, roasted Bambara groundnuts are served as a salty snack. In Africa, the cooked seeds are also canned and they have a high market value[42, 44]. The following are some of the goods made from Bambara groundnut.

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Figure 2.5: Products from Bambara Groundnut
Source: Ismaila Muhammad *et. al.*, 2021

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Nasima: The flour of dehulled Bambara groundnut seeds is used to produce "nasima," a thick, sticky porridge[45].

Chipele: The Bambara groundnut seeds are roasted first and then boiled. After that, the cooked seeds are crushed and consumed as a delicacy[45].

Supplementation in a Variety of Foods: Bread, confectionaries, and pasta can all benefit from the usage of legumes like Bambara groundnut as a wheat replacement[45,46]. They may also be used as a supplement in cereals for a variety of food items such as bread, porridge, bakery, and extrusion goods, as the amino acid profile of legume proteins is similar to that of cereals[47]. Bread made with a mixture of Bambara groundnut flour (up to 50% Bambara flour) and wheat flour had greater protein content and was well received by customers[37.]

Tubani, Okara, and Moin Moin: Bambara flour, which is rich in protein, is used to make pancakes, soups, and purees. Traditional Ghanaian dishes like "akla" (also known as koose) and "tubani" are made using Bambara groundnut flour paste, which is steamed or fried. It's also made into a bean fritter known as "okara" and a savory pudding known as "moin moin." To make "okpa," a doughy paste of Bambara flour is steamed or boiled[42,45].

Milk from Bambara Groudnut: Bambara groundnut milk is a high-nutrient, consumer-acceptable, and tasty product[24]. Bambara Groundnut are soaked overnight, then dehulled and resoaked for 24 hours. Soaked beans are homogenized with hot water, then strained through muslin to get raw milk. The resulting milk has a better acceptance rating than pigeon pea, soybean, or cowpea milk, and its lighter colour is well valued. It also has a lower concentration of trypsin inhibitor than other legume milks, allowing it to be used in a wider range of culinary applications[38]. Furthermore, Bambara groundnut

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milk has excellent emulsion stability, and curds made from soymilk may be readily made from Bambara groundnut milk. Consumers have showed a high level of acceptance for Bambara groundnut milk fermented with lactic acid bacteria[45].

Consumer acceptance of extruded meat analogs or texturized vegetable proteins produced from Bambara groundnut flour has been high. It could be a good source of cheap protein to replace some of the meat proteins in your diet, and it can also be used to replace minced meat in a variety of recipes. Extruded foods are lower in fat and sodium, and they contain significant amounts of dietary fiber[43,45]. Consumers have also shown interest in extruded snacks made from a combination of Bambara groundnut flour, cassava starch, and corn bran flour.

Production of Bambara Groundnut Flour: All foreign elements, including as dust, dirt, tiny branches, and immature seeds, are removed from the Bambara groundnut seeds during sorting and cleaning. The seed coats of the chosen seeds are separated from the cotyledons and winnowed off. The cotyledons are hammer milled and then sieved to produce fine flour[21].

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2.3.8 Advancement in Bambara Groundnut Processing and Product Development

Because the seeds of Bambara groundnut are particularly hard and take longer to cook commercial processing is limited[45]. Antinutritional factors and dehulling are additional important problems of Bambara groundnut for culinary applications, in addition to the longer cooking time[48].

The current pre-treatment approach for improving the nutritional quality of Bambara groundnut has to be enhanced further. It's also crucial to look into efficient, cost-effective, and environmentally friendly pre-treatment methods for Bambara groundnut

processing. Furthermore, the development of appropriate post-harvest machinery, such as a Bambara groundnut sheller and a solar dryer for drying pods, as well as proper storage facilities, such as the use of a silo bin, will relieve processors of the tedium of current methods, reduce seed damage, and prevent mold, aflatoxin, and other microorganism attacks. Sulfur-containing amino acids are absent in Bambara groundnut[38]. As a result, combining Bambara groundnut with a staple food like Maize, which contains higher levels of cysteine and methionine, is a nutritional strategy that is recommended[38]. Plant-based protein sources are generally thought to be of lower quality, which limits their use in various food products and formulations. However, for environmental, economic, and religious reasons, isolated plant proteins are increasingly being used instead of animal proteins[49]. However, the ability of synthetic proteins to partially or completely replace animal proteins in diverse dietary applications is dependent on their functioning. Despite the fact that Bambara groundnut proteins have lower functional properties (foaming, emulsification, and gelling) than egg white and soy proteins, succinylation and acetylation have shown promising results in improving the foaming, emulsifying, and solubility properties of Bambara proteins[50]. However, further study is needed to confirm the Bambara proteins' functional relevance in model food systems[38]. Bambara groundnuts have the potential to replace soybean in animal feed (fish, rabbits, dairy cattle, and so on); however, further research is needed to determine the recommended quantity that these farm animals can endure without compromising their performance[46]. Pre-treated Bambara can be used in chicken and pig diets since it contains the least level of antinutritional elements[48].

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2.3.9 Evaluation of Important Traits

Bambara groundnut as a drought-resistant crop: Plants respond to drought in a variety of ways, both above and below ground. Because of these responses, drought resistant plants can avoid escape orwithstand drought[30].

1. Avoidance: this is most typical in plants and is triggered by intermittent stress. Plants' capacity to sustain essentially normal physiological functions during mild, moderate, and intermittent drought stress is referred to as drought tolerance. Plants use an extensive and prolific root system (biomass, length, density, and depth as main characteristics), stomatal regulation, reduction in canopy size and duration, leaf rolling, increased wax accumulation on the leaf surface, and heliotropism, among other mechanisms, to increase soil water capture and reduce water losses[30]. These responses are accompanied by a down regulation of photosynthesis due to decreased UV capture and a reduction in antioxidant accumulation[30].

2. Escape: Phenological plasticity is linked to escape. Plants may speed up their life cycles in order to reproduce before water stress kills them. This is prevalent in annual crops, when blooming and maturation times are accelerated. However, because of the shorter growing period, this method has drawbacks, such as decreased biomass accumulation and harvest index build-up. In agro geographical locations where rainfall is scarce during planting cycles and irrigation is not possible, breeding for earliness might be a breeding emphasis for Bambara groundnut[30]. Early maturing genotypes of Bambara groundnut have been found, including 'Zebra coloured,' which matures in 90 days, and 'Mottled cream,' which matures in 98–100 days and produces 12.5–23.6 g pods per plant. Furthermore, when Bambara groundnut plants were stressed at 30% of crop water use, landraces 'Red' and 'Brown' from Jozini, South Africa, showed a

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significantly earlier maturity date [mean: 123 days after planting (DAP)], demonstrating developmental plasticity[51].

3. Tolerance: Among the drought-stressed plants' mechanisms, tolerance is likely the least prevalent. It refers to a plant's capacity to maintain a particular level of physiological activity in the face of severe drought by regulating hundreds of genes and their networks. To sustain tissue turgidity, plants enhance their osmotic adjustment capacity and increase cell-wall flexibility. Plants also change a number of metabolic pathways to prevent or repair stress-related damage by increasing osmoregulatory molecules in cells and changing the activity of cell defense enzymes to reduce the buildup of hazardous by-products[30].

All three drought tolerance mechanisms are present in Bambara groundnut: avoidance, escape, and tolerance[52, 53, 54]. As a result, Bambara groundnut is an ideal crop since it can withstand a wide range of climatic conditions and stress durations[55]. Furthermore, the Bambara groundnut has evolved to a variety of habitats, ranging from freezing temperatures at night and high temperatures during the day in a dry environment in Botswana to much warmer and more humid conditions in Indonesia. As a result, it’s an essential crop to promote in regions that are now drought-prone, as well as a future crop in locations where climate change forecasts suggest greater drought frequency and intensity. In response to anticipated climate change in South Africa, Bambara groundnut output and water productivity would rise by 37.5 percent and 33 percent, respectively[56]. Furthermore, it has been revealed that, as a result of climate change, the regions appropriate for Bambara groundnut production in South Africa will grow, demonstrating the crop's resistance to climate change[57].

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Several researches in the last 30 years have clarified drought resistance mechanisms in Bambara groundnut[30]. Researchers found that osmotic adjustment, decreased leaf area, and operational stomatal control can help Bambara groundnut maintain leaf turgor pressure. According to research, Bambara groundnut can sustain turgor at a water potential of 2.0 megapascal (MPa), which is much less than groundnut (1.2 to 1.6 MPa). Others have documented changes in a variety of Bambara groundnut growth and physiological features in response to water shortage stress, including: Canopy formation, size, and duration, Biomass accumulation and partitioning, Phenological plasticity, Gas exchange, Osmoregulation and regulation of photosynthesis and Leaf temperature-transpiration (leaf orientation/paraheliotropism) and epicuticular wax[30].

Testa color has also been linked to drought tolerance traits in Bambara groundnut, with dark-colored seeds having higher emergence rates than light-colored seeds due to the presence of tannins, which are polyphenols that act as antioxidants under stress conditions, though this may not be a desirable trait for a commercial crop[52]. The intensity and rapidity of the drought, as well as the phenological stage impacted, all affect the degree of drought resistance of landraces and their origins. Scientist, for example, tested a number of Bambara groundnut landraces for drought and heat tolerance and found that certain landraces could tolerate 120 days of dryness when irrigation was halted at 30 DAP. Burkina, the most drought-resistant landrace discovered, was a landrace taken from 'Burkina Faso, a Sahelian country. It had the greatest leaf and root biomass of all the landraces studied, and few pods were formed, while others failed to produce any pods. Other landraces, such as 'NAV 4', 'NAV Red,' and 'Black eye,' have also withstood extended drought and temperatures as high as 40.2°C throughout the growth season[58]. In the dry climate of Botswana, Bambara groundnut has evolved to a wide range of temperatures, from chilly nights and hot days

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(about 10–20 °C difference between day and night, depending on month) to considerably milder and more humid conditions in Indonesia (days around 29°C and night 23°C). Drought-resistance mechanisms found in Bambara groundnut might aid the species' ability to deal with moderate, intermittent, and terminal drought stress[30].

Despite the fact that drought reduces the production of Bambara groundnut and most other crops, Bambara groundnut was still able to provide a respectable yield of dried pods of up to 1.7 t ha1 with a range of 1.3–2.1 t ha1[59]. These yields are equivalent to drought resistant groundnut cultivars under similar drought stress conditions and are greater than chickpea cultivars (0.3–0.5 t ha1). In general, the Bambara groundnut's high efficiency of resource collection and conversion might be important contributors to crop yield under drought. Despite drought stress reducing the radiation conversion coefficient(s) of Bambara groundnut from 1.51 to 1.02g MJ1, between the dry matter of Bambara groundnut and soybean, the dry matter of Bambara groundnut was reported to be higher than that of soybean (*Glycine max*), which ranged from 0.52 to 0.92 g MJ1[59]. For high potential yield output, the efficiency with which plants convert water into dry matter is critical. The dry matter of Bambara groundnut (1.65 g kg1) under drought stress was higher than that of other grain legumes cultivated in low rainfall Mediterranean settings, such as lentil (Lens culinaris Medik; 1.37 g kg1) and chickpea (1.11 g kg1)[59]. While Bambara groundnut has been demonstrated to be drought resistant, there are still substantial gaps that must be filled via crop development before this feature may be of greater use to farmers[59].

A recent study of an F5 segregating population produced from a hybrid between single genotype parents Tiga Nicuru and DipC under increasing mild drought stress in a controlled setting revealed significant genetic diversity for several morpho-physiological characteristics[54]. For future breeding programs in Bambara groundnut,

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this study demonstrated the possibility of choosing individual lines with increased potential drought tolerance while maintaining appropriate yielding characteristics[30].

2.3.10 The Importance of Root Studies

Drought adaptations above ground (i.e., increased water usage efficiency) are frequently linked to lower yields due to reduced biomass buildup. While below-ground adaptations have been linked to increased water and nutrient capture and, as a result, the ability to maintain higher rates of gas exchange and biomass accumulation under stress, above-ground adaptations have been linked to increased water and nutrient capture and, as a result, the ability to maintain higher rates of gas exchange and biomass[60]. Roots and their associated traits play a variety of important tasks, including water and nutrient absorption, among others. Drought stress decreases root growth, which diminishes the root systems' nutrient acquisition capability, affecting growth, development, yield, and yield quality traits, even if water is later restored. Before employing root traits as selection criteria in breeding programs, it's critical to have a deeper knowledge of them. Because crops with deeper roots may reach water and nutrients contained in deeper soil layers, rooting depth is one of the most often evaluated traits[30].

Cowpea cultivars with well-distributed and deep roots, for example, tend to withstand drought stress better than those with shorter roots[61]. Given the technical challenges of setting up and conducting root research, as well as the intricate root–soil interaction, the concentration on shoot traits over root traits is justified. Root observation and excavation techniques range from laboratory (paper and gel media) to field and/or greenhouse (soil media) methods for phenotyping root characteristics. Most of these phenotyping methods are time-consuming, necessitate plot damage to reach the roots,

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and, most critically, do not replicate or provide an accurate depiction of actual field circumstances, such as soil physical property heterogeneity[62]. Emerging technologies and developments in the field of phenomics, on the other hand, are beginning to alleviate some of the technical challenges and create chances for root trait analysis. Recent advancements have also enabled high-throughput phenotyping by tracking the root phenotype and its relationship to other characteristics or stressors using computational models and tools[30]. These include RootReader 3D, RootScan, RootNav, WinRhizo, X-ray micro-computed tomography (CT), X-ray micro-computed tomography (µCT) and image. Bambara groundnut root phenotypic investigations may be made more efficient by using the above tools and algorithms, which would help us better understand how the plants' root growth characteristics vary in response to drought[30].

2.3.11 Soil and Climate Requirement for Bambara Groundnut

Bambara groundnut is a versatile plant. It is drought tolerant and may be cultivated on hot, dry, marginal soils where other leguminous crops like sorghum, Maize, and peanuts cannot. Its growth is most suited to climatic zones with latitudes of 20-30° and altitudes of 2000m, i.e. tropical wet and dry climate zones and subtropical dry summer climate zones[63]. Temperature below 16°C and over 38°C is not conducive to plant development[63]. Optimal growth conditions include germination at 30-35°C, average daytime temperatures of 20-28°C in full sun, average annual rainfall of 600-750mm that should not exceed 3000mm (optimum yields are obtained when rainfall is higher), good phosphorus (P) and potassium (K) soil content, and light sandy-loams with a pH of 5.0-6.5. It thrives on acidic laterite soils, which are prevalent in Africa, but it struggles in calcareous soils[64]. Bambara groundnut is mostly grown in Northern Nigeria, notably in the North-East, where the climatic and environmental circumstances are

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more conducive to the crop's development. The crop is most commonly produced as a mixed crop with cowpea, Maize, millet, sorghum, and groundnut in the southern guinea savanna region. The Bambara groundnut is grown in Nigeria from Jos, Plateau State, and Yola, Adamawa State, all the way to Garoua, Cameroon[21].

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Figure 2.6: Bambara Groundnut cultivation
Source: Department of Agriculture, Forestry and Fisheries, 2017

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2.3.12 Planting and Harvesting

Bambara groundnut is planted in November/December on flat land, but occasionally on mounds or ridges, which may be advantageous in rainy places because the crop does not tolerate waterlogging[65]. Bambara Groundnut is sometimes planted atop yam mounds in Ghana to protect them from erosion[21]. 5-6 months after planting, mature and ripe Bambara groundnut pods are manually picked at the farm, depending on genotype, ecological circumstances, and the farmers' goal[64]. The pods are formed underground and the plant grows close to the earth. As a result, harvesting it mechanically is challenging. Harvesting is done using a hoe, cutlass, digger, bare hands, or any other rudimentary agricultural tool. The plant is removed from the earth, revealing the nut-bearing pod. To gather the seed, the pods are removed from the plant roots, dried, and shattered[21].

2.3.13 Photoperiod Requirement in Bambara Groundnut

The Bambara groundnut was first discovered near the, where the daylength (photoperiod) is rather constant. During the planting season, however, the crop is extensively produced across Africa's varied latitudes, with increasing day durations at higher and lower latitudes. Previous research has indicated that the crop requires a photoperiod of about 12 hours for maximal pod set and seed output, whereas lengthy photoperiods result in more leaves being generated at the price of pod formation[66]. Long daylengths exceeding 14 h might delay the beginning of blooming, the progress of flowering, the onset of podding, and the development of pod growth. Under photoperiods of 14 h and 16 h, however, the influence on podding was larger than on

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blooming, and some plants did not produce any pods. Because of the Bambara groundnut's photoperiod sensitivity, it can only be grown in areas closer to the equator. Selecting a suitable daylength for planting during the season may be an option for allowing pod filling, but it may not be the best option in terms of water availability[30]. Vegetative growth period extension, bud set, transition to flower development, fruit set and seed production, tuberisation, changes in winter hardiness, changes in chemical composition, and variations in enzyme activity are all examples of photoperiodic responses in plants[67]. The impacts on flowering have been widely investigatedand the time of flowering start is the foundation for classifying plants that blossom in either long- or short-day circumstances[68].

Under extended daylengths, Arabidopsis thaliana and other temperate longday species such as lentils, pea *(Pisum sativum* L.), chickpea, and barrel medic (*Medicago truncatula*) flower sooner[69]. Short daylengths, on the other hand, stimulate flowering in other species including rice (*Oryza sativa*), cowpea, soybean, and common bean (*Phaseolus vulgaris*)[70]. Aside from flowering, photoperiod influences several other aspects of reproductive development, including pod and seed yield, and seed filling, which are among the most important agronomic traits for agricultural research and policy[71]. Understanding the photoperiodic and genetic regulation of pod set, as well as its link to seed development, may therefore become useful tools for increasing Bambara groundnut output[30]. It is generally assumed that both photoperiod and temperature are important in the phenological development of most annual crops, due to the difficulties in uncoupling photothermal effects in fluctuating field environments[30]. Indeterminate soybeans, for example, were exposed to extended photoperiods, which delayed the reproductive period from flowering to maturity, resulting in more pods and seeds, and this was linked to increases in the quantity of radiation accumulated over the crop

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cycle[71]. Pod development in the Bambara groundnut responds significantly to photoperiodic variations, even when temperatures in tropical areas are high[30].

