MOLECULAR MECHANISM OF RESISTANCE IN KENYAN GROUNDNUT (Arachis hypogea) AGAINST Aspergillus flavus INFECTION.

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DECLARATION AND APPROVAL

Declaration

This thesis is my original work and has not been presented for an award of a diploma or conferment of degree in any other university or institution.

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DEDICATION

This dissertation is dedicated to my late father Mr. Martin Okayo and my mother Mrs. Rose Okayo for their love, affection and sacrifice.

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ABSTRACT

Pathogenesis of *Aspergillus flavus* on groundnut is a key concern on human health. Much success has been achieved by the application of atoxigenic strains of *A. flavus*, improvement of cultural practices and chemical application for controlling aflatoxin contamination in groundnuts. Development of aflatoxin resistant cultivars is more sustainable strategy for ensuring quality food. However, there is lack of sources of resistance and biological information for molecular and biotechnology applications. This study was therefore conducted to identify the sources of resistance and contribute to the understanding of genes involved in *A. flavus* resistance in groundnuts. Pure isolates of *A. flavus* was isolated using morphological and molecular approaches. Thirty gene bank accessions were evaluated in the green house and the pods harvested for bioassay in the laboratory. Differentially expressed genes in response to *A. flavus* infection were identified through microarray. Eight genes were selected for addition scrutiny using real time PCR at a seedling stage at an interval of 2 days within a 7-day period. Five isolates were identified to be pure toxigenic strain of *A. flavus*. Screening the genotypes reveal a highly significant ($P<0.001$) difference of aflatoxin contamination, incidence and severity. The incidence had a mean of 23.5% and a range between 7.5% to 48.5%. All except genotype 12 (GBK005111) had percentage incidence greater than 10%. The severity index had a mean of 0.25 and a range of 0.049 to 0.58. Genotype 12 (GBK005111) had the lowest mean rating for severity while genotype 1 (GBK000423) had the highest. Hierarchical clustering based on the incidence, severity and aflatoxin contamination grouped the genotypes into 3 with cluster 3 containing 7 promising genotypes that had lower levels of infection and contamination. A large-scale microarray analysis identify total of 163 genes that were differentially expressed as determined by log2 ratio > 1.5 of the fold change out of which 63 genes were upregulated while 100 genes were down regulated. 57 genes differentially expressed had a putative function assigned to them while 106 genes were hypothetical. The upregulated genes with putative functions were grouped into 9 functional categories based on dominant biological function. The eight selected genes reveal an intricate network of gene expression patterns in a sequential order in both resistance and susceptible lines at a seedling stage with the peak level indicating the crucial time for gene action. This study successfully cultured and isolated 5 toxigenic isolates of *A. flavus* and submitted them to the gene bank where they were allocated accession numbers LC567154, LC567155, LC567156, LC567157 and LC567158. Different response to *A. flavus* and aflatoxin contamination exist in Kenyan groundnuts germplasm held in gene bank. GBK005111 (genotype 12) exhibited low values for incidence, severity and lower aflatoxin contamination accepted by the Kenya Standards. This genotype represents a relevant tool for the breeding program for resistance to *A. flavus* as a potential gene donor. The transcriptomics analysis reveals differential gene expressions and the transcripts upregulated are confirmed to be involved in resistance mechanisms. These genes are key tools in identifying biomarkers for breeding, potential candidate genes for transgenic manipulation, and will help in understanding complex plant-fungal interaction for future downstream research.

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ACRONYMS

AFB1:	Aflatoxin B1.
AFB2:	Aflatoxin B2.
AFBO:	AFB1-exo-8,9 epoxide.
AFG1:	Aflatoxin G1.
AFG2:	Aflatoxin G2.
CPA:	Cyclopiazonic acid.
DNA:	Deoxyribonucleic acid.
DRR:	Disease resistance response proteins.
ELISA:	Enzyme linked immunosorbent assay.
ERF:	Ethylene responsive transcriptional factors.
EST:	Express Sequence Tags.
EU:	European Union.
HCC:	hepatocellular carcinoma.
IARC:	International Agency for Research on Cancer.
ITS:	Internal transcribed spacer.
KEBS:	Kenya Bureau of Standards.
KEPHIS:	Kenya Plant Health Inspectorate Services.
LOX:	Lipoxygenase.
Mb:	Mega bites.
MPK:	Mitogen protein kinase.
NCBI:	National Centre for Biotechnology information.
NOR:	Norsoloric acid.
PAMP:	Pathogen associated molecular patterns.
PCR:	Polymerase chain reaction.
PDA:	Potato dextrose agar.
PPB:	Parts per billion.
PTI:	PAMP Triggered immunity.
rpm:	Revolution per minute.
RNA:	Ribonucleic acid.
RT-PCR	Real Time- PCR.
ul:	Microlitres.
YEP:	Yeast extract peptone.
YES:	Yeast extract sucrose.