Photoperiod has a greater influence on pod filling than flowering time, according to combined photoperiod and temperature studies[72]. The Mali genotype "Tiga Necuru" has been described as day-neutral for flowering time and photoperiod-sensitive for podding, whereas the Nigerian genotype "Ankpa 4" appears to be extremely photoperiod sensitive in terms of both time to flowering and pod-set. A group of 102 landraces from Tanzania (East Africa) were cultivated in a greenhouse under extended photoperiod (16–17 h) to find photoperiod neutral lines for pod set in another experiment. This study found that three landraces, Mtwara (TZA-1498), Lindi (TZA-1505), and Shinyanga (TZA-2114), exhibited appropriate photoperiod insensitivity when compared to widely distributed landraces such Tiga Necuru and DipC (Botswana)[72]. Scientists found that canopy sizes and leaf area within landraces were larger under a 14 h photoperiod than under a 12 h photoperiod when they evaluated the performance of 13 Bambara groundnut landraces in Ghana under 12 and 14 h photoperiods[73].s Five landraces that produced some pods and were less sensitive to photoperiod were likewise early maturing during a 14-hour photoperiod. The Uniswa Red landrace was used in field tests in Botswana, which revealed that the beginning of flowering was photoperiod-insensitive, but the pace of progress from flowering to pod-set was photoperiod-sensitive[74]. As such, crop variability is thought to have increased as plants were cultivated further from the equator[74]. Because pod and/or seed are significant components in pulses, photoperiodic regulation of fruit growth is critical. As a result, the creation of day-neutral lines, which are defined as having the potential to establish pods and seeds during daylengths more than 13 hours, will be extremely important[30]. At the latter phases of fruit and seed development, pod set is more

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responsive to endogenous and external factors than vegetative growth. Long photoperiod, for example, hindered the allocation of assimilates to seeds and increased the allocation to vegetative organs in common bean[30]. In 1989, a researcher proposed that the degree of determinateness (preference for partitioning to seed) influences the relative partitioning of assimilates into reproductive or vegetative organs after the start of flowering in tropical legumes, and that photothermal regimes have a direct influence on the expression of determinateness[75]. Even in early flowering varieties, long photoperiods, either alone or in combination with high temperatures, appear to promote indeterminacy by lengthening the flowering period, decreasing flowering and pod-set synchronization, and delaying pod ripening[30].

Fertilization was detected in the Bambara groundnut landrace "Ankpa-4," and viable embryos were discovered in the ovaries up to 32 days after anthesis under a photoperiod of more than 14 hours. This evidence suggests that during extended photoperiods, embryo abortion and lack of pod development in Bambara groundnut are largely caused by a lack of or competition for photo assimilates and nutrients among vegetative and reproductive sink organs. The quantitative response to increased critical period duration owing to photoperiodic effects in terms of pod and seed quantity is not well understood[30]. A greater knowledge of photoperiod responses would aid in the development of cultivars that were suitable for a larger geographical range in addition to drought tolerance, potentially facilitating the effective interchange of germplasm across various latitudes[30].

2.3.14 Pests and Diseases

Bambara groundnut is known for its pest and disease resistance. However, Bambara Groundnut is not as pest and disease resistant as originally thought, especially when it

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is not well dried. Although the nut's shell may protect it from insects, stored Bambara Groundnut seed is particularly vulnerable to bruchid infestation[76].

The following are some of the Bambara groundnut pests and diseases that have been reported:

Pests include: Aphids, Bruchids (*Callosobruchus sp.*), Leafhoppers (*Hilda patruelis*), Termites, Root-Knot Nematodes (*Meloidogyne javanica*), Rodents, Weevils, Parasitic Plants (*Alectra vogelii* and *Striga gesnerioides*)[21].

Diseases include: Leaf spot (*Cerscospora canescens* and *Phyllosticta voandzeia*), powdery mildew (*Erysiphe sp.*), wilt (*Fusarium sp.*), leaf blotch (*Phomopsis sp.*), stem rot (*Phomopsis sp.*), leaf blotch (*Phomopsis sp.*), and stem rot (*Phomops (Scleorotium rolfsii*)[21].

2.3.15 Germplasm Resources Bambara Groundnut

Cultivated Bambara groundnuts are landraces that have most likely developed straight from their wild cousins that have adapted to dry and semi-arid settings, and the crop is popular among farmers for its production stability under a variety of environmental circumstances[30]. In 1988, it was established that the Bambara groundnut originated in the region of North-Eastern Nigeria and northern Cameroon, and RS Pasquet found severe spreading kinds of wild. In comparison to domesticated bunchy varieties, spreading growth habit in a wild line, VSSP11, has been verified as a dominant feature[77]. A recent study on agro morphological variety in Bambara groundnut gathered in Benin revealed a significant degree of morphological heterogeneity as well[78]. Assuming a single area of domestication, accessions from West Africa are predicted to exhibit more genetic variability. Based on a comprehensive analysis of the genetic

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population structure, Researchers proposed that the Southern/Eastern African area might be a secondary hub of domestication or diversification for the crop[79].

The accessions from West Africa and Southern/Eastern Africa were divided into two groups[79]. 4500 Bambara groundnut accessions have been reported to be conserved ex-situ across the world[80]. There are 16 nations with collections, as well as three regional or international genebanks. The International Institute of Tropical Agriculture's (IITA) Genetic Resources Center (GRC) holds 1910 accessions from several nations in Sub-Saharan Africa. Landraces assist to retain genetic variety, which can aid in agricultural genetic improvement. Under low-input farming methods, Bambara groundnut landraces have preserved a substantial degree of genetic diversity[30]. Traditional Bambara groundnut farmers have relied heavily on the existing variety within the landraces that they plant, which has resulted in on-farm genetic diversity conservation, with agro-ecological and farmer-driven selection on Bambara groundnut landraces (e.g., practices in Zimbabwe)[81]. Ex-situ conservation of Bambara groundnut landraces, as well as on-farm conservation by farmers, are critical for the crop's future genetic improvement programs. Landraces, on the other hand, are difficult to comprehend the genetic basis of features of relevance for breeding since they are a mix of various genotypes, which can lead to misunderstanding between environmental impacts and those that have a genetic source. Even if the final "product" required is a combination of improved lines—an artificial landrace, developing controlled crossings and an association genetics panel might allow molecular breeding, including marker-assisted selection[30]. A well-characterized germplasm collection with high-quality genotype data will be a valuable resource for plant breeding and agricultural improvement programs, since scientists and breeders may utilize the information to choose parental genotypes for breeding programs[79]. Accessions obtained from various nations or sections of a country may

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share a common ancestry or may be the same genotypes with different names in different areas of the world. As a result, IITA is using a genome-wide genotyping-by-sequencing technique to characterize the Bambara groundnut collection with the objective of detecting duplicate accessions[30].

2.3.16 Genetic Improvement of Bambara Groundnut

Through a mix of natural and artificial selection, domesticated varieties of the Bambara groundnut were developed from their wild cousins[82]. The occurrence of genetic diversity in all drought-resistance pathways is encouraging for selection and breeding under drought circumstances, with a focus on harvest index. In Sub-Saharan Africa, Bambara groundnut yields range from 0.5 to 3 t ha1 seed. With a production potential of over 3 t ha 1, the focus should be on developing high yielding cultivars for personal use or as a cash crop for resource-poor or arid-zone farmers. Many crop species have found it difficult to select for grain production in drought circumstances. Under drought circumstances, significant genotype x environment interactions (GEI) leading to QTL x environment interactions have frequently resulted in poor heritability. High-yielding traits may be less vulnerable to GEI and so might be targeted for indirect selection[83]. Incorporating a specific characteristic into molecular breeding programs and finding candidate genes underlying traits of interest for forward genetics processes require reliable phenotyping methodologies[30]. In addition to grain yield, breeding efforts on the Bambara groundnut might focus on traits like seed nutritional quality, disease resistance, heat tolerance, high leaf volume, and foliage palatability, so it can be used as a grazing crop. There is a need to learn more about the trade-offs involved in developing drought resistance and other desirable traits[30]. The segmentation of traits for

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a future breeding program in underutilized crops is highly reliant on having strong genetic population patterns. This allows for the partitioning of trait variation into genetic and non-genetic components, as well as the subsequent localization of the impact to specific genomic regions. This type of research can reveal if a trait gene has pleiotropic effects and, more importantly, whether distinct QTL are genetically connected or unrelated[30]. In summary, the creation of segregating populations in Bambara groundnut, as well as recent genetic mapping and QTL studies, mark the beginning of controlled breeding and trait dissection. With the use of an association genetics panel, this screening will be taken to a new level, with the same material being tested in various settings and promising lines being made available to farmers and research institutes[30].

2.4 Aflatoxins in Bambara Groundnut

Scientists have investigated the behavior of *Aspergillus flavus* in Bambara groundnut as a result of processing and storage. Bambara groundnut samples meant for human consumption were discovered to be infected by several fungal species at retail stores and open marketplaces in Durban, South Africa[2]. *Aspergillus flavus, Aspergillus niger, Aspergillus tamarii, Penicillium citrinum*, and *Penicillium oxalicum* were isolated from the seeds of the Bambara groundnut. Bambara groundnut pods grow and mature in the soil or slightly above the ground, with the soil acting as a source of harmful fungus inoculums[19]. Subsequent studies found that toxigenic strains of *A. flavus* also contaminated Bambara groundnut. Despite the fact that additional fungal species were isolated from the seed, a multi-mycotoxin analysis revealed that only aflatoxin B and G derivatives had accumulated. It has been shown that fungal infection does not always imply the formation of mycotoxin, and vice versa[84]. Both aflatoxin derivatives are produced by the S-strain of *A. flavus*, *A. parasiticus*, and other fungi[85]. A study found

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contamination by a wide range of filamentous fungus species, including *Aspergillus*, *Penicillium*, *Fusarium*, *Purpureocillium*, *Paecilomyces*, *Debaryomyces*, and *Byssochlamys* in Bambara groundnut samples. The most common genera were *Aspergillus* (100%) and *Penicillium* (70.6%), whereas *Fusarium sp.* (5.9%), *Paecilomyces sp.* (5.9%), and *Byssochlamys sp.* (5.9%) were uncommon. With rates of 64.7 and 100 percent respectively, *A. flavus* and *A. niger* were found to infect Bambara groundnut in both retail stores and open markets[2]. Contamination by *A. tamarii* was found in Bambara groundnut purchased from the open markets at a rate of 5.9%. *Penicillium citrinum* (64.7 %) and *Penicillium oxalicum* (5.9%) were exclusively found in Bambara groundnuts purchased from open markets and retail stores, respectively. *Purpureocillium lilacinum* was identified from both sources of samples, contaminating 11.8%. Only Bambara groundnut from open markets yielded *Debaryomyces hansenii* and *Fusarium oxysporum* (6.3% each)[2]. *A. toxicarius*, *A. nomius*, *A. bombycis*, *A. parvisclerotigenus*, *A. minisclerotigenes*, and *A. arachidicola* are among the species that generate both AF derivatives[2]. The average mold count varies significantly between the two sources' samples. The fungal contamination in samples from open markets was somewhat greater than in samples from retail shops[2]. *A. flavus*, *A. niger*, *Penicillium citrinum*, and other fungi have been identified from Maize and Bambara groundnut collected in the Limpopo area of South Africa in an investigation[86]. *A. flavus* and *A. niger*were also found on various colored seeds of Bambara groundnut[2].

A significant prevalence of *A. flavus*, *A. niger*, and *Penicillium citrinum* contamination have also been found in Bambara groundnut. Aflatoxin and ochratoxin, two harmful metabolites produced by the isolated fungus, have been linked to their production. Few researchers have researched the mycobiota of Bambara groundnut, and its safety for human consumption has not been thoroughly investigated[2]. The identification of

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mycotoxigenic fungus in Bambara groundnut raises some concerns for consumer health, particularly in poor nations where the nut is frequently utilized as a supplemental meal in cereal-based diets, particularly for growing children[2]. Exposure to mycotoxin-contaminated foods at a young age, through supplemental meals, relates to newborn and young child development issues in Africa[87]. Scientists found a strong link between aflatoxin exposure and slowing of growth and malnutrition in children. A total of 480 youngsters (aged nine months to five years) from Benin and Togo had their blood samples tested to see if they had been exposed to aflatoxin through their diet. In 99% of the samples, aflatoxin-albumin adducts were found. Aflatoxin-albumin concentrations were 30-40% higher in children with stunted development or low body weight[88].

According to the European Commission (2006), the concentration of AFB1; of 2 µg/kg for AFB1 and 4 µg/kg for total AF in groundnuts and nuts, and 0.1 µg/kg for baby foods for infants and young children, the most frequent and dangerous of the AFs, discovered in the samples screened surpasses the maximum acceptable levels permitted in groundnuts, other nuts, and baby meals for babies and young children[2,89]. Even at low quantities or over a lengthy period of time, eating aflatoxin-contaminated foods can have health consequences. AFs, particularly AFB1, have been extracted from the bodily fluids of malnourished children and cancer patients in previous investigations[90]. The natural fungal contaminants of Bambara groundnut and during storage were affected differently by traditional processing procedures such as dehulling, milling, roasting, and spontaneous fermentation. Although roasting caused AF to degrade, the heat treatment did not completely destroy the infection in the seed's interior tissues, resulting in AF formation and deposition during storage[2].

Lactic acid bacteria (LAB) fermentation had a significant impact on the development of *A. flavus* and the generation of AF by fungal species in Bambara groundnut. Within 24

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hours of inoculation, *A. flavus* was completely eradicated, and the concentration of AFB1 was significantly reduced[2]. The generation of anti-fungal metabolites, organic acids, and the competitive inhibitory effect have all been linked to the efficacy of LAB fermentation in decreasing fungal infection[91, 92]. LAB fermentation has a significant impact on *A. flavus* growth and survival during storage. The inhibitory mechanisms of LAB can also be responsible for this[2]. Roasting and fermenting with *L. fermentum* or *L. plantarum* can decontaminate Bambara groundnut flour. A similar research over a longer period of storage is recommended to further understand *A. flavus* behavior in Bambara groundnut and its Maize composite flour[2].

Under the storage conditions described, Bambara groundnut flour is vulnerable to fungi and AF contamination. Water activity, fungal count, AF generation, and general stability of Bambara groundnut flour during storage were all affected by dehulling, milling, and roasting, although fungal and AF contamination was not eliminated[2]. The presence of aflatoxigenic *A. flavus* and AFs in Bambara groundnut flour raises concerns about its use as a supplement in cereal-based meals, particularly for babies and young children. Prevention studies, such as farmer training and improved drying conditions, effective fungus removal and detoxification technologies, and laws regulating AF levels in Bambara groundnut are also needed[2].

Aspergillus flavus population recovered from all of the stored samples did not follow the typical linear trend of microbial survival plots, which is determined by graphing the logarithm of survivors against time. Several variables, including cell clumps or chains, cell damage, and the physiological age of the culture, may have influenced fluctuations in *A. flavus* growth and AF production[2]. Further research into the growth kinetics of *A. flavus* and its AF production routes as they are impacted by processing activities in Bambara groundnut might assist in the development of anti-*A. flavus* approaches[2].

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The survival or death of an organism depends on its introduction into a new environment. During cell development, the organism adapts to a two-stage process including synthesis and enzyme activity in order to live. The development and survival of fungus in foods is influenced by the nutrient status of the substrate as well as other variables such as pH, water activity, processing temperature, and storage temperature[2]. Fungal metabolism is better adapted to carbohydrates-rich substrates[2]. The larger population of *A. flavus* recovered in BGF during the storage period when compared to FBGF and RBGF shows that BGF provides a more suitable substrate for *A. flavus* growth. Water-binding characteristics, pH, nutritional potential, Eh, and the presence of antimicrobial chemicals all affect a microorganism's ability to survive in a low water activity environment. Over the storage period, *A. flavus* growth and survival were considerably reduced in *L. plantarum* -FBGF. The presence of antifungal compounds in the flour can be linked to the decrease in fungus population. Phenyllactic acid and 4-hydroxyphenyllactic acid, two antifungal substances generated by *L. plantarum*, have been identified[2].

A physical examination conducted revealed that the seed coat had started to shrink due to a fungal infection[2]. The infection pattern of *A. flavus* on Bambara groundnut was discovered using scanning and transmission electron microscopy[2]. The seed coat was colonized quickly by fungal spores, and *A. flavus* grew on it, causing the seed coat structure to be disrupted. The underlying cotyledonal cells were penetrated by *A. flavus* through the torn seed coat. Tissue invasion by *A. flavus* resulted in cell wall collapse, rupturing of storage cells, and the formation of holes in parenchymatous cells[2]. Because fungi are heterotrophic, they digest other complex organic compounds to satisfy their nutritional needs[7]. Seed coat and cellular structures were disrupted as a result of fungal penetration through the seed coat and release of hydrolytic enzymes necessary for

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storage cell destruction. During fungal metabolism, cell wall disintegrating enzymes such as cellulases, xynalases, and pectinases are generated[2]. *A. flavus* infection caused comparable cellular disturbances and tissue disintegration, as demonstrated by transmission electron microscopy. The enzyme-linked immunosorbent assay was only capable of detecting low levels of aflatoxin (2.5 ng/g) in the samples after a 96-hour incubation period. Under ideal conditions, *Aspergillus flavus* can generate AF in less than 24 hours[93]. After roasting experimentally infected Bambara groundnut at 140 °C for 20 minutes, the fungus was no longer detectable in the flour after a 96-hour incubation period. During storage of flour derived from comparable processing of naturally contaminated Bambara groundnut, *A. flavus* growth was observed. This discrepancy can be due to spores that survived roasting and were not removed from the seed's interior tissues[2]. Cleaning and drying seeds to a low moisture content before storage, monitoring temperature and moisture during storage, and educating smallholder farmers about the health risks of mycotoxin contamination of food commodities, as well as good agricultural practices like crop rotation, avoiding overcrowding during planting, seed handling to minimize insect damage, and avoiding planting during periods of hibernation. Legislation regarding aflatoxin contamination in Bambara groundnut is required, as is the creation of surveillance mechanisms to check mycotoxin contamination in agricultural food commodities on a regular basis[2].