CHAPTER ONE: INTRODUCTION

1.1 Background Information

The domesticated groundnuts is a significant crop in attaining food security in majority of households in the developing countries (Reddy *et al*., 2011). It is ranked 13th as the most important food crop and 4th important oil crop globally (Reddy *et al*., 2011). Virtually all parts of the crop are utilized either as food or feed. The kernels are largely consumed as raw, roasted or processed into groundnut butter and is a good source of vitamins, fats and proteins (Reddy *et al*., 2011). The kernels form an alternative protein source to the most households who cannot afford a regular supply of animal proteins (Tsafiyo *et al*., 2022). The haulms and seed cake provide a good source of protein feeds for the animals and can be stored as feeds during long dry season (Tsafiyo *et al*., 2022).

However, the optimal cultivation of the crop is limited by erratic rainfall patterns, terminal drought, heavy weed pressure, nutrient deficiency and diseases such as leaf spot, collar rot, rust, bud necrosis, stem necrosis rosette virus, gray moulds among others and aflatoxin contamination caused by *Aspergillus flavus (A. flavus*) (Reddy *et al*., 2011).

Aspergillus flavus infection at a pre and post-harvest period is a major factor that hinders the profitable production of groundnuts (Mutegi *et a*l., 2012). It leads to poor germination percentage and reduction in quality of nuts and its products (Reddy *et al*., 2011). Additionally, the fungus has a great impact on human and animal health in that it can lead to *aspergillosis* and/or *aflatoxicosis* (Arunyanark *et al*., 2010). *Aspergillosis* occurs when a susceptible individual inhale *Aspergillus* spores (Guto *et al*., 2016).. Although *Aspergillosis* is uncommon, it can lead to aspergic reaction, aspergilloma, fever, chills and shortness of breath (Guto *et al*., 2016). *Aflatoxicosis* occurs when aflatoxin contaminated groundnuts and/or its products are consumed (Arunyanark *et al*., 2010).

Currently there are approximately 18 know aflatoxin molecules (Benkerroum 2020). Out of these, the most studied molecules are aflatoxin B1, B2, G1 and G2 (Mutegi *et al*., 2012). Aflatoxin B1 (AFB1) is the most toxic and frequently present in many foods (Mutegi *et al*., 2012). Exposure to aflatoxin molecules can lead to malabsorption of nutrients, immune suppressions and endocrine problems, aflatoxin-

driven hepatocellular carcinoma (HCC) or liver cancer (Liu and Wu, 2010), stunted growth in children and reproductive problems (Voth-Gaeddert *et al*., 2018). The human and animal system can metabolize these molecules to yield derivatives which are toxic to the body (Benkerroum 2020). The aflatoxin BI molecule, for example, is metabolized to form an intermediate AFB1-exo-8,9 epoxide (AFBO) metabolite (Benkerroum 2020). This molecule is highly unstable and reacts with nucleic acids, proteins and phospholipids to disrupt cell structure and numerous genetic, metabolic, signaling activities of the cell (Benkerroum 2020). AFB1 can also lead to the induction of oxidative stress (OS) thereby affecting the cell integrity and functioning (Benkerroum 2020). Additionally, precursors within the aflatoxin biosynthesis pathway such as versicolorin A has toxic effects that pose a potential health risk (Liu and Wu, 2010).

Studies conducted by Mutegi *et al*., (2012) revealed as high as 7525ppb aflatoxin contamination on groundnuts samples from Western Kenya. This is higher than the locally accepted tolerant level of 10 ppb set by the Kenya Bureau of Standards (KEBS). This underscores the need for greater scrutiny of aflatoxin in groundnuts.
Considering health effects, it is extremely necessary to manage aflatoxin contamination. Both pre- and post-harvest methods have been used to reduce aflatoxin contamination in groundnuts and other vulnerable crops such as cotton, and maize. Fungicide application, use of biological control agents and various cultural practises that maintains the integrity of the pod and limits biotic and abiotic stresses have been used to minimise the aflatoxin contamination. Maintaining cultural practices had been a challenge to many resource poor farmers in Asia and Africa that contributes to 95% of the production area (Janila *et al*., 2013). The application of fungicide is also too costly for these farmers (Upadhyaya et al., 2000). Biological control through the application of atoxigenic strains of *A. flavus* that are more aggressive to outcompete the toxigenic strains had a great impact in reducing the aflatoxin contamination in groundnuts (Yu, 2012). Recent studies demonstrated that biocontrol non-aflatoxigenic strains reduced aflatoxin concentrations in treated crops by more than 80% under both field and storage conditions (Lewis *et al*., 2019). However, the employed biocontrol product's efficacy depends on several factors, including inoculum rate, formulation, application of herbicide, the soil's temperature, and the availability of water and substrate (Mohale *et al*., 2013).

For a sustainable control of the *A. flavus* infections and aflatoxin pollution, it is necessary to advance a resistant cultivar that could exclude the fungal invasion and shut the toxin synthesis (Yu, 2012). Regrettably, there is paucity of information on the groundnut gene pool that is resistant or tolerant to the *A. flavus* infection in Kenya. Screening of groundnuts genotypes held in the gene bank which have high genetic variability against *A. flavus* is therefore necessary. For adequate screening, toxigenic strains need to be identified which are specific to the groundnut host. In Kenya there is lack of report of *A. flavus* isolation specific to groundnuts host or gene bank collections holding *A. flavus* strains isolated from groundnuts.

In order to unravel the molecular mechanism of the groundnut host and *A. flavus* pathogen interaction, the candidate genes and pathways involved have to be identified. There is no documented study of gene action on Kenyans groundnuts germplasms against *A. flavus* and this may be attributed to the complexity of the groundnut genome (Yu, 2012). Molecular investigation into the groundnut and *A. flavus* interaction could therefore offer insights into the pathways responsible for the pathogen resistance. The express sequence tags, microarray and RNA seq technologies have enable the identification of gene differentially expressed in *A. flavus* infection response in crops such as maize, cotton and groundnuts (Guo *et al.*, 2011and Yu, 2012). In this study, microarray analysis was conducted that provided additional understanding of the molecular mechanisms of groundnut and *A. flavus* interaction.

1.2 Statement of the Problem

The contamination of agricultural products with aflatoxin has in recent times created a great alarm on food security in Africa (Leslie, 2015). In Kenya, the aflatoxin concentration on groundnuts has been reported to be as high as 7525ppb contrary to the standard requirement of 20ppb (Mutegi *et al*., 2012). Thus, the development of resistance in Kenyans groundnuts to *A.flavus* is essential to improve food quality and human health. The main challenge encountered in breeding for aflatoxin resistance has been the scarcity of resistant germplasm with elite agronomic traits (Liao *et al*., 2010). Additionally, due to gene mutations in the aflatoxin biosynthesis pathway (Navya et al., 2013), there is no guarantee that the isolates held in the depositories are toxigenic. There is no documented study into transcriptomics analysis of Kenyans

groundnuts in response to *A. flavus* invasion. Furthermore, there is no documented evidence to validate the involvement of transcripts from Kenyan groundnuts to defence mechanism. This study was therefore formulated to obtain pure culture of *A. flavus*, mine the sources of resistance and identify the key genes involved in resistance mechanisms.

1.3 Significance of the study

This study has the potential of identifying groundnut genotypes tolerant or resistance to *A. flavus* infection. These genotypes could provide safe foods to the populace and acts as critical gene contributor in various breeding programs. The contrasting genotypes could provide a relevant tool in generating mapping populations for quantitative trait loci and in differential gene expression analysis. The isolation and characterization of aflatoxigenic strains of *A. flavus* pathogen from groundnuts could provide a useful tool in establishing control strategies of the fungus in food products. The pure culture identified could be stored in the depositories for future characterization and for continued studies. Additionally, the methodologies used could be adopted by other studies for future characterization of the fungus within the *Aspergillus* species. This study could also provide information on the transcript abundance in host pathogen interaction on the Kenyans groundnut germplasm. The resistance genes identified could acts as genetic markers in screening for *A. flavus* resistance in groundnuts and this accelerates the breeding program.

1.4 Overall Objectives.

To identify the sources of resistance and contribute to the understanding of genes involved in *A. flavus* resistance in groundnuts.

1.5 Specific Objectives.

1. To characterize toxigenic *A. flavus* isolates from Kenyans groundnuts germplasm.
2. To identify sources of resistance for *A. flavus* colonization and aflatoxin contamination in groundnuts from the Kenyan gene bank accessions.
3. To identify differentially expressed genes in resistance verses susceptible groundnuts to *A. flavus* infection.
4. To determine the involvement of the putative groundnut genes to *A. flavus* resistance.