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2.5 Health Effects of Aflatoxins on Human and Animals (Aflatoxicosis)

Aflatoxicosis is a disease that affects both people and animals and is caused by aflatoxins. It comes in two forms: It comes in two forms: It comes in two forms:

1. Acute primary aflatoxicosis, which happens when moderate to high amounts of aflatoxins are ingested; and

2. Chronic primary aflatoxicosis, which occurs when moderate to high levels of aflatoxins are consumed. Hemorrhage, abrupt liver injury, edema, changes in digestion, absorption, and/or metabolism of nutrients, and potentially mortality are all possible symptoms of acute illness[116]. Acute dietary exposure to AFB1 has been linked to hepatic damage outbreaks[94]. Human cases of acute aflatoxicosis have been recorded all over the world, particularly in third-world nations like Taiwan, Uganda, India, Kenya, and others[95]. Ingestion of low to moderate amounts of aflatoxins causes persistent primary aflatoxicosis[95]. The effects are typically subtle and difficult to detect. Impaired food conversion and decreased development rates, with or without the creation of an overt aflatoxin syndrome, are some of the most prevalent signs[112]. The chronic forms of aflatoxicosis include:

1. Teratogenic effects associatedwith congenital malformations.

2. Mutagenic effects where aflatoxins cause changes (mutations) in the genetic code, altering DNA and these changes can be chromosomal breaks, rearrangement of chromosome pieces, gain or loss of entire chromosomes, or changes within a gene.

3. The carcinogenic effect in which the carcinogenic mechanisms have been identifiedsuch as the genotoxic effect where the electrophilic carcinogens alter genes through interaction with DNA and thus becoming a potential for DNA damage and the genotoxic carcinogens that are sometimes effective after a single exposure, can act in a cumulative manner, or act with other genotoxic carcinogens which affect the same organs. Chronic effects of aflatoxin have been shown in animal models to disrupt normal body immune function by decreasing phagocytic activity or diminishing T cell quantity and function. In a dose-response connection between aflatoxin exposure and rate of growth in babies and children, aflatoxin has also been shown to interfere with

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nutrition[99]. Aflatoxins can promote nutrient alteration in animal models, rendering vitamins A and D unavailable for normal human physiology, resulting in nutritional deficits[95]. Aflatoxin contamination of foods and feeds can have serious health consequences for humans and animals. More than 5 billion people in developing countries are at risk of chronic aflatoxin exposure as a result of consuming aflatoxin-contaminated foods, with more than 4 billion developing aflatoxin-related liver cancer, particularly hepatocellular carcinoma[116]. Aflatoxin exposure is mostly a concern in impoverished and emerging nations, where food processing and storage regulations are lax and malnutrition rates are high. In most Sub-Saharan African nations, aflatoxins have also been associated to kwashiorkor and marasmus in children. Chronic aflatoxicosis is common in these nations, and it's linked to long-term exposure to low to moderate amounts of aflatoxin in the food supply chain. The proteins AFB1, AFB2, and AFM have been found in the liver, gall bladder, spleen, heart, muscle, and kidney[104]. Aflatoxin B1 causes steatosis, or fat buildup, as well as necrosis, or cell death, in liver cells. Consumption of aflatoxins adds to the body's mutagenic, carcinogenic, teratogenic, and immunosuppressive health consequences. Aflatoxin poisoning in humans may cause everything from acute hepatic toxicity to chronic diseases including liver cancer, haemorrhages, oedema, and even death. Aflatoxins have also been linked to poor immunological function, malnutrition, stunted development, and a variety of impairments in children, as well as mortality[95].

According to human studies, aflatoxins increase the amount of circulating alpha tumor necrosis factor, implying that these mycotoxins are also immunotoxic in humans. It has been linked to HIV and tuberculosis because of the body immunosuppressant aflatoxin[115]. Aflatoxins are also harmful to developing fetuses, and they are passed from mother to child through breast milk. In Thailand, New Zealand, Czechoslovakia, the

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United States, Malaysia, Venezuela, and Europe, aflatoxins have been linked to Reye-like Syndrome. Animals of all species are vulnerable to aflatoxicosis, and individual animals' vulnerability varies greatly depending on dose, length of exposure, species, age, sex, and diet. When protein and protein-free parts of the diet were provided separately, AFB1, AFB2, and AFM were found in the liver, gall bladder, spleen, heart, muscle, and kidney of developing pigs. Because of the production of adducts, chronic exposure to aflatoxins in animals suppresses the immune system and interferes with protein metabolism and numerous micronutrients that are essentialhy for health[104]. Mutations, cancer, immunological suppression, lung damage, and birth abnormalities are all caused by these adduct. In animals ingesting modest dietary quantities of aflatoxins, the aflatoxins induce liver damage, decreased milk supply, reproductive problems, and immune suppression[104]. Animals with aflatoxicosis may experience vomiting, abdominal pain, pulmonary oedema, convulsions, coma, and death, as well as cerebral edema and fatty liver, kidney, and heart involvement. Anorexia, depression, a dramatic drop in milk production, weight loss, lethargy, gastrointestinal dysfunctions such as ascitis, icterus, tenesmus, abdominal pain, bloody diarrhoea, decreased feed intake and efficiency are all signs of acute toxicosis in dairy and beef cattle; weight loss, jaundice, abortion, hepatoencephalopathy, blindness, walking in circles, ear twitching, frothy mouth, photosensitization, bleeding and death[96]. In poultry, in addition to inappetance, weight loss, decreased egg production, leg and bone issues, poor pigmentation, fatty liver, renal dysfunction, bruising and mortality, natural immune reduction and susceptibility to parasitic, bacterial, and viral infections are possible[97].

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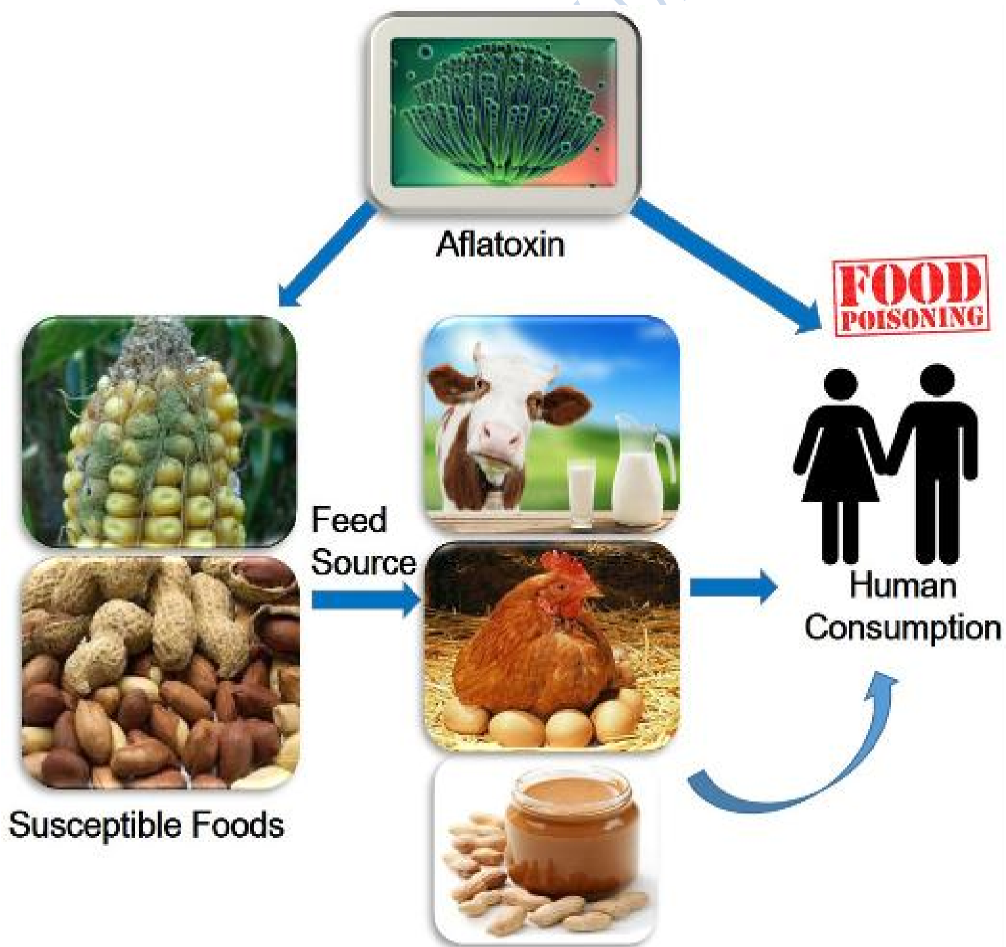

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Figure 2.7: Aflatoxin in Food and Feed
Source: Kumar, Pradeep, Dipendra K. Mahato, Madhu Kamle, Tapan K. Mohanta, and Sang G. Kang, 2017

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2.5.1 Mechanism of Toxicity and Health Effects of Aflatoxins

Aflatoxin is a toxin that targets the liver[98]. Fever, malaise, and anorexia are the first indications of aflatoxins-induced liver toxicity, followed by stomach discomfort, vomiting, and hepatitis; nonetheless, cases of acute poisoning are uncommon and rare[99]. Aflatoxin-induced chronic toxicity has immune-suppressive and carcinogenic consequences. In male F344 rats, the effects of AFT- B1 on splenic lymphocyte morphologies and inflammatory cytokine expression were investigated[100]. The anti-inflammatory cytokineIL-4 was lowered by AFT-B1, whereas the pro-inflammatory cytokines IFN-g and TNF-a were raised by NKcells. These findings suggest that AFT-B1 regulates cytokine gene expression and thereby promotes inflammatory reactions[99]. Researchers also discovered that AFT-B1 disrupts the antigen-presenting function of porcine dendritic cells, suggesting that this could be one of AFT-immunological B1's toxicity mechanisms[101]. Aflatoxins impair the effectiveness of childhood vaccinations, increasing the risk of infection. The fundamental cause of aflatoxins' hepatocarcinogenicity is lipid peroxidation and oxidative DNA damage[99]. AFTs-B1 is activated in the liver by cytochromep450 enzymes and transformed to AFTs-B1-8,9-

epoxide, which has carcinogenic effects in the kidney. Aflatoxins, among all main mycotoxins, provide a considerable danger in dairy since their derivative, AFTs-M1, is present in milk, providing a possible health threat for human consumption. AFTs-B1 is quickly absorbed and processed by the liver, which transforms it to AFT-M1 for further release in milk and urine[99].

Despite the fact that AFTs-M1 is less mutagenic and carcinogenic than AFTs-B1, it has a significant genotoxic potential. Other AFTs-M1 side effects include liver damage, decreased milk output, immune suppression, and lower oxygen delivery to tissues due to anemia, all of which affect appetite and growth in dairy calves[102]. Several investigations have demonstrated that aflatoxins have negative effects on the liver, epididymis, testis, kidney, and heart[99]. Aflatoxin was discovered in post-mortem brain tissue, indicating that it has the ability to pass the blood-brain barrier[103]. AFTs also affect mitochondrial DNA and brain cells, causing changes in their structure and function. Scientist examined the effects of aflatoxin on brain chemistry in depth[104]. Furthermore, few studies have looked at the impact of AFTs-B1 on the anatomy of the central nervous system in rodents[105].

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2.5.2 Effets of Aflatoxins on Mitochondrial DNA

During hepatocarcinogenesis, the reactive aflatoxin-8,9-epoxide preferentially binds to mitochondrial DNA (mitDNA) rather than nuclear DNA, inhibiting ATP production and FAD/NAD-linked enzymatic functions, resulting in mitochondrial dysfunction in various parts of the body that require ATP production[106]. Aflatoxin damage to mitochondria can cause mitochondrial disorders and may play a role in the aging process[106]. Certain mitochondrial illnesses have been linked to the nucleus' ability to detect energy imbalances in its region. The nucleus tries to compensate for ATP

shortages by inducing replication of any nearby mitochondria, but unfortunately, this response encourages replication of the mitochondria that are generating the local energy shortfall, exacerbating the problem[104]. AFB1 binds to DNA and causes structural DNA modifications that result in gene mutations, as well as changes in the length of telomeres and cell cycle checkpoints. The binding of AFB1 to DNA at the guanine base in liver cells tampers with the genetic code that controls cell development, resulting in tumor formation[104]. Adduction and mutations of mitochondrial membranes damage mitDNA, resulting in increased cell death (apoptosis) and interruption of energy generation (ATP synthesis)[107]. By disrupting the various check points that regulate cell cycle development and proliferation, the reactive aflatoxin-8, 9-epoxide can affect the mitotic (M) phase, growth process (G1 and G2), and DNA synthesis (S phase) in the cell cycle, leading to deregulation of the cell and thus cancer development[104,107]. Nevertheless, in resistant rodents, aflatoxins from DNA adducts that affect mitochondrial transcription and translation are protected against mitDNA[108]. Energy-linked functions of ADP phosphorylation, FAD- and NAD-linked oxidizing substrates, and-ketoglutarate-succinate cytochrome reductases are all altered by the mycotoxin[104,108].

2.5.3 Effects of Aflatoxins on Mitochondrial Structure

Aflatoxin promotes ultrastructural alterations in mitochondria and mitochondrial directed apoptosis, which reduces mitochondrial function. Aflatoxins may also disrupt telomere length and other checkpoints in the cell cycle, causing severe damage to the cell cycle's regulatory systems[108, 109]. Also, the extent of aflatoxin binding to DNA and its damage, as well as the level of different proteins from cell cycle and apoptotic pathways such as c-Myc, p53, pRb, Ras, protein kinase A (PKA), protein kinase C (PKC), Bcl-2, NF-kB, CDK, cyclins, and CKI, all contribute to the life or death

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decision making process, which may contribute to the deregulation of the cell proliferation leading to cancer development.

2.5.4 Effects of Aflatoxins on Protein synthesis

Aflatoxin binds to and interferes with enzymes and substrates required for protein synthesis's initiation, transcription, and translation processes. They create adducts with DNA, RNA, and proteins and interact with purines and purine nucleosides, impairing protein synthes Aflatoxin also suppresses RNA synthesis by interfering with DNA-dependent RNA polymerase activity, leading to endoplasmic reticulum degranulation. Increased liver and renal necrosis could also explain the decrease in protein content in bodily tissues such as skeletal muscle, heart, liver, and kidney[110]. AFB1 is mutagenic, carcinogenic, teratogenic, and immunosuppressive, and all of these properties can interfere with normal protein synthesis as well as block multiple metabolic systems, resulting in damage to various organs, particularly the liver, kidney, and heart[111].

2.5.5 Roles of Aflatoxins in Cancer

Aflatoxins, particularly AFB1, AFG1, and AFM1, are the most potent, naturally occurring carcinogens, with AFB1 being the most hepatocarcinogenic[106]. They cause malignancies of the liver and other bodily organs in humans and animals. Aflatoxin's propensity to build altered types of DNA adducts contributes to its cancer-causing potential. Hepatocellular carcinoma is the most common illness linked to aflatoxin consumption (HCC, or liver cancer). With around 550,000–600,000 new cases each year, this illness is the third-leading cause of cancer death worldwide[106]. In most nations, the incidence of liver cancer has continuously been greater in men than in women, with a sex ratio ranging from 2 to 3[112]. East Asia and Sub-Saharan Africa account for 83 percent of cancer mortality[113]. Hepatocellular carcinoma (HCC) is a type

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of cancer that affects the liver and has a bad prognosis. The majority of cases occur in Southeast Asia and Sub-Saharan Africa, where chronic hepatitis B and C virus (HBV and HCV) infection, as well as dietary aflatoxins exposure, are key risk factors[112].

In human HCC, aflatoxins B1 and B2, the most frequent and powerful aflatoxins, are linked to a particular AGG to AGT amino acid transversion mutation at codon 249 of the p53 gene, giving mechanistic support for a causal connection between exposure and illness[114, 115]. The rising prevalence of liver cancer corresponds to the growth in chronic hepatitis B (HBV) and hepatitis C (HCV) infection[115]. Chronic hepatitis B or C virus (HBV/HCV) infection can lead to severe liver disease, such as cirrhosis and hepatocellular carcinoma (HCC), a type of primary liver cancer HCC is the world's third biggest cause of cancer-related death. According to the statistics, those who have hepatitis B and are exposed to aflatoxin in their diet have a 60-fold increased chance of developing hepato-biliary carcinoma or liver cancer, especially in impoverished developing nations throughout the world[115]. According to reports, HBV and aflatoxins have a variety of interactions in the development of hepatocellular carcinoma in humans[104]. They could include the fixation of AFB1-induced mutations in the presence of liver regeneration and hyperplasia caused by chronic HBV infection, the predisposition of HBV-infected hepatocytes to aflatoxin-induced DNA damage, an increase in susceptibility to chronic HBV infection in aflatoxin-exposed individuals, and oxidative stress exacerbated by co-exposure to aflatoxins and chronic hepatitis infected individuals.

In humans, epidemiological studies in Africa, Southeast Asia, the United States, and other western nations where hepatocellular carcinoma is common have found a link between cancer incidence and aflatoxin concentration in the diet. In Asia and Sub-Saharan Africa, aflatoxin B1 (AFB1) is a key risk factor in the etiology of liver

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cancer[117]. In a number of experimental animals, aflatoxin B1 is a powerful liver carcinogen. Following delivery via different methods, it produces liver tumors in mice, rats, fish, marmosets, tree shrews, and monkeys. Hepatocellular carcinoma (rats), colon and kidney cancer (rats), cholangiocellular cancer (hamsters), lung adenomas (mice), and osteogenic sarcoma, gall bladder adenocarcinoma, and pancreatic carcinoma (monkeys) are among the malignancies identified in study animals.

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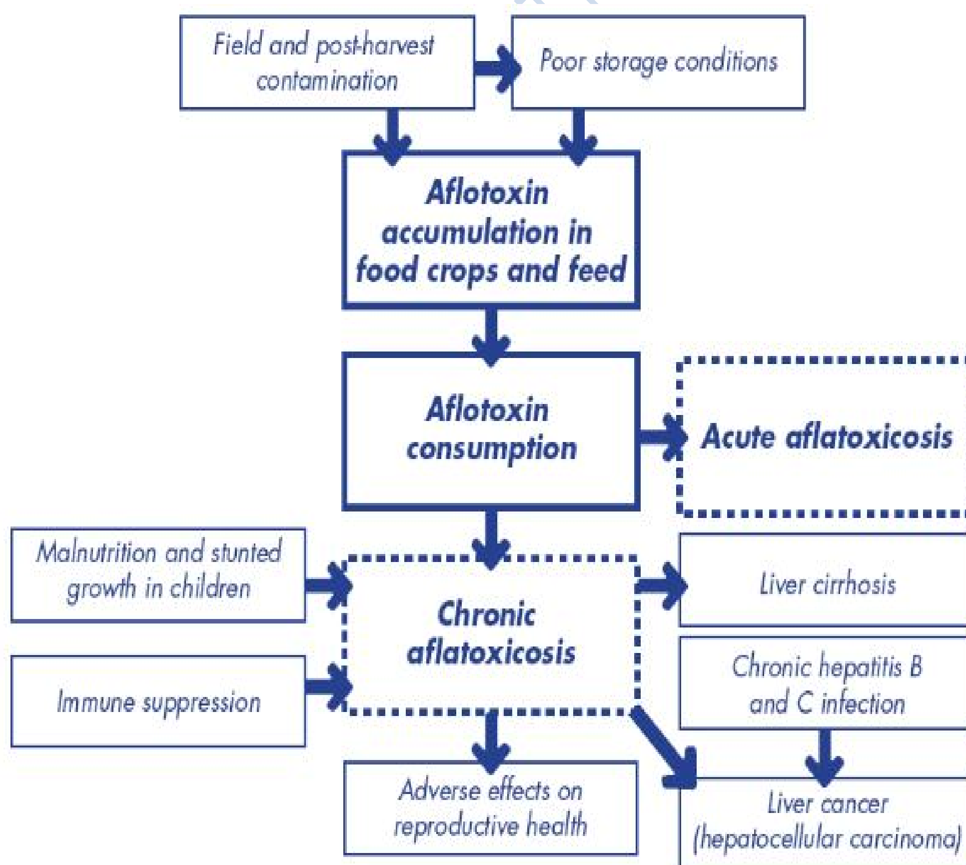

Figure 2.8: Effects of Aflatoxin

Source: Bbosa *et al,* 2013

2.6 Molecular Techniques

Molecular techniques are major modern tools for the analysis of microorganisms from biological samples; the techniques can ease the process of screening broad range of organisms in single analysis[118].

Molecular techniques emerged in the 1960s and allow fingerprinting and characterization of microorganisms which provide details of their genus, species, and their vulnerability to antibiotics. Diagnostic information is most often gained via (i) the direct characterization of the rRNA, (ii) the amplification of the genes and characterization of the amplicons, or (iii) the direct sequencing of the ribosomal genes. Molecular techniques are effective because of their high specificify and sensitivity especially in the identification of microorganisms that has slow growth and cannot be easily isolated on culture because of their special growth requirement[119].

2.6.1 DNA Extraction Techniques

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Use DNA for scientific diagnosis or for research-driven modification typically allows it to be separated and to a degree cleaned. DNA is typically retrieved from cells by methods that involve cell rupture which prevent mechanical shearing of the DNA from fragmenting. It is typically achieved with the use of EDTA, which chelates the magnesium ions needed as cofactors for enzymes that degrade DNA called DNase. Ideally, cell walls should be enzymatically digested, if present (e.g., lysozyme in the bacteria). In addition detergent can be used to solubilize the cell membrane. In addition, where physical disruption is required, it should be held to a minimum and should require smashing or squashing of cells, rather than use shear powers. Cell destruction and the remainder of corresponding actions should be performed at 4°C, utilizing autoclaved glassware and solutions to eliminate DNase operation. Upon removing nucleic acids from the cells, RNA may be extracted by treatment with ribonuclease (RNase), which has been heat treated to inactivate any DNase contaminants; owing to its disulfide bonds, RNase is fairly resistant to heat, which means that the molecule is quickly renaturated after cooling[120].

Fungal tissues are used for DNA extraction. For culturable fungi, cultures derived from 'single spore isolation' is commonly used and for unculturable fungi, fruiting bodies present on thehosts are carefully checked and removed to subject for 'direct DNA sequencing'. Isolates obtainedfrom single spore isolation are grown on culture media (MEA, PDA etc.). To extract DNA, myceliaare scraped from pure cultures. If the attempt of culture isolation failed, the fruiting bodies on thehost are carefully surface sterilized before they undergo molecular treatments. Fungal DNAextraction can be done in many ways. Most commonly and widely used method is the CTAB (cetyltrimethyl ammonium bromide) based extraction buffers. However, nowadays

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Genomic DNA Extraction Kits (e.g. QIAGEN GmbH, QIAGEN Strasse 1, BioFlux®)are commonly used.[121].

If DNA is to be extracted from a single organelle or viral sample, it is best to isolate the organelle or virus before extracting its DNA, as recovery from a mixture with a specific form of DNA is typically very difficult. Where a high degree of purity is required, DNA may be subjected to ultracentrifugation by density gradient via cesium chloride, which is particularly useful for plasmid DNA preparation. The purity of DNA can be tested by agarose gel electrophoresis and the concentration of DNA can be measured by using the assumption that 1 absorbing unit is equal to 50 µg/ mL of DNA: $50A260$ = Concentration of DNA sample (µg/mL). Contaminants can also be identified through ultraviolet (UV) scanning-spectrophotometry from 200 nm to 300 nm. A ratio of 260 nm: 280 nm of approx. 1.8 shows the sample is safe from protein degradation, which firmly absorbs at 280 nm[122].

2.6.2 Electrophoresis of Nucleic Acids

The process of electrophoresis in an agarose or polyacrylamide support gel is normally undertaken in order to analyze nucleic acids by size. Electrophoresis may be used either analytically or in practice, and can be either qualitative or quantitative. Large DNA fragments such as chromosomes can also be separated by a modification of electrophoresis called pulsed field gel electrophoresis (PFGE), utilizing alternating DNA migration directions. To visualize the DNA, it is essential to undergo staining, typically with a dye such as ethidium bromide. This dye binds to DNA by insertion between stacked base-pairs, called intercalation, and when illuminated with UV light it exhibits a strong orange / red fluorescence. Also accessible are substitute stains such as SYBRGreen or Gelstar that have identical sensitivities, and are less dangerous to use. Electrophoresis is typically used to test the purity and intactness of a DNA preparation,

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or to determine the degree of an enzymatic reaction during, for example, the steps involved in DNA cloning. "Mini-gels" are especially useful for these tests, as they need minimal planning, use small quantity samples, and produce results quickly. Agarose gels may be used to separate molecules greater than about 100 basepairs (bp). Polyacrylamide gels are the preferred method for greater resolution or for the effective separation of shorter DNA molecules. A variety of acrylic gels were produced in recent years and can be used as an alternative to agarose and polyacrylamide[123,124].

The fragment of gel holding the target DNA molecule is manually extracted using a scalpel if electrophoresis is used preparatively. Instead, the DNA is extracted in various forms from the gel part. This can involve crushing in a limited volume of buffer with a glass handle, utilizing agarase to digest the agrose leaving the DNA, or by electroelution. In this method, the piece of gel is sealed in a buffer-containing length of dialysis tubing and then placed between two electrodes in a tank with more buffers. Passing an electrical current through the electrodes allows DNA to move out of the gel piece, but it stays stuck inside the tubing of the dialysis, which can thus be quickly retrieved[121] .

2.6.3 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions of more copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. PCR, a technique for amplifying DNA regions using thermostable DNA polymerase and oligonucleotide primers pairs, was developed in 1988 and has been commonly used by microbiologists for the detection and identification of both culturable and non-culturable species. The specificity of the

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process is close to that of hybridization probes, since DNA amplification relies on hybridization of the oligonucleotide primers with their target sequences, however the intensity is even greater since the target area is amplified as much as a million fold. In addition, adequate copies of the target region are made to enable for more thorough study, such as identification of nucleotide sequences. This effective technique has been used in clinical samples to classify uncultured pathogens to more fully characterize the microbial diversity in natural environments by identifying unculturable bacteria in a viable but non-cultural state[125].

Detection, identification and classification of uncultured organisms has been advanced through the amplification and sequencing of phylogenetically informative portions of rRNA genes present in DNA directly extracted from clinical and environmental samples. This strategy uses PCR primers which anneal to highly conserved regions in eubacterial or archaeal rRNA genes, and prime amplification of the less conserved sequence between the primer annealing sites. The amplification products are cloned, and individual clones (each of which carries a single amplification product) are selected for DNA sequence determination. In certain bacteria the sequence will then be compared with homologous sequences to establish the taxonomic status of the uncultured organism. This technique has proved effective in detecting unculturable pathogens in clinical samples[126].

Individual PCR products in the gel can then be identified by Southern hybridization or removed from the gel to determine their nucleotide sequences. The simplicity of usage of PCR and the universality of eubacterial and archaeal primers render amplification of broad spectrum an appealing path to the detection of unculturable bacteria. However, the use of broad-spectrum primers in PCR reagents can produce artifactual amplification products that are derived from contaminating bacterial DNA[125].

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Commercial preparations of thermostable polymerases have been shown to be a source of contamination, and therefore the standard precautions in the laboratory to prevent contamination are ineffective. The usage of negative control reactions, when there is no reference DNA applied to the PCR, is a safe approach to prevent such contamination. A scientist suggested the use of 8-methoxypsoralen and long-wave UV light reagents is an efficient way of eliminating contaminating DNA from reagents. Bias in the amplification of mixtures of rRNA genes has been shown and although PCR amplification of rRNA genes may be used to characterize microbial diversity in natural environments, quantitative analysis should be interpreted with caution[127].

For a variety of sample types, DNA amplification utilizing primers unique to the target individual, or community of target species, was used for direct detection. Using rRNA sequence data, primers can be designed that will selectively amplify rDNA from the target group to group-specific regions of rRNA genes[126]. Usually the identity of the amplification products is confirmed with a group-specific probe by Southern hybridisation after PCR. PCR amplification of genes other than rRNA genes is frequently used in heterogeneous samples for direct detection of particular organisms. The most common approach to the detection of pathogenic bacteria using PCR is designing primers to amplify genes that encode pathogenic determinants. Not only is this approach highly specific but it also correlates the detection of an organism with its pathogenicity potential. Specific amplification of DNA targets in large quantities of DNA extracts from environmental and clinical samples enables the identification of different species or classes of similar organisms without the need for cultivation. Because DNA recovery procedures do not discriminate between the target organisms' cultureable and unculturable forms, all cells with intact amplification targets will be detected[128].

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In addition, it should not be predicted that bulk DNA recovery and amplification by PCR discriminates between viable and dead cells with intact amplification targets. As a result, few researchers have focused on the relative effectiveness of their PCR procedures to detect culturable and uncultural forms. Direct detection methods based on PCR are generally developed and tested using cultured cells, either in pure culture or by using natural samples that have been spiked with dilutions of freshly grown cells. However, some studies specifically addressed the detection of uncultural forms and demonstrated that both viable and non-viable uncultural cells are detected using PCR-based assays[126].

The fact that PCR methods in the viable but nonculturable state can readily detect organisms makes it a useful method for directly determining the presence of target organisms in samples. The fact that targets may also be enhanced in nonviable cells indicates vigilance should be exercised while interpreting the PCR-based detection findings. If the goal of a PCR-based assay is to detect only viable organisms in both the cultural and non-cultural state, then additional methods must be applied to assess viability. One suggestion is to amplify DNA from mRNA targets instead of genomic DNA targets[129].

Since cellular mRNA is much less stable than DNA, the chances of finding targets for intact amplification in nonviable cells would be much lower. Amplification of mRNA targets requires reverse transcriptase treatment to generate a duplicate DNA (cDNA) that will then act as a guide for PCR amplification. The prerequisites for this strategy's success would include selecting a constitutively expressed target and finding a good method for extracting intact mRNA from the sample of interest[129].

The Essential Components of PCR

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The essential components of polymearse chain reaction (PCR) are as follows: (i) a thermostable DNA polymerase to catalyse templates dependent synthesis of DNA. (ii) A pair of synthesis Oligonucleotides to prime DNA synthesis. (iii) Deoxynucleotide triphosphates (dNTPs). (iv) Divalent cations: Magnesium or manganese ions generally mg^{2+} is used. (v) Buffer to maintain pH (VI) Thermus aquaticus polymerase (Taq polymerase) Vii) Template DNA containing the Target sequence[130].

Principle of PCR

PCR is commonly carried out in a reaction volume of upto 25ul in reaction tubes of about 0.5ml in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperature requirement at each step of the reaction. The technique involves three stages[13]:

1. Denaturaton Stage: This step is the first regular cycling event and consists of heating the reaction to 94˚C-98˚C for 20-30 seconds. It causes separation of DNA template and primers by distrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strand of DNA[130].

2. Annealing Stage: The reaction temperature is lowered to 50˚C-65˚C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically, the annealing temperature is about 3^0C-5^0C below the temperature of primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesi[130].

3. Extension/Elongation Stage: The temperature at this step depends on the DNA polymerase used. Taq polymerase has its optimum activity temperature at 75˚C-80˚C and commonly a temperature of 72˚C is used with this enzyme. At this step, the DNA

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polymerase synthesizes a new DNA strand complementary to the DNA template in 5’ to 3’ direction, condensing the 5’ phosphate group of the dNTPS with the3’ hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified[130].

2.6.3.1 DAF and RAPD

DAF and RAPD are fingerprinting strategies focused on amplification of nucleic acid (concurrent identification of several loci without genotype assignment) that use an in vitro enzymatic reaction primarily to amplify a multiplicity of target sites in one or more nucleic acid molecules[130]. In general, the amplification reaction is driven by arbitrary or semi-arbitrary short synthetic oligonucleotides which produce a collection of largely non-allelic amplified products. DAF uses a single (5-10 bp) primer to randomly amplify genomic DNA[131]. Data from DAF and its other studies suggest a remarkable degree of homogeneity among contemporary isolates of *S. enterica* serotype typhimurium DT104 at a molecular level. RAPD method was first employed in 1990 to analyze samples of human DNA from unidentified people. Several authors have since reported on the use of RAPD technique in microorganisms. It uses random primers and can be applied to any species without requiring any nucleotide sequence information. In this study, the amplification products display polymorphism and may therefore is used as genetic markers. However, the existence of a RAPD band does not provide for the differentiation between hetero- and homozygous states. The fragments are scored as Mendelian dominant elements, and the protocols are relatively simple. The band pattern produced in the study represents the characterisation of a specific bacterial strain by the genome. However, the approach has the ability to examine phylogenetic relationships between closely related organisms and may discern strains within a population[132].

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The procedures include DNA extraction, DNA digestion with restriction enzymes, electrophoresis fractionation of the resulting DNA fragments, DNA transition from electrophoresis gel matrix to membrane, preparation of radiolabellated and chemiluminiscent samples, and hybridization to membrane-bound DNA. RFLP fingerprinting technique is known to be the most effective tool for strain detection, and this technique has been extensively used to test many bacterial strains. In the Restriction Endonuclease Analysis (REA), one of the first methods of Restriction Fragment Length Polymorphism (RFLP), a bacterial chromosome undergoes a digestive process in which restriction enzymes cut the chromosome into smaller fragments, which are then separated by size through use of gel electrophoresis[132]. Under a structured procedure, this approach is quite straightforward, systematic and easy to replicate, but the complicated nature of the patterns generated makes it difficult to interpret the findings and hampers the sharing of data between various study groups. A synthesis of RFLP and ribotyping may be used to maximize the analysis of results, where a second stage is applied in addition to genome digestion, which hybridizes a gene-complementary rRNA probe to the genome fragments. Certain species-specific hybridization probes may be used, for example during typing IS6110, where standardized typing of Mycobacterium tuberculosis can be achieved[133]. Despite these improvements, conversely, studies showed that RFLP clusters lack predictive accuracy and can be further subdivided by new typing methods based on the WGS[18] .The higher resolution of such WGS methods could allow clinicians to make a better distinction between outbreak strains and non-outbreak strains[134].

2.6.3.2 Amplified Fragment Length Polymorphisms (AFLPS)

AFLP is a variant RAPD and is capable of detecting restriction site polymorphisms without prior understanding of the sequence utilizing PCR amplification to identify

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restriction fragment. A restriction enzymedigested genomic DNA is the basis for a PCR reaction in this regard. The primers contain the site for the recognition of restriction enzymes as well as additional 'arbitrary' nucleotides that extend beyond the restriction site. The fixed portion provides consistency to the primer, and the random portion enables identification of many loci. Amplified products are resolved by electrophoresis of the polyacrylamide gel. AFLP analysis is one of the rigorous multi-locus fingerprinting techniques among genetic marker techniques evaluated for genotyping[135]. AFLP is based on the selective amplification of a subset of genomic restriction fragment using PCR. DNA is digested with restriction endonucleases and double stranded DNA adapters are ligated to the ends of the DNA fragment to generate template DNA for amplification. Thus, the sequence of the adapters and the adjacent restriction site serves as primer binding sites for subsequent amplification of the restriction fragments by PCR, selective nucleotides extending into the restriction fragments are added to the 3' ends of the PCR primers such that only subset of the restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified. The subsets of amplified fragment are then analysed by denaturing polyacrylamide gel electrophoresis to generate fingerprinting[135].