1.5 Hypotheses.

1. There is no aflatoxigenic *A. flavus* in the Kenyans groundnuts germplasm.
2. There is no variation in groundnut response to *A. flavus* infection and aflatoxin contamination.
3. There are no differential genes expression in resistance groundnuts to *A. flavus* infection.
4. The differential genes in groundnut response to *A. flavus* are not involved in resistance.

1.6 Outline of the thesis.

This thesis is arranged as per the rules and regulation of JOOUST post graduate doctoral thesis writing guidelines. It follows option 3 of doctoral thesis writing contained in section 4.0 subsection 4.2.3.

It has seven chapters. Chapter 1 has introduced the research presented in this dissertation by delineating the background of the study, problem statement, justification of the study, goal, objectives and limitation.

Chapter 2 presents the general literature review on the topic and brings out the groundnut crop and its role in food security; *A. flavus* and its classification, aflatoxin biosynthetic pathways, factors affecting *A. flavus* infection in groundnuts, health effects associated with aflatoxins, tolerance limits established for aflatoxin in groundnut and groundnut products, methodologies for aflatoxin determination, management strategies for aflatoxin in peanuts and milestones in high throughput strategies on groundnuts resistance against *A. Flavus*.

The subsequent four chapters are presented as a series of papers that address the four specific objectives of the study. Chapter 3 addresses the isolation and characterization of *A. flavus* from groundnuts. Chapter 4 examined the tolerance levels of groundnuts germplasm against *A. flavus* infection and aflatoxin contamination. Chapter 5 explored the transcriptomics changes in groundnuts upon *A. flavus* infection while Chapter 6 investigated the involvement of putative groundnut genes to *A. flavus* resistance. Chapter 7 has an appendix information.

1.7 Limitations

The study was limited to the evaluation of groundnut germplasm from the Kenya Agricultural and Livestock Research Organization gene bank for resistance to *A. flavus*. The agronomical attributes of these genotypes and the performance of the superior genotypes were not evaluated against the cultivars under production. This was not possible due to financial constraints. In addition, the study investigated the host-plant interaction but was limited to the host plant strategies without investigating the strategies of the pathogen to overcome the plant defence mechanism.

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CHAPTER 2: LITRATURE REVIEW

2.1 Introduction

Groundnut (*Arachis hypogaea* L.) is believed to be a native of South America in southern Bolivia and North West Argentina region on the slopes of the Andes (Talawar *et al*., 2005). The Portuguese traders and explorers assisted in the dispersal of the crop to the rest of the world (Gibbons *et al*., 1972). Global trade in the new world has led to the convergence of different groundnuts genotypes in Africa. The hybridization and selection in various ecological zones in Africa have given rise to new and distinct types compared to those found in its centre of origin (Janila *et al*., 2013). This has made Africa an important source of secondary genetic variation for groundnuts (Gibbons *et al*., 1972). Currently, groundnut cultivation is mainly done in latitude between 40^0 North and 40^0 South of the equator. These regions are characterized by a mean temperature of approximately 20 ^{0}C and an erratic rainfall range between 500 mm to 1200 mm annually (Singh, 2016). Asia and Africa continent contributes approximately 95 % of the world production area and in most cases by small scale farmers (Janila et al., 2013).

Groundnuts is an herbaceous plant belongs to the genus *Arachis* of the family Fabaceae and a subfamily *Papilionaceae*. The Arachis consists of annual and perennials cleistogamous plants with around 80 species (Krapovickas and Gregory, 1994). The *Arachis* species are unique in that the flower are borne above ground while the fruits form below ground (Krapovickas and Gregory, 1994). This species are grouped into 9 sections according to their distributions, cross compatibilities and character clustering (Krapovickas and Gregory, 1994). These section are *Arachis, Trierectoides, Erectoids, Extranervosae, Triseminate, Heteranthae, Caulorhizae, Rhizomatosae* and *Procumbentes* (Krapovickas and Gregory, 1994). Approximately 40 % of the groundnut species belongs to the *Arachis* section. This section consists of 29 diploid species and two tetraploid species known as the *A. monticola* and *A. hypogaea*. The *A. hypogea* is the most extensively cultivated species globally and it comprises of two subspecies: *Hypogaea* and *Fastigiata*. These species are distinguished by branching patterns, nature of vegetative distribution and reproductive nodes along the main stem and lateral branches (Krapovickas and Gregory, 1994)