When DNA fingerprints of related samples are compared; common bands as well as bands that differs (DNA polymorphism) are observed in an otherwise identical fingerprint. Less DNA polymorphism detected in the fingerprints obtained by restriction cleavage can result from alterations in the DNA sequence, mutations abolishing or creating a restriction site and insertions, electrons or inversions between two restriction sites[135].

2.7 Sequencing

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Ever since the first report of a complete genome sequence in 1995, sequencing technologies have rapidly improved. Second generation sequencing platforms allow whole bacterial genomes to be sequenced within hours, while third-generation sequencing platforms that provide longer reads and additional information, such as methylation sites, with even higher speed have been developed[135].

NCBI BLAST: The National Center for Biotechnology Information (NCBI) has a Basic Local Alignment Search Tool (BLAST) service available (https://blast.ncbi.nlm.nih.gov/ Blast.cgi). Multiple BLAST variants are available via a Web-based interface. To identify the species origin from single or multiple contigs, megaBLAST is the most advisable tool, which is also the default when standard nucleotide BLAST is performed. The default database is the Nucleotide Collection (nr/nt) which is a large database containing all sequences present in the NCBI database[136].

Phylogeny

Genomic characteristics that are obtained by genome characterization and comparison tools can be used to estimate the phylogeny of pathogenic isolates. Estimated phylogenies allow clinicians to establish detailed networks of transmission of outbreak strains between different patients and inform appropriate patient isolation protocols. Here clinicians might want to address the following questions. (i) Are bacterial isolates from different patients nearly identical or only distantly related? (ii) Are different pathogenic isolates from the same outbreak cluster or from separate transmission events? (iii) Which patient harbors the initial outbreak source strain? Several phylogeny algorithms that address these questions through computing phylogeny estimates via either Bayesian methods or ML methods are available. In biological studies and field research, quantitative measurements and binary data are frequently gathered. The

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objects of the study (species or other taxonomic entities) and the measurement or binary criteria (morphometric measurements, results of metabolic tests, etc.) are typically organized in corresponding rows and columns of a table or matrix. This data can then be compared directly, for instance, to support the uniqueness of one or more taxa or as support for the discovery of one or more novel species[137].

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Chapter Three

Methodology

3.1 Sample Collection and Preparation

A total of 15 samples of Bambara groundnut (BG), *Vigna subterranean* sample*s* were purchased from three major markets namely: Abakpa, New market and Ogbete in Enugu, Enugu State, Nigeria. The samples were collected in the sterile polythene bag and then transferred to the Microbiology laboratory in Lead City University, Ibadanfor analysis. The samples were ground into powder using a cleanIwatani Blender, IFM-800 (Made in Japan).

3.2 Preparation of Culture Medium for Fungal Enumeration

Potato dextrose agar (PDA) was used for the growth and maintenance of the fungi isolates. The manufacturer's recipe was followed to prepare the PDA, 19grams of the PDA powder was weighed and dissolved in 500mL of water. The medium was homogenized by bringing it to boil for 15 minutes and then sterilized by autoclaving at 121°C for 15 minutes, it was then allowed to cool to temperature between 42-45 °C. 1% of reconstituted streptomycin sulphate BP was added to the media prior to pouring on the plate to prevent the growth of bacteria and to ensure the growth of fungi only[1].

3.3 Sterilization of Materials

All the glass wares used in this research were cleaned by washing them with detergent, and then rinsed and covered with aluminum foil. The covered glass wares were sterilized for 30 minutes in a ventilated oven to avoid being damaged during the sterilization process. The media used were also sterilized by autoclaving at temperature of 121°C for 15 minutes. Inoculating needles and Scalpel were sterilized by immersing them into 70% ethanol and passing them over a spirit lamp flame until red hot.

3.4 Fungi Isolation

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The work bench was disinfected by applying ethanol to ensure that there are no contaminations. One gram of each thoroughly mixed ground sample was weighed and transferred into a conical flask containing 100mL of sterile distilled water to form the mother inoculum[2]. This was mixed very well to resuspend the organisms into the sterile distilled water. Serial dilution was performed for each sample by transferring 1mL of each of the mother inoculum into their respective 9ml of sterile distilled water contained in sterile bottles to make one in ten dilutions i.e.10^{-1}. 1mL of the mixture from 10^{-1} dilution was aseptically transferred into another bottle containing 9ml of sterile distilled water for each sample to make one in hundred dilutions i.e. 10^{3}, this process was repeated for all the samples until a 10^{-5} dilution was obtained. Pour plate method was used for the fungi enumeration. 1ml each of the dilution of 10^{-1}, 10^{-3}, and 10^{-5} was transferred using pipette into three sterile petri dishes respectively, after which 15mL of the prepared sterilized molten PDA medium was poured into the petri dishes containing the serial dilutions. These were homogenized by rocking the petri dishes on the work bench. The media in the petri dishes were allowed to set and then incubated in an inverted position at room temperature 25°C for 72 hours. At the end of the 72 hours of incubation at 25 °C, visible growths were sub-cultured into a freshly made media and this was repeated until a pure culture was obtained. 15mL of the molten PDA was also poured into McCartney bottles, autoclaved and kept in a slanted position until it solidified. These were used as slants to preserve the isolates.

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3.5 Morphological Identification of the Fungi

The morphology of the isolates grown on the petri dish were observed and characterized according to the formation of spore, color, hyphae and shape.

Microscopic examination was performed by transferring a tiny portion of the culture on the plate to a microscope slide using a sterile needle. A drop of cotton blue-in-lactophenol was used to stain the culture on the slide after which the slide was gently teased and then covered using a coverslip. The covered slide was then viewed under the 10x and 40x objectives of the light microscope. This was done repeated for all other isolates. The isolates were identified by comparing their characteristic[3].

3.6 Quantification of the Aflatoxin Load of the Bambara Groundnut

Aflatoxin content of the Bambara groundnut was measured using AgraQuant® Total Aflatoxin Assay 4/40.

3.6.1 Assay Principle

The AgraQuant® Total Aflatoxin Test is an enzyme-linked immunosorbent assay that is directly competitive (ELISA). Aflatoxins are extracted with 70% methanol from a pulverized sample. The enzyme-conjugated aflatoxin and the extracted sample are combined and applied to the antibody-coated microwell. Aflatoxins in samples and control standards are allowed to compete for antibody binding sites with enzyme-conjugated aflatoxin. An enzyme substrate is introduced after a washing stage, and a blue hue emerges. The color intensity is inversely related to the aflatoxin concentration in the sample or standard. The color is then changed from blue to yellow by adding a stop solution. The microwells are optically measured using a microwell reader with a 450nm absorbance filter and a 630nm differential filter. The samples' optical densities are compared to the standards' ODs, and an interpretive result is determined[4].

3.6.2 Aflatoxin Quantification Procedure

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The Bambara groundnut was ground to obtain representative samples. 20g of the ground sample was weighed and transferred into a cleaned jar that can be tightly sealed, 100 mL of 70/30 (v/v) methanol/water extraction solution was added to the ground sample in the ratio 1:5 and then the jar was tightly sealed. This was shaken and thoroughly mixed after which it was allowed to stand for 3 minutes. After three minutes of standing, the sample extract was filtered through a Whatman #1 filter paper. The filtrate was collected and used for the aflatoxin assay. 100µL each of the sample filtrate was transferred accordingly using a single pipette into corresponding Dilution Well containing 200 µL of Conjugate. The content of the dilution well was mixed using an 8-channel pipettor with fresh tips for each 8-well strip, by carefully pipetting it up and down 3 times, after which 100 µL of the contents from each Dilution Well was immediately transfer into a corresponding Antibody Coated Microwell stripe. This was incubated at room temperature for 15 minutes by allowing it to stand. The contents of the Micro well strips were then emptied into a waste container. The Micro well was washed filling each well with distilled water, and then dumping the water from the micro well strips. This step was repeated for a total of 5 washes. The Micro well was tapped on several layers of absorbent papers towers laid on a flat surface to expel as much residues as possible after the wash step. The bottom of the Micro wells was dried with a towel. 100 µL of Substrate was pipetted into each Micro well strip using an 8-channel pipettor and then incubated at room temperature for 5 minutes. 100 µL of Stop Solution was dispensed into each Micro well strip using an 8-channel pipettor. A change in color from blue to yellow was observed. The Micro well strips were read using a Micrp well reader at 450nM. The OD readings for each Micro well was read and recorded[4]. This procedure was repeated for all the samples.

3.7 Molecular Identification

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3.7.1 DNA Extraction

DNA extraction was carried out using Quick DNA fungal/bacterial miniprep kit (catalog number D6005) following manufacturer's instructions. 2mL each of fungal cells subcultured into Yeast Extract was transferred into 2mL microfuge tube and then centrifuged at 10,000rpm for 10 minutes, after which the supernatant was decanted into waste bottle containing 1x of hypo chloride. The cells were then transferred into corresponding ZR BashingTM Lysis Tube respectively. 750ul Lysis Solution was added to each ZR BashingTM Lysis Tube respectively, the respective tubes were secured in a bead fitted with 2 ml tube holder assembly and processed at maximum speed for 5 minutes. The ZR BashingBeadTM Lysis Tubes containing the isolate solution were then centrifuged in a microcentirifuge at 10,000 x g for 1 minute. 400 ul supernatant was Transferred into corresponding Zymo-SpinTM IV Spin Filter (orange top) in a Collection Tube and centrifuged at 7,000 x g for 1 minute. Each of the filtrates from the centrifugation process were collected into a collection tube. This was followed by the addition of 1,200 ul of Fungal/Bacterial DNA Binding Buffer to each of the filterates in the Collection Tubes. 800 ul of the mixture transferred into corresponding Zymo-SpinTM IIC Column in a Collection Tube and centrifuged at 10,000 x g for 1 minute. The flow through from each of the Collection Tubes were then discarded and the step repeated for the remaining mixture. 200 ul of DNA Pre-Wash Buffer was added to each of the Zymo-Spin TM IIC Column in new Collection Tube and centrifuged at 10,000 x g for 1 minute. 500 ul Fungal/Bacterial DNA Wash Buffer was then added to the Zymo-SpinTM IIC Column and centrifuged at 10,000 x g for 1 minute. Each of the Zymo-SpinTM IIC Column were transferred into a clean 1.5 ml microcentrifuge tube and 100ul of DNA Elution Buffer was directly added to each of the column matrix and then centrifuged at 10,000 x g for 30 seconds to elute the DNA[5].

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3.7.2 Polymerase Chain Reaction

The fungal genomic DNA was amplified using polymerase chain reaction (PCR) to obtain an approximately 260-bp fragment of the 28s rRNA gene. PCR was performed in tubes containing 48 µL of standard PCR mix, 10 µL each primer (U1 5′-GTGAAATTGTTGAAAGGGAA–3′ and U2 5′- GACTCCTTGGTCCGTGTT–3′), 0.25 µL Taq polymerase (Promega, Madison W1 USA) and 2 µL of the extracted DNA. Amplification was performed in a thermal cycler (Bio-Rad Laboratories, USA) programmed for 30 cycles of denaturation at 94 °C for 45 s (initialization at 94 °C for 3 min), annealing at 50 °C for 50 s, polymerization at 72 °C for 5 min and a final extension at 72 °C for 5 min. To evaluate the quality of the amplicons, 5 µL aliquots of the PCR products were analyzed by electrophoresis in 2% (w/v) standard agarose gel (Eurobio, France) with 1X buffer Tris Acetate-EDTA. A 100 bp DNA Marker ladder (Promega, Madison, W1 USA) was used as the standard. Gels were electrophoresed at 100 V for 30 min, then observed and photographed using a CCD camera and Gel Smart system software (ClaraVision, France). The PCR products were stored at 4 °C till sequencing. Amplicons were sequenced by IITA, Bioscience Center (Ibadan, Nigeria)[2].

3.7.3 Statically Analysis

The fungi were enumerated in three sets for each sample. Mean CFU/g of samples was calculated using SPSS for Windows version 24.0 (IBM Corporation, New York, USA). DNA base sequences were compared with the GenBank databases of the National Centre for Biotechnology Information. The GenBank was searched using BLAST program to determine the closest known relatives of the partial 28s rRNA gene sequences[2].

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Endnotes

[1.] A. O., Osibona, O. O., Ogunyebi & T. O. Samuel. "*Storage fungi and mycotoxins associated with stored smoked Catfish (Clarias gariepinus)*." **Journal of Applied Sciences and Environmental Management** 22, no. 5 2018: 643-646.

[2.] F.O. Omotola, "*Incidence of mycotoxigenic fungi during Processing and Storage of Bambara Groundnut (Vigna subterranea) Composite Flour*," Ph.D diss., Durban University of Technology, Durban, South Africa, 2019.

[3.] Da Silva, Neusely, H. Marta Taniwaki, Valéria C. A Junqueira, Neliane Silveira, Margarete Midori Okazaki, & Renato Abeilar Romeiro Gomes. Microbiological examination methods of food and water: a laboratory manual. CRC Press, 2018.

[4.] AgraQuant® *Total Aflatoxin ELISA Test*; https://www.romerlabs.com/shop/inter_en/agraquant-r-total-aflatoxin-elisa-test

[5.] *Quick-DNA Fungal Bacterial Isolation Kits* | ZYMO RESEARCH; https://www.zymoresearch.com/collections/quick-dna-fungal-bacterial-kits

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Chapter Four

Results and Discussion of Findings

4.1 Results of the Findings

4.1.1: Occurrence of Fungal Isolates from Bambara Groundnut Samples in Different Locations in Enugu Metropolis

Molecular characterization of aflatoxin producing fungi in Bambara groundnut were investigated by visiting five different sampling points from three markets in the eastern part of Nigeria. The markets are Abakpa (A, B, C, D and E), New Market (F, G, H, I and J) and Ogbete (K, L, M, N and O). Eleven different species of molds were isolated from the market using Potato Dextrose Agar (PDA) culture medium. Isolated molds were subjected to preliminary physical and microscopical investigation before being sub cultured into Yeast extract sucrose (YES) medium for molecular aflatoxin production and subsequent characterization. From the preliminary investigation, the following suspect moulds were isolated: *Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus, Aspergillus orchraceous, Penicillum spp, Rhizopus stolonifer, Curvularia spp, Trichothecium spp, Alternaria macrospora, Scolecosporae inflata* and *Colletotrichum lindenuthian.*

The results of the fungi isolates obtained from the 5 different points across the 3 markets are presented in table 4.1. *Aspergillus niger* and *Trichothecium* spp were isolated from New main market and Ogbete market. *Aspergillus flavus* was isolated across the three selected markets. *Colletotrichum lindemuthian, Aspergillus fumigatus, Rhizopus stolonifera* and *Penicillium* sppwere isolated from Ogbete in addition to *Trichothecium* sppmentioned above. *Aspergillus orchraceous, Scolecosporae inflate* and *Alternaria macrospara* were isolated only from Abakpa market. *Culvularia spp* was isolated from Abakpa and New main market

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Table 4.1: Occurrence of Fungal Isolates from Bambara Groundnut Samples in Different Locations in Enugu Metropolis

Fungal isolate	Abakpa A					New Main Market					Ogbete				
	A	B	C	D	E	G	H	I	J	K	L	M	N	O	
Aspergillus niger	-	-	-	-	-	+	+	-	-	+	-	-	-	+	-
Aspergillus flavus	+	+	-	+	-	+	-	+	+	+	-	+	-	+	+
Aspergillus fumigatus	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Aspergillus orchraceous	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Curvularia sp	-	-	+	+	-	+	-	+	-	-	-	-	-	-	-
Trichothecium sp	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
Scolecosporae inflata	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Alternaria macrospara	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Colletotrichum lindemuthian	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-
Penicillium sp	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
Rhizopus stolonifer	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-

Key note: + = present; - = absent

Source: Laboratory Analysis, 2021.

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4.1.2: Frequency (%) of Occurrence of Fungal isolates on Bambara Groundnut from three Major Markets in Enugu Metropolis

The percentage frequency of occurrence in Table 4.3 shows that Bambara groundnut being sold in New Market has the highest mold isolate contributing 45% (35 out of 77 colonies) of the total isolates with location F having the highest mold isolates (16% of the figure). In the same view, total molds isolated from Abakpa market was investigated to follow the new market value, having 29% (22 out of 77) of the accurate value, while Ogbete market was observed to have the lowest close figure (25%), indicating frequent stock replenishment.

4.1.3 Average Frequency (%) of Occurrence of Fungi Isolated on Bambara Groundnut Samples in three Selected Markets in Enugu Metropolis

The average frequency (%) of occurrence of the fungi isolated (Table 4.3) showed that *Aspergillus flavus* has 32% (highest) occurrence of the total isolates. It was closely followed by *Culvulanaria* (30%). Others in descending order are *Aspergillus niger* (12%), *Aspergillus fumigatus* (6%), *Penicillum spp* (5%), *Colletotrichum* spp (4%), *Rhizopus stolonifer* (3%), *Aspergillus orchaceous (3%)* and *Trichotothecium* spp (3%), *Alternaria macrospora* and *Scolecosporae* spp was 1% each as they were isolated only from Abakpa A and Abakpa D locations respectively.

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Table 4.2: Average Frequency (%) of Occurrence of Fungi Isolated on Bambara Groundnut Samples in three Selected Markets in Enugu Metropolis

	Abakpa A					New Main Market						Ogbete			Total
Fungal isolate	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
Aspergillus niger	-	-	-	-	-	4	1	-	-	2	-	-	-	2	-	
Aspergillus flavus	4	3	-	3	-	2	-	3	1	5	-	1	-	1	2	
Aspergillus fumigates	-	-	-	-	-	-	-	-	-	-	-	2	3	-	-	
Aspergillus orchraceous	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	
Curvularia sp	-	-	2	6	-	7	-	8	-	-	-	-	-	-	-	
Trichothecium sp	-	-	-	-	-	-	-	-	1	-	-	-	-	-	1	
Scolecosporae inflate	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	
Alternaria macrospara	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Colletotrichum lindemuthian	-	-	-	-	-	-	-	-	-	-	1	-	2	-	-	
Penicillium sp	-	-	-	-	-	-	-	-	-	-	1	-	-	3	-	
Rhizopus stolonifer	-	-	-	-	-	-	1	-	-	-	1	-	-	-	-	
Occurrence	6	3	2	10	1	13	2	11	2	7	3	3	5	6	3	=77
% occurrence	8	4	3	13	1	17	3	14	3	9	4	4	7	8	4	=100

Key note: n = 5; - = absent

Source: Laboratory Analysis, 2021

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Table 4.3: Average Frequency (%) of Occurrence of Fungi Isolated on Bambara Groundnut Samples in three selected Markets in Enugu Metropolis

Fungal isolate	No (%)
Aspergillus flavus	25.0 (32%)
Curvularia sp	23 (30%)
Aspergillus niger	9.0 (12%)
Aspergillus fumigatus	5 (6%)
Penicillium sp	4 (5%)
Colletotrichum lindemuthian	3 (4%)
Trichothecium sp	2 (3%)
Rhizopus stolonifer	2 (3%)
Aspergillus orchraceous	2 (3%)
Scolecosporae inflate	1 (1%)
Alternaria macrospara	1 (1%)

Source: Laboratory analysis, 2021

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4.1.4 Aflatoxin Contamination Load of the Bambara Groundnut from Three selected Markets in Enugu Metropolis

The aflatoxin analysis as shown in Table 4.4 revealed that the Bambara groundnut samples produced aflatoxins. The sample from Obete markets (Ogbete N) produced the highest aflatoxin with concentration at 80 ppb, this is followed by new main market with concentation at 72 ppb (averaged) and then Abakpa market with concentation at 69 ppb (averaged). The presence of aflatoxin in the Bambara groundnut samples at concentrations higher than the WHO recommended limits for foods indicates that it is highly hazardous.

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Table 4.4: Total Aflatoxin Concentration of the Samples from three Markets in Enugu Metropolis

Sample	Total Aflatoxin Concentration (µg/kg)
Abakpa A	70
Abakpa D	68
New Main Market F	69
New Main Market H	75
Ogbete N	80

Source: Laboratory analysis, 2021

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4.1.5 Molecular Characterization of the Aspergillus Species Isolated from the Bambara Groundnut

From the preliminary analysis results obtained, 41 out of the total of 77 fungi isolated belongs to *Aspergillus* species, 25 which were identified morphologically as *A. flavus* were characterized using molecular techniques. DNA extraction results showed high molecular weight DNA (figure 4.1) and the result for the amplification of the internal transcribed spacer region of the fungal isolates are shown figure 4.2. Sequence alignment of the isolate sequences on NCBI Genebank database identified 14 out of the 25 isolates as *A, flavus*, 6 isolates as other species of *Aspergillus* and 5 isolates as other genera of fungi. Table 4.5 shows the BLAST results of the isolate sequences. Unique accession numbers for the isolates were generated for the 14 isolates identified as *A. flavus*. The *A. flavus* isolates were characterized for Aflatoxigenicity and all 14 isolates showed positive results indicating the presence of the Aflatoxin regulatory gene in the isolates (figure 4.1).

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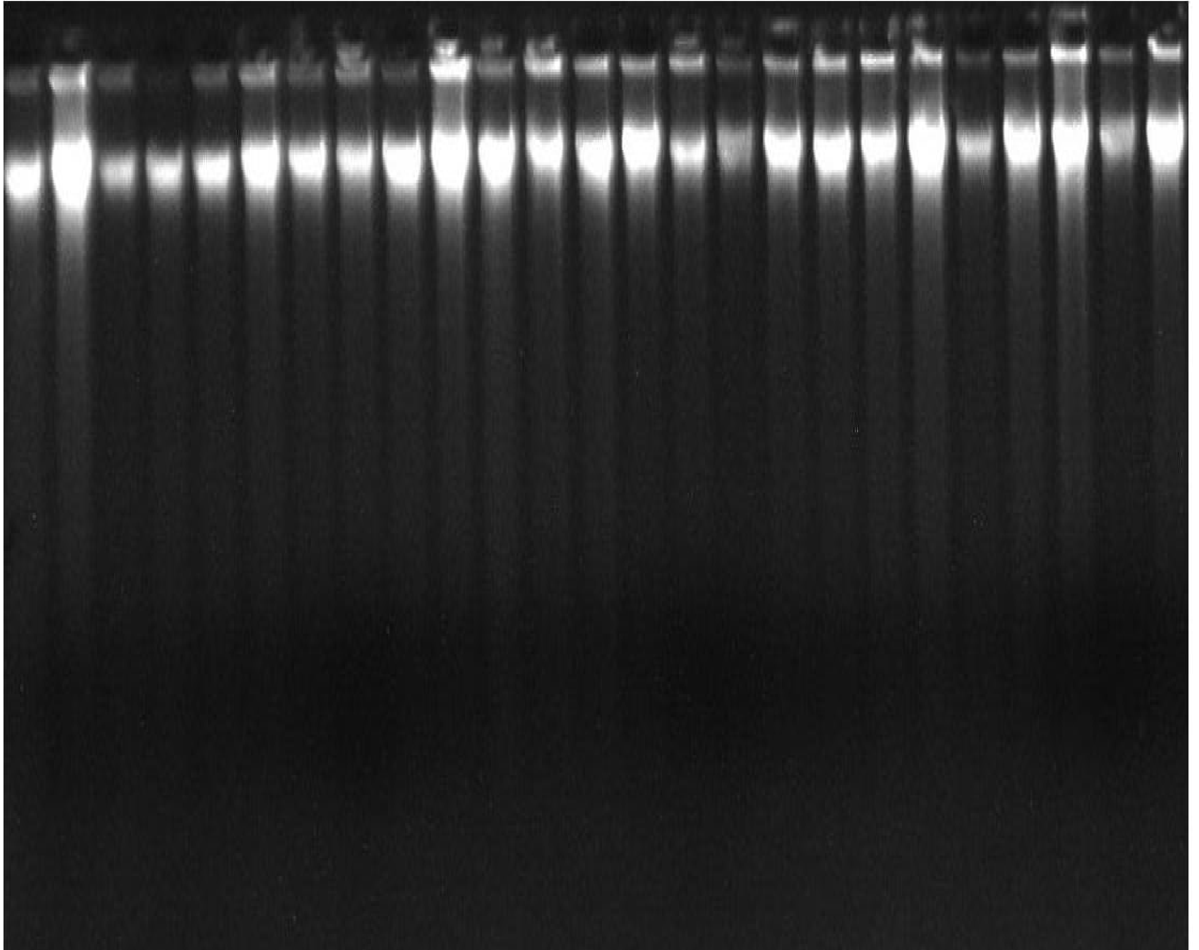

Figure 4.1: Gel Image of High Molecular Weight DNA Extracted from the Isolates.
Source: Laboratory Analysis, Bioscience Center, IITA, 2021.

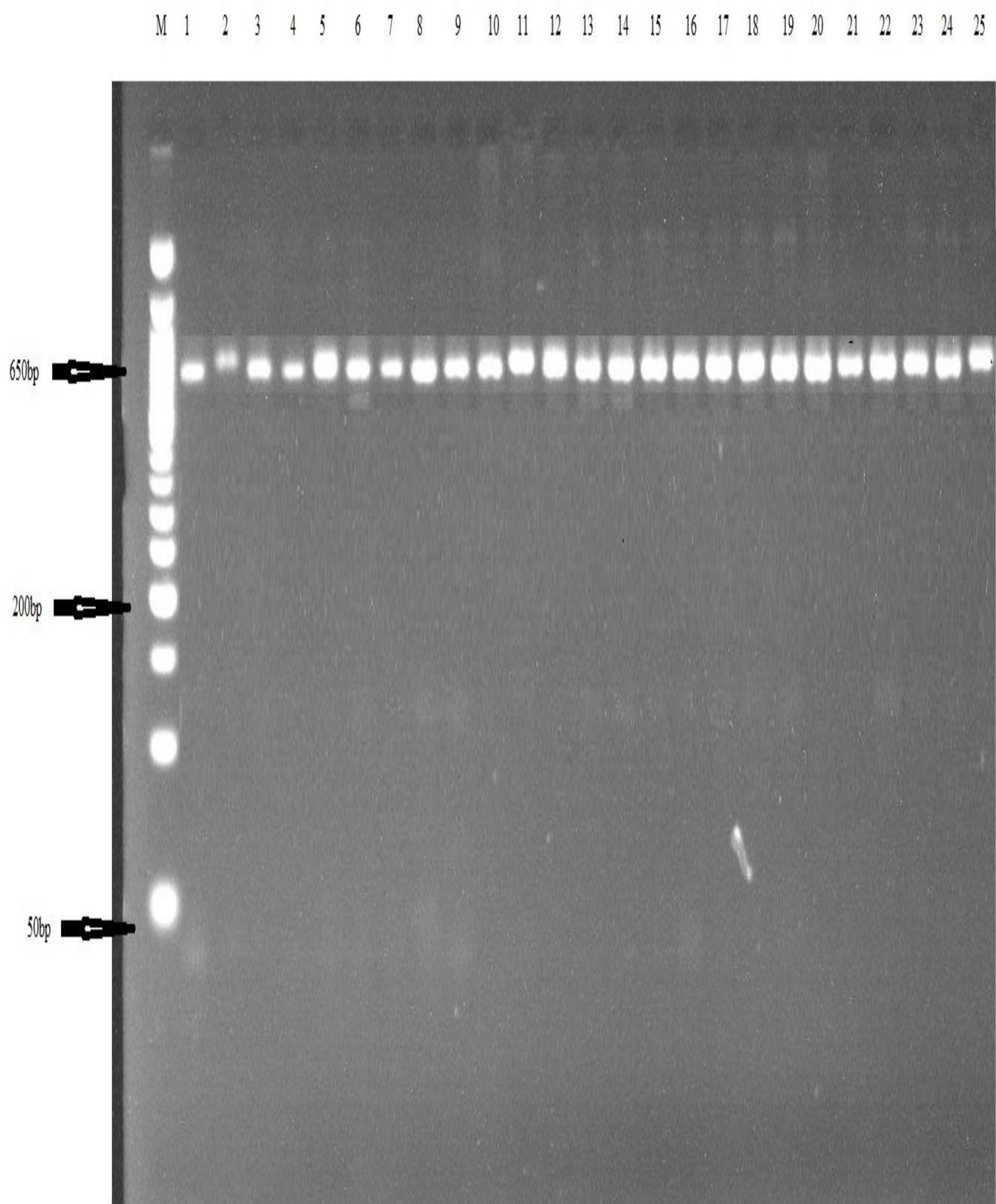

Figure 4.2: PCR Amplification of the Internal Transcribed Spacer (using ITS1 and ITS4 Primers) Region of the DNA Isolated at about 650pb.
Source: Laboratory Analysis, Bioscience Center, IITA, 2021.

Table 4.5: BLAST Results of Aligned Sequences of 25 Selected Isolates on NCBI

Isolate Code	Accession	Identified Organisms
1	MH511110	*Aspergillus flavus* strain L.A-1
2	KX098195	*Fungal sp.* strain Xmf236
3	MT139632	*Aspergillus sp.* strain 2F3F_AM
4	MG745384	*Aspergillus parvisclerotigenus* strain Maci262
5	MF668183	*Aspergillus parvisclerotigenus* strain CBS 121.62
6	MT446181	*Aspergillus flavus* strain ZMXL12
7	MT446181	*Aspergillus flavus* strain ZMXL12
8	MK226236	*Clavispora lusitaniae* strain D36
9	MT492458	*Aspergillus flavus* strain bpo4
10	MT594359	*Aspergillus flavus* strain 64-A1
11	MN565937	*Aspergillus flavus* strain omo2
12	KR611590	*Aspergillus flavus* strain PKM24
13	MK461562	*Aspergillus flavus isolate* strain BRM051244
14	MW011355	*Aspergillus aculeatinus* strain RCBBR_AEANW9
15	MG554264	*Alternaria sp.* strain GB-MG-3-3
16	MT669258	*Fungal sp.* strain BTf_8-1
17	MT446145	*Aspergillus sp.* strain ZMGL1
18	MG554231	*Aspergillus flavus* strain JN-YG-3-5
19	KP992924	*Penicillium indicum* strain zy24
20	MT594359	*Aspergillus flavus* strain 64-A1
21	MH863632	*Aspergillus alabamensis* strain CBS 125691
22	MH864265	*Aspergillus flavus* strain CBS 126856
23	KR611590	*Aspergillus flavus* strain PKM24
24	JX232269	*Aspergillus flavus* strain SGE22
25	MT072061	*Aspergillus flavus* strain QH06-08

Source: Laboratory Analysis, 2021

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Table 4.6 Unique Accessions Numbers Generated for the *Aspergillus flavus* isolates from the Bambara Groundnut Samples

Isolate Code	Accession No	Identified Organism
1	OL336880	*Aspergillus flavus* strain BV1
6	OL336881	*Aspergillus flavus* strain BV2
7	OL336882	*Aspergillus flavus* strain BV3
9	OL336883	*Aspergillus flavus* strain BV4
10	OL336884	*Aspergillus flavus* strain BV5
11	OL336885	*Aspergillus flavus* strain BV6
12	OL336886	*Aspergillus flavus* strain BV7
13	OL336887	*Aspergillus flavus* strain BV8
18	OL336889	*Aspergillus flavus* strain BV10
20	OL336890	*Aspergillus flavus* strain BV11
22	OL336891	*Aspergillus flavus* strain BV12
23	OL336892	*Aspergillus flavus* strain BV13
24	OL336893	*Aspergillus flavus* strain BV14
25	OL336894	*Aspergillus flavus* strain BV15

Source: Laboratory Analysis, Bioscience Center, IITA, 2021.

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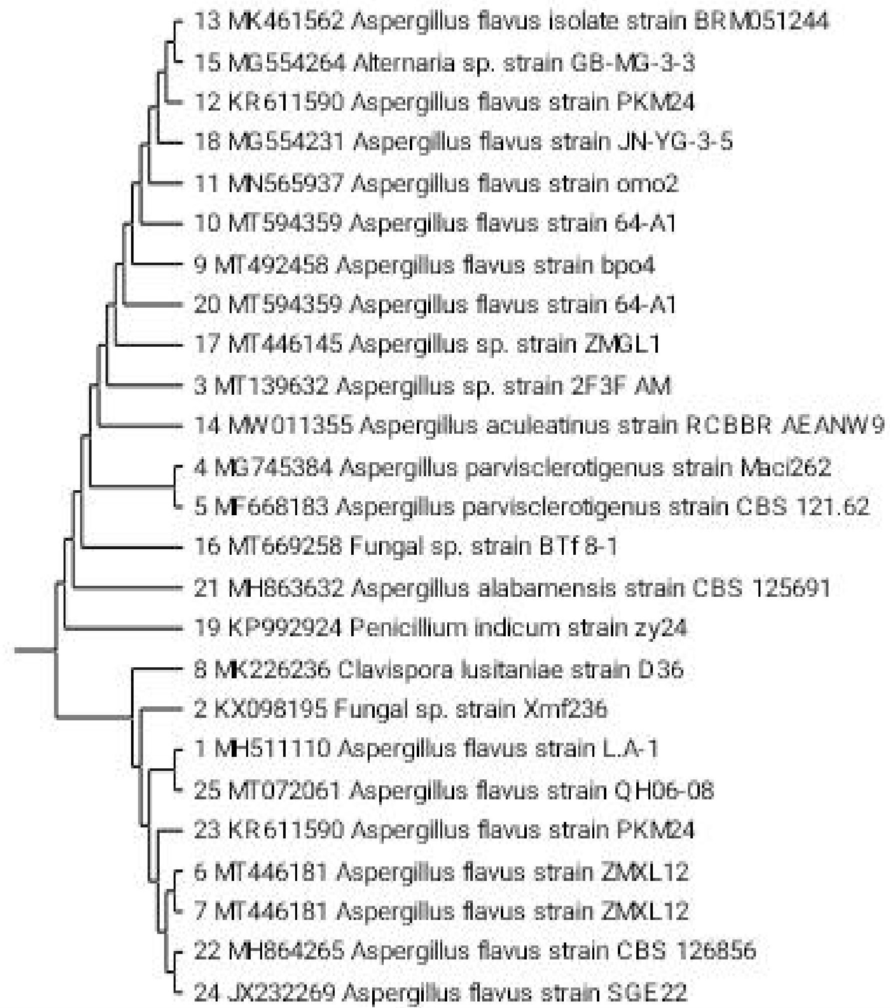

Figure 4.3: Phylogenetic Relationship between the 25 Isolates identified using Molecular Techniques

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The evolutionary history was inferred using the UPGMA method[1]. The optimal tree is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method[2] and are in the units of the number of base substitutions per site. This analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 926 positions in the final dataset. Evolutionary analyses were conducted in MEGA11[3].

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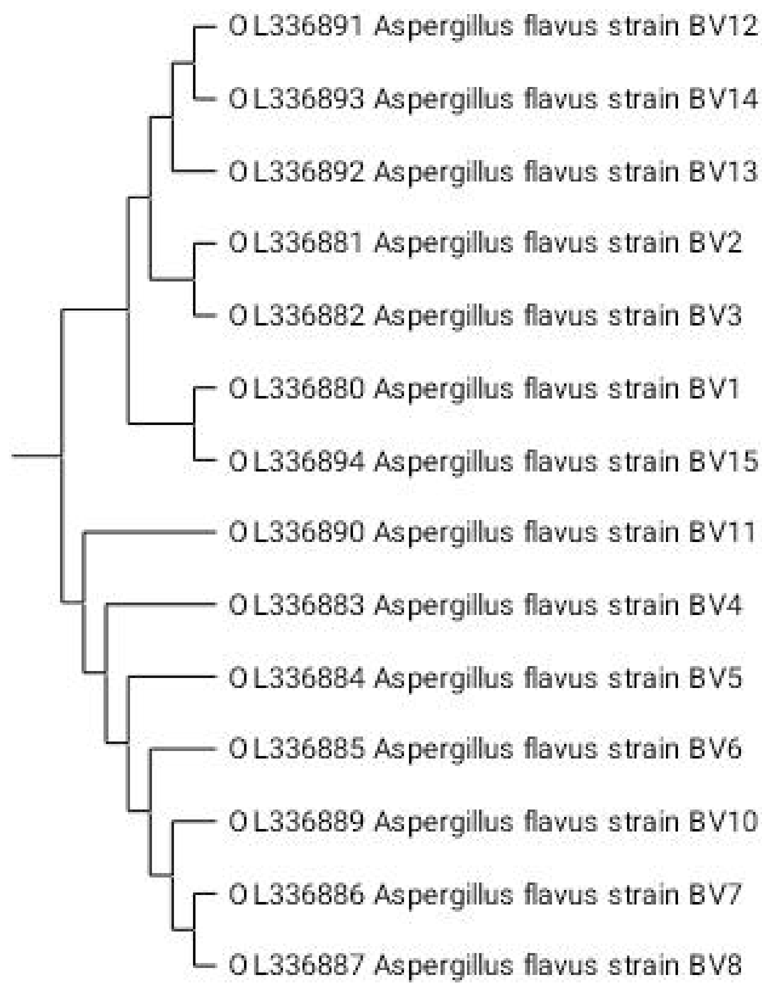

Figure 4.4: Phylogenetic Relationship between the 14 Isolates Deposited on NCBI Gene Bank

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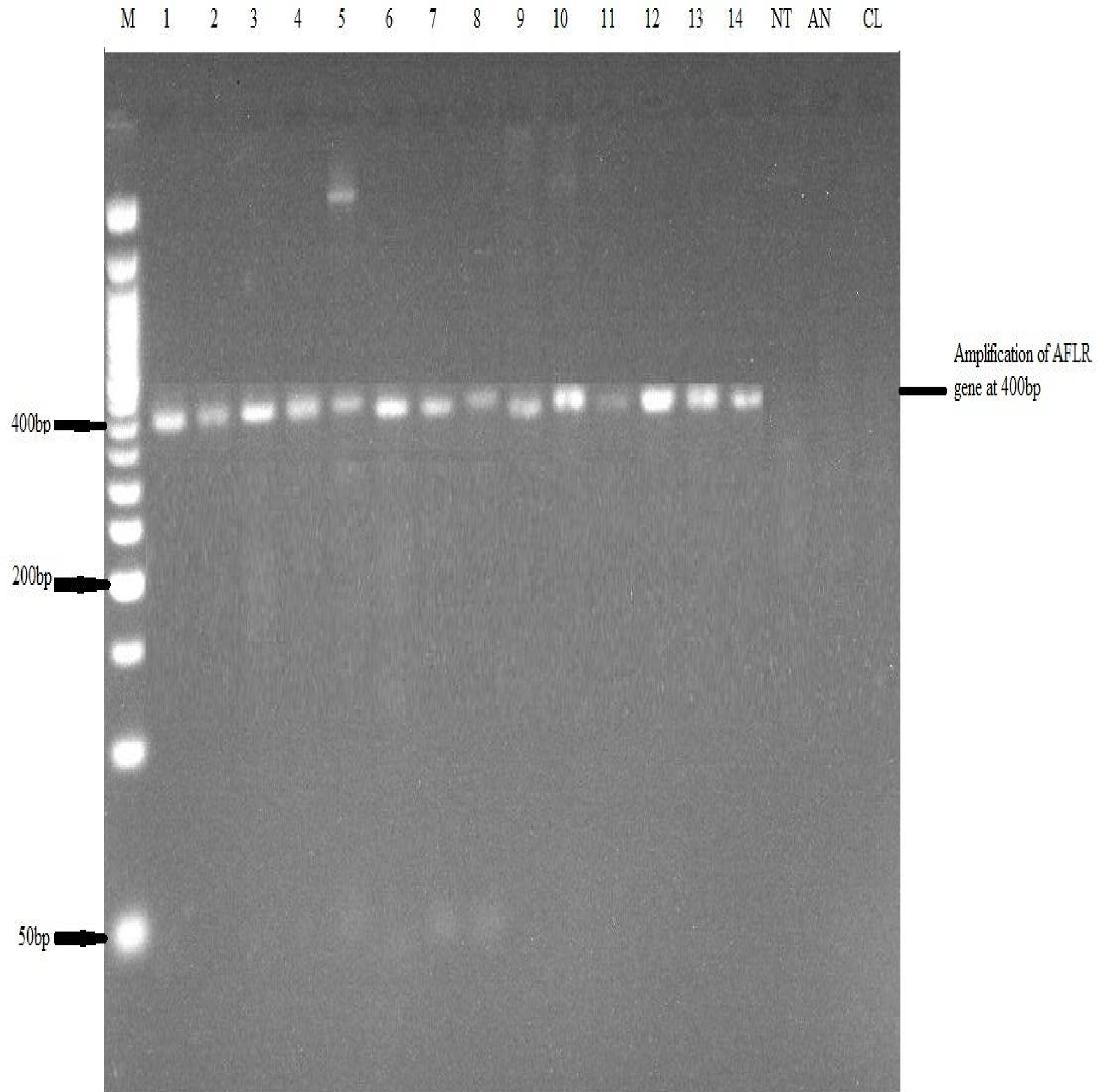

Figure 4.5: PCR amplification of the aflR gene of the isolated DNA.

Lane M is a 50bp ladder, lane 1-14 are *A. flavus* isolates, lane NT is a No Template (control), lane AN is *A. niger* (control) while lane CL is *Curvularia* sp (control).

Source: Laboratory Analysis, Bioscience Center, IITA, 2021.

4.2 Discussion of Findings

This study revealed the association of different species of fungi. The most dominant species among these fungal isolates as shown in my findings were *Aspergillus*. The occurrence of these fungal isolates might be due to the ability of fungal isolates to adapt to the prevailing conditions in the environment of the Bambara groundnut, and this in line with the report of[4,5,6] who stated that bambara nut provides significant quantity of nutrients and enabling environment for fungal growth.

A sum total of eleven (11) fungi isolates including seven different genera were isolated from the Bambara groundnut in this study. Four (4) of these fungi were different species of the genus *Aspergillus* (*niger, flavus, fumigatus* and *orchraceous*). One fungus isolated from the Bambara groundnut, which was consistent across the three markets, is *Aspergillus flavus*. *Aspergillus flavus* is referred to as a mould and is characterized with abundance of spores/conidia in the environment. The high frequency of fungal isolates found in this study corresponds to the report of[8] who reported similar fungal isolates with frequencies from Bambara nuts sold at Nsukka Nigeria, and this may be as a result of same geographical location and method of isolation.

The majority of fungal species were found in greater abundance in new main market than in other locations, this is followed by Abakpa Market and then Ogbete market. *A. parasiticus* was not isolated from any of the Bambara groundnut sampled and this is in accordance with the study conducted by Olagunju *et al*[7] who failed to isolate *A. parasiticus* from Bambara nut. He stated that this might be as a result of better sanitary practices in some parts of South Africa.

Aspergillus flavus found in this study is a known mold that produces afaltoxin and and aflatoxin have been linked to human and animal health issues.Aflatoxins detected in

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this study might be as a result of a number of factors, including the use of infested Bambara groundnut seeds for planting, infestation of the farm land prior planting, insufficient post-processing handling practices, storage of the seed in an unconducive environment and open display of the seeds in basins and bowls to the messy of these ubiquitous molds.According to the findings of this study, all market samples tested positive for aflatoxins, with overall aflatoxin contamination levels ranging from low to high, Abakpa (67 ppb), New main market (72 ppb) and Ogbete (80 ppb). The high level of aflatoxin contained in the Bambara groundnut samples found in this study corresponds to the report of[8], and this may be a result of low storage temperature of 25°C. It was found that lower temperature of 25 °C favours the synthesis of aflatoxin.

The mucorales are responsible for the majority of human sickness, *Rhizopus* spp., *Aspergillus* spp., and *Penicillium* spp. and *Altenaria* spp causes the majority of human sickness. Diabetes mellitus, neutropenia, long-term immunosuppressive medication, chronic prednisone use, iron chelation therapy, and severe malnutrition are all risk factors for the host. Fungi belonging to the genera *Altenaria* is the most frequent mold that causes asthma and increases disease severity and mortality, they primarily induce allergies in adults who are susceptible to respiratory infections[9]. Aflatoxins are important because some are extremely toxic to a variety of animals; for example, 10ppb AFB1 consumed regularly by rats can lead to fatal liver cancer, and aflatoxin in slightly higher concentrations of a few hundred Parts per billion (ppb) — can cause a wide range of ill effects in wild and domestic animals. In the United States, an aflatoxin contamination of 20ppb is allowed in feed grains and feeds; however, there is no tolerance for aflatoxin contamination in foods meant for human consumption.

Total aflatoxins (the sum of AFB1, AFB2, AFg1, and AFG2) have a guideline level of 20g/kg in food in the United States. For total aflatoxins in food, the European

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Community has a more restrictive ML of 4g/kg. Aflatoxin-free foods for infants are also required in Europe, as is an ML of 0.1g/kg for processed cereal-based foods and baby foods for newborns and young children[3]. Continuous exposure to aflatoxins-contaminated food and feed can cause immune system suppression in humans and animals, increased viral load in HIV and AIDS patients, infertility in men, liver cancer, and even death. Since their discovery, evidence has accumulated that aflatoxins causes liver cancer in humans and are linked to up to 28% of all incidences of liver cancer globally.

Cancers caused by aflatoxins are the fifth and seventh most prevalent cancers in men and women, respectively. Due to deliberate rejections of export crops and animal products with unacceptable levels of aflatoxins, Africa loses roughly 1.5 billion Naira (about $500 million) in export trade each year. The EU rejected 24 Nigerian agricultural products due to aflatoxins and pesticide residues, including groundnuts, which were specifically rejected due to aflatoxins. In addition, the EU's restriction on the import of five (5) Nigerian commodities resulted in a loss of $671.1 billion in import revenue. As a result, attempts to export aflatoxins-contaminated crops have resulted in significant trade losses and diplomatic embarrassment for Nigeria[10].

In this study, nested PCR of the aflR gene which regulates the aflatoxin biosynthesis by controlling the expression of the nor-1 and ver-1 gene in *A. flavus*, gave a positive result for all 14 *A. flavus* isolates screened for aflatoxin production by amplification of the aflR gene at the expected base size of 400bp (figure 4.6). This result is in accordance with previous studies[11,12]. In this study, it was discovered that the persistence of *Aspergillus flavus* in all three market samples might be due to high nutrient level and thriving environment for these isolates.

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Chapter Five

Conclusion

5.1 Summary of Findings

Bambara groundnut is an important underutilized indigenous crop which is used in a variet of food applications because of it nutritional profile. It is used as a food supplement in cereal-based diets and also as whole meal. It grows near or under the soil hence it ability to act as an inculum for harmful fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* which are fungi producing aflatoxin. Bambara groundnut purchased form three different markets within Enugu Metropolis were screened for the presence of aflatoxin producing fungi. The three markets were selected because the inhabitants of Enugu metropolis eat different delicacies prepared from Bambara groundnut.

The finding from this present study showed that the Samples were heavily contaminated with different fungi genera including *Aspergillus* spp, *Penicillium* spp, *Rhizopus* spp among other fungi genera. *Aspergillus flavus* ranked the highest of the *Aspergillus* species contaminating the sampled Bambara groundnut. Total aflatoxin quantification of the Bambara groundnut carried out in this present study showed that the samples were having concentrations of aflatoxin above the recommended concentration for nuts (20 ppb); Abakpa (67 ppb), New main market (72 ppb) and Ogbete (80 ppb).

25 fungal isolate morphologically identified as *A. flavus* were characterized using molecular techniques. 14 out of the 25 isolates were identified as *A. flavus* with sequence alignement of the isolate sequences on NCBI Genebank database. Unique accession numbers for these isolates were generated. The *A. flavus* isolates were

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characterized for Aflatoxigenicity and all 14 isolates showed positive results indicating the presence of the Aflatoxin regulatory gene in the isolates.

5.2 Conclusion

This research work showed that the Bambara groundnut purchased from the three markets in Enugu Metropolis contains high level of Aflatoxin producing fungi. The most prominent among these fungal isolates as shown with the results of this study were *Aspergillus flavus*, *Curvularia* spp and *Aspergillus niger*. Molecular Characterization of the fungal isolates, confirmed the presence of the Aflatoxin Regulatory Gene in the *A. flavus* screened using Molecular techniques. The consumption of Bambara groundnut contaminated by Aflatoxin producing fungi species poses major health risks as aflatoxin is harmful to the human health due to it carcinogenic and teratogenic nature.

5.3 Recommendations

I. There is a need to enhance knowledge about the potential risk involved in the use of Bambara groundnut as supplementary feeding for infants as well as adults.

II. Research has shown that Bambara groundnut serves as an inoculum for fungi growth, hence, biocontrol products should be developed for the control of Aflatoxins in Bambara groundnut.

III. Farmers should be trained and educated on the use of bio-control products on farmland in order to drastically reduce the presence of the pathogenic fungi on the farmland.

5.4 Contribution to Knowledge

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The data generated from this study contributes to existing knowledge on the use of molecular technique for the characterization of aflatoxin producing fungi. It also contributes to existing knowledge on the possible contamination of Bambara groundnut with aflatoxin.

5.5 Area of Further Research

There is the need to focus on the development of Bio-control micobes for combacting Aflatoxin problem on Bambara groundnut.

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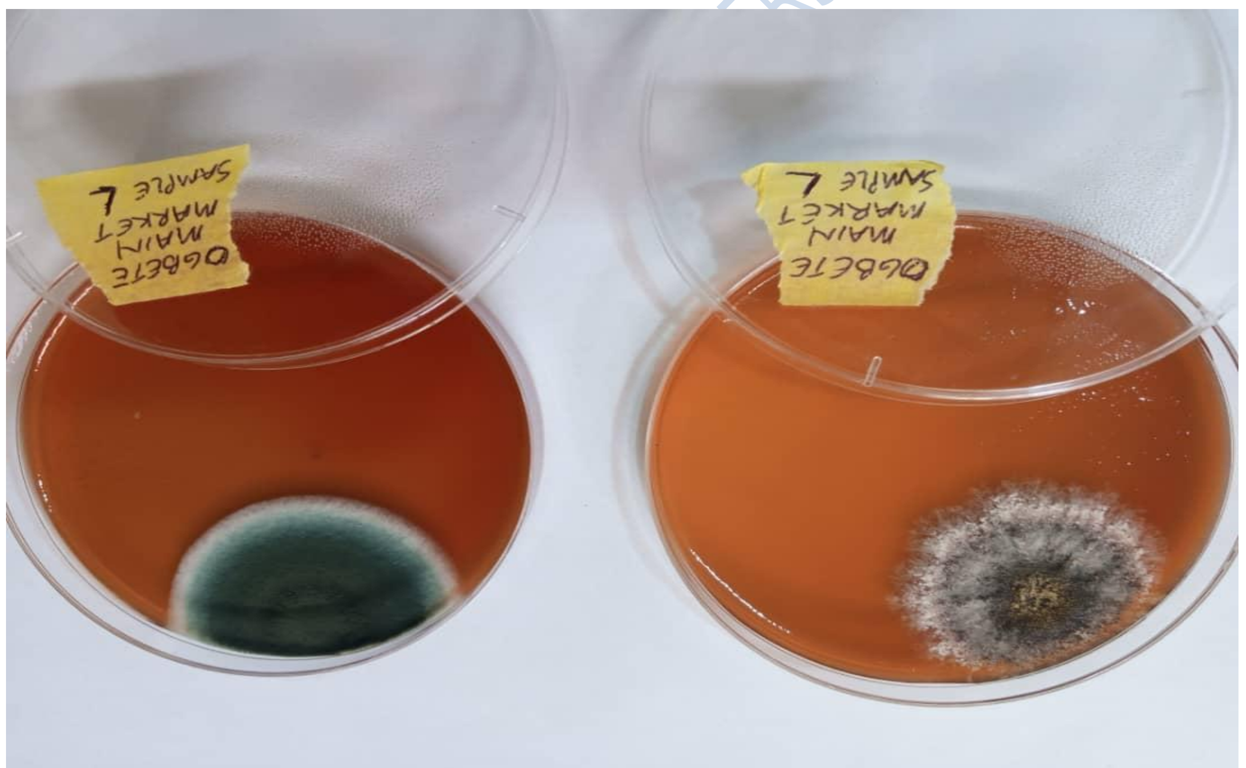
Appendices



Fungi Isolate on SDA Agar.
Source: Laboratory Analysis, 2021

Fungi Isolated from Ogbete Market.
Source: Laboratory Analysis, 2021

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Fungi Isolated from New Market.
Source: Laboratory analysis, 2021

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Fungi Isolated from Abakpa Market.
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Fungi Isolated from Abakpa Market.
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Isolation of Genomica DNA from Fungi Isolates
Source: Laboratory Analysis, Bioscience Center, IITA, 2021.

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Preparation of Samples for Electrophoresis
Source: Laboratory Analysis, Bioscience Center, IITA, 2021.

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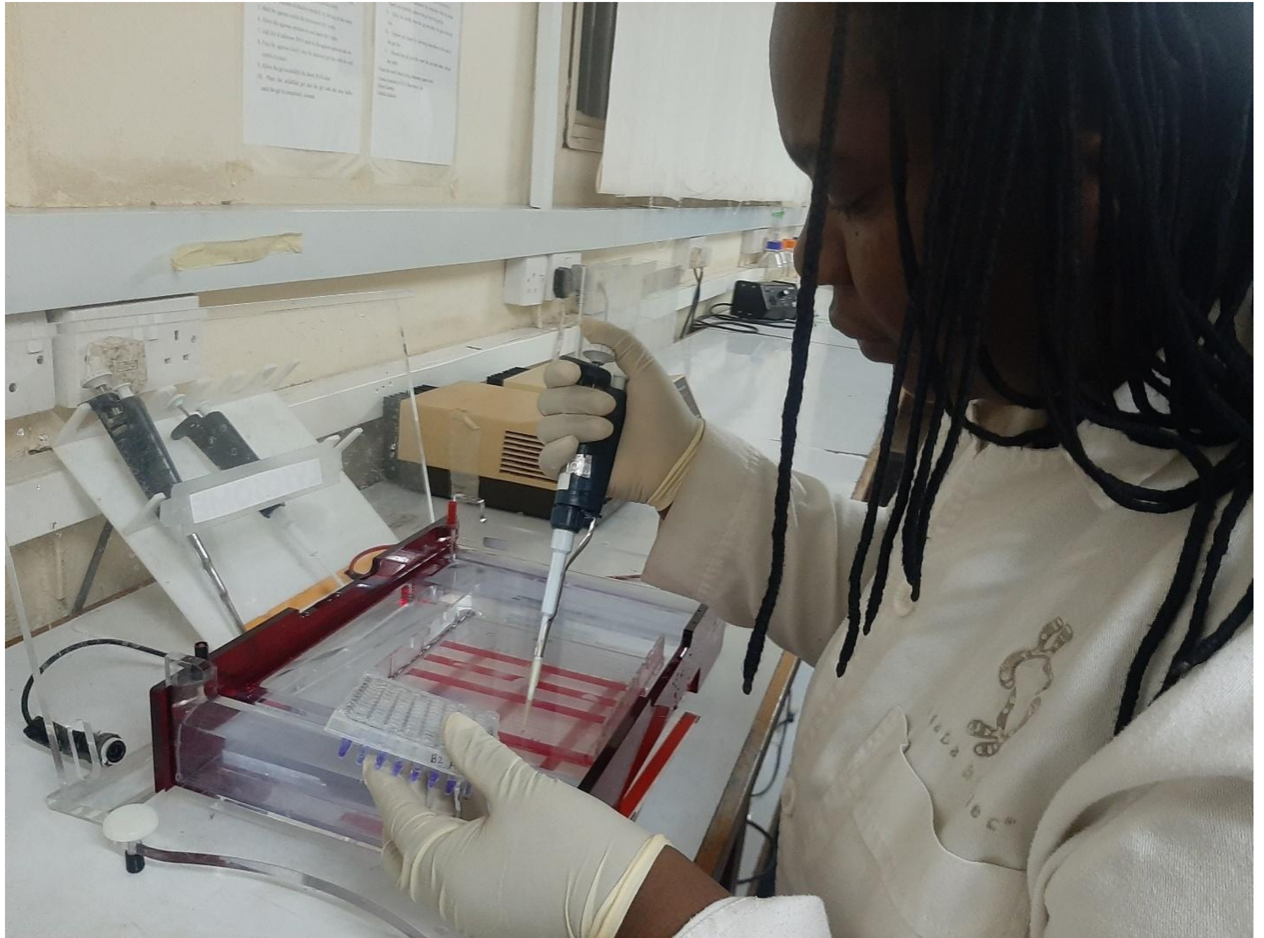

Gel Electrophoresis
Source: Laboratory Analysis, Bioscience Center, IITA, 2021.

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Gel Documentation of Electrophoresis Analysis
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Polymerase Chain Reaction setup
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Preparetion of Samples for Polymerase Chain Reaction
Source: Laboratory Analysis, Bioscience Center, IITA, 2021.

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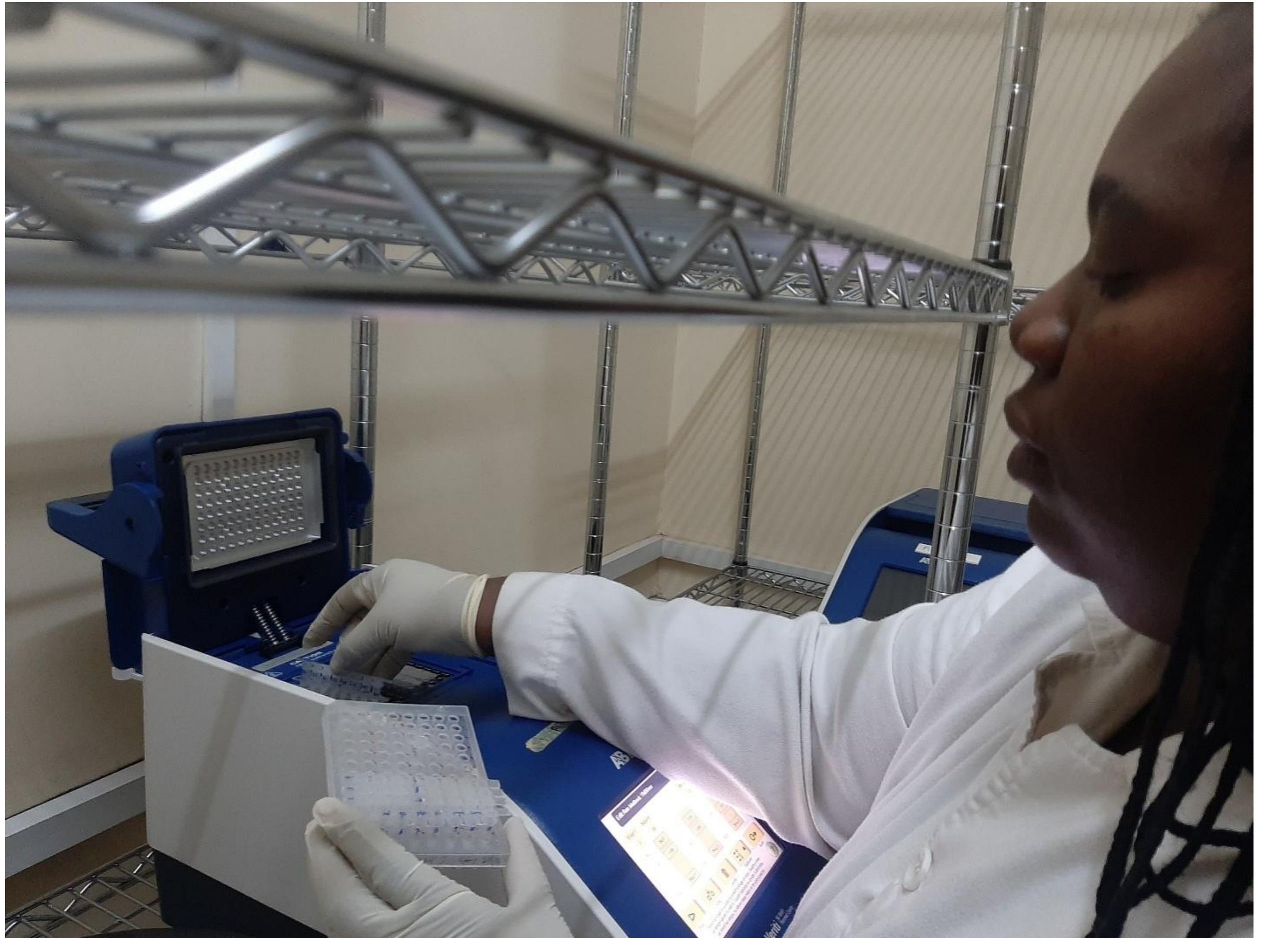

Loading of Samples on Thermal Cycler
Source: Laboratory Analysis, Bioscience Center, IITA, 2021.

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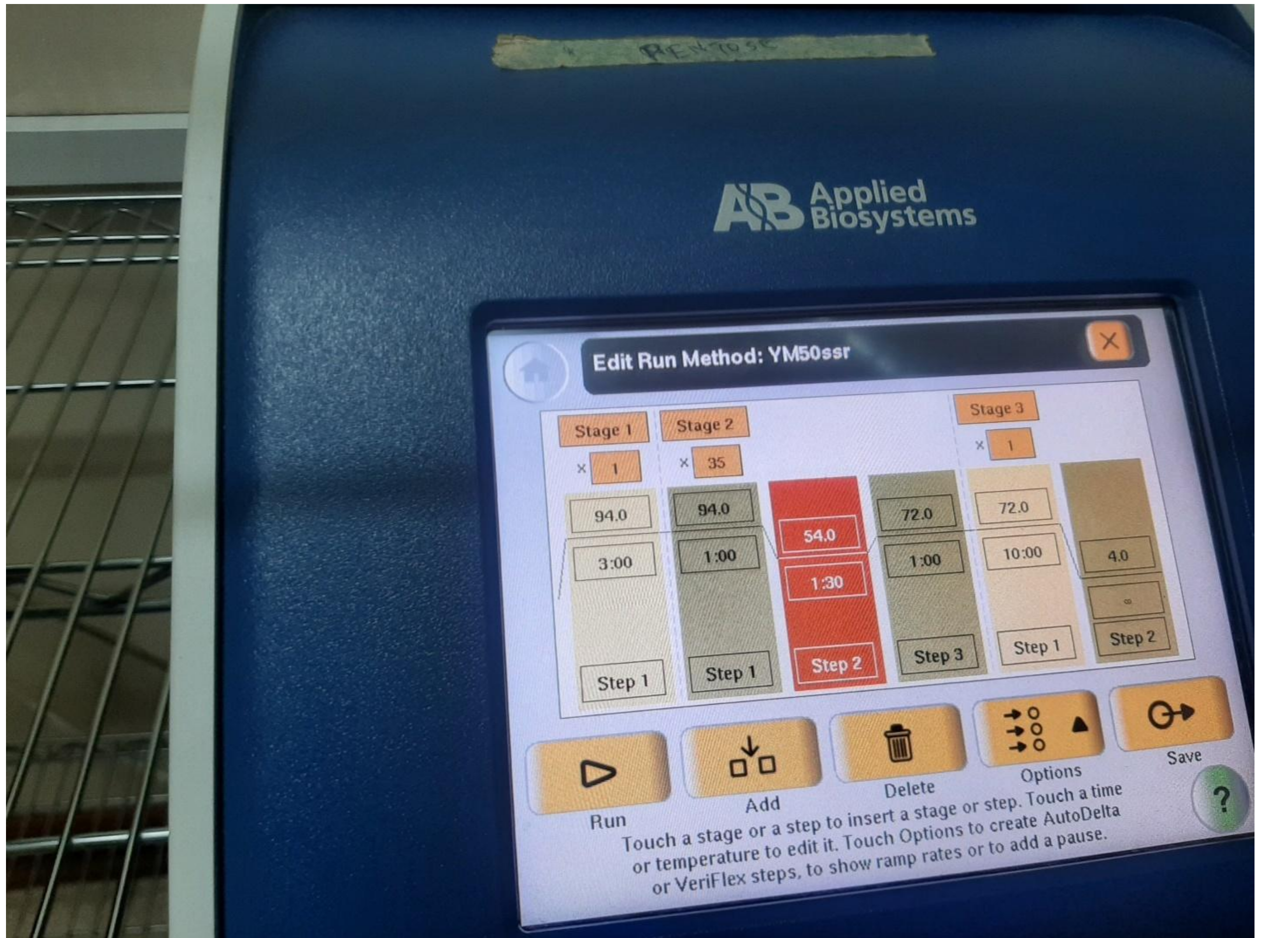

Polymerase Chain Reaction Profile used for Analysis
Source: Laboratory Analysis, Bioscience Center, IITA, 2021.

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(DISEASE DIAGNOSIS/CONTROL AND FEED ANALYSIS LABORATORY)
Plot 17, Block 1, 19th Avenue, Oluyole, Ibadan, Oyo State

13th August, 2021.

TO: Mrs. Iwu Victoria,
Lead City University,
Ibadan.

Dear Ma,
Below is the result for Aflatoxin test carried out on your samples of Bambara groundnut submitted for analysis.

A. Aflatoxin Test Results

SN		AFLATOXIN STANDARDS	OPTICAL DENSITY (Reads@ 450nm)	TOTAL AFLATOXIN CONCENTRATION IN THE SAMPLE (ppb)
1	AFLATOXIN TEST STANDARDS	0	2.643	
2		4	2.537	
3		10	1.435	
4		20	0.595	
5		40	0.370	
1	TEST SAMPLES	Sample A	1.947	70
2		Sample D	1.994	68
3		Sample F	1.978	69
4		Sample H	1.811	75
5		Sample N	1.753	80

AFLATOXIN REGULATIONS

EUROPEAN UNION LIMITS FOR HUMAN FOODS	COMMODITY	MAXIMUM LEVEL
	Groundnuts (peanuts) and other oilseeds for sorting or processing	15 ppb
	Groundnuts (peanuts) and other oilseeds intended for direct human consumption	4 ppb
	Almonds, pistachios and apricot kernels for sorting or processing	15 ppb
	Almonds, pistachios and apricot kernels intended for direct human consumption	10 ppb
	Hazelnuts and Brazil nuts for sorting or processing	15 ppb
	Hazelnuts and Brazil nuts intended for direct human consumption	10 ppb
	Tree nuts, other than those listed for sorting and processing	10 ppb
	Tree nuts, other than those listed intended for direct human consumption	4 ppb
	Dried fruit for sorting and processing	10 ppb
	Dried fruit intended for direct human consumption	4 ppb
	Maize and rice (including brown rice) for sorting and processing	10 ppb
	All cereals, including maize and rice, intended for direct human consumption	4 ppb
	Spices (capsicum, pepper, nutmeg, ginger, turmeric) **including white and black pepper);** ***Myristica fragrans*** **(nutmeg);** ***Zingiberofficinale*** **(ginger);** ***Curcuma longa***	10 ppb
	spices: ***Capsicum spp.*** **(dried fruitsthereof, whole or ground, includingchillies, chilli powder, cayenne andpaprika);** ***Piper spp.***	5ppb
	Milk (raw milk, milk for manufacturing of milk based products and heat treated milk)	0.05 ppb
	Baby foods and processed cereal based foods for infants and young children	-
	Infant formulae and follow-on formulae, including infant milk and follow-on milk	0.025 ppb
	Dietary foods for special medical purposes intended specifically for infants	0.025 ppb

USA LIMITS FOR HUMAN FOOD	COMMODITY	MAXIMUM LEVEL
	All foods except milk	20 ppb
	Milk	-

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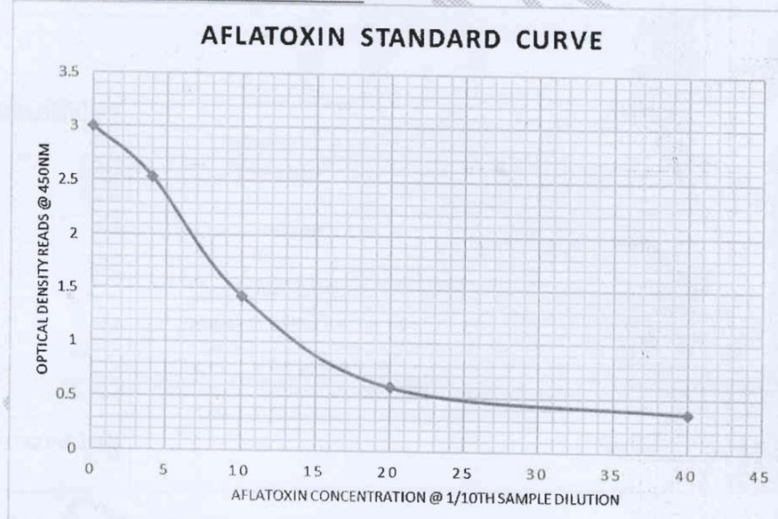
	NAFDAC/EU/USFDA/CFIA/ANAC Limits For Animal Feed and Feed Raw Materials	COMMODITY	MAXIMUM LEVEL
		Animal Finished Feed	20ppb
		Animal feed Raw Materials	20ppb

Note:
ppb: Part per billion
NAFDAC: National Agency for Food and Drug Administration and Control
EU: European Union
USFDA: United States Food and Drug Administration.
CFIA: CANADIAN Food Inspection Agency
ANAC: Animal Nutrition Association of Canada

AFLATOXIN STANDARD CURVE



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Biodata

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Plateau High School, Laranto, Jos.	(1991-1997)	SSCE
Plateau State Polytechnic, Plateau state.	(2001-2003)	National Diploma
Federal Polytechnic Bauchi	(2004-2006)	Higher National Diploma
Lead City University	(2017-2018)	Post graduate Diploma
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C. Working Experience:

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International Institute of Tropical Agriculture

II. Research Supervisor — 2014-2016
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D. Awards and Fellowships (if any) Non

E. Professional Membership:

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Biotechnology Society of Nigeria
Microbiology Society of Nigeria

F. Publications (if any)

3rd FASCON Conference 2022

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Signature **Date**

University Compliance Certification

This is to certify that the thesis by Iwu, Chiyaka Victoria with matric number LCU/PG/001001 in the Department of Biological Sciences, Faculty of Basic Medical and Applied Sciences, Lead City University, Ibadan is in full compliance with the approved University Format and Style.

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Signature **Date**

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