The subsp. *hypogaea* is further divided into two botanical varieties: *Hypogaea* and *Hirsuta.* The *hypogaea* has no flower on the mainstem, has alternating floral and reproductive nodes on the lateral branches with short and relatively few trichomes. It has two market type, Viginia and Runner types (Krapovickas and Gregory, 1994). The Virginia has large seeds while Runner types are small seeded with both exhibiting less hairy characteristics. The *hirsuta* have the same characteristics as the hypogea however, their seeds are hairier. The sub species fastigiata is divided into four botanical varieties: *Fastigiata, Peruviana*, *Aequatoriana* and *Vulgaris* (Singh and Nigam, 2016). Their flowers are borne on the mainstem and have sequential pairs of floral and vegetative axes on branches. The botanical variety *fastigata* has a market type Valencia which are characterized by little branches and curved leaves (Stalker *et al*., 2016). The *peruviana* are less hairy and have deep pod reticulation while *aequatoriana* are very hairy, has deep pod reticulation, heavily branched and has purple stems. The *vulgaris* are more branched and upright and have a market type Spanish which have small kernels with reddish brown skins (Stalker *et al*., 2016).

The cultivated groundnut is a polyploid that resulted from the single hybridization event of two closely related wildtype diploid progenitors *A. duranensis* and *A. Ipaensis.* The hybridization was followed by chromosome doubling leading to allotetraploid (2n = 2x = 40). This gave rise to a large genome size of approximately 2.7GB (Bertioli *et al*., 2016). The hybridization event occurred approximately 3500 years ago and allotetraploid nature has prohibited the successive hybridization with other wild relatives (Janila et al., 2013).

Groundnut is a key crop in ensuring the food security in millions of families globally (Setimela *et al*., 2004). It is considered the second, third, fourth and thirteenth critical crop in terms of cultivated legume, source of vegetable oil, source of edible oil and food crop respectively (Taru *et al*., 2008; Shilman *et al*., 2011). Nutritionally, it has 44-56%, 22-30% and 9.5-19% oil, proteins and carbohydrates contents respectively (Shilman *et al*., 2011). Additionally, it is a rich source of dietary fibre, minerals, and vitamins (Surendranatha *et al*., 2011). Owing to its high protein contents, it has an incredible potential to mitigate the protein nutrition deficiency in poverty ridden region of the world (Surendranatha *et al*., 2011). The crop has additional benefits in

that the haulms and seed cake can be used as animal feeds and ecologically, it improves the soil fertility through nitrification (Tsafiyo *et al*., 2022).

In Kenya, the crop is predominately grown in the western part of the country and to a smaller extend in the Rift valley region, Coastal strip and Eastern region (Anonymous, 2004). In these regions, the crop is consumed or sold directly as row, roasted, boiled or fried kernels (Reddy et al., 2011). The value addition at the farm level is usually rudimentary and thus the nuts are mostly sold as whole kernels (Tsafiyo *et al*., 2022).

2.2 Production challenges

The climatic change has led to upsurge of various biotic and abiotic factors that hinders groundnuts production. An estimated yield losses running to billions of dollars annually have been attributed to sporadic rain and high solar radiation (Waliyar *et al*., 2005). Low soil fertility as a result of mineral depletion has also led to reduction in yields (Janila *et al*., 2013). A bacteria, *Ralstonia solanacearum* causing bacterial wilt and a nematode, *Meloidogyne arenaria* infection contributes to approximately 12% global yield reduction (Janila *et al*., 2013). Fungal diseases such as *Aspergillus niger* (Collar rot), *Phaeoisariopsis personata* (Late leaf spot), *Cercospora arachidicola* (Early leaf spot), *Puccinia arachidis* (Rust), *Aspergillus flavus* (Yellow mold), and *Sclerotium rolfsii* (Stem rot) also contributed significantly to yield reduction (Sharma and McDonald, 1990; Singh *et al*., 1997). *Aspergillus flavus* and *Aspergillus parasiticus* are known to be a major aflatoxin producing fungi in groundnuts (Wagacha *et al*., 2008)

2.3 Aspergillus flavus.

Aspergillus flavus (*A. flavus*) belongs to the genus *Aspergilli*. It is a filamentous fungus that exist in the soil as conidia or sclerotia and as a mycelium in plant tissue or debris. It is the most virulent saprophyte pathogen within the genus (Amaike and Keller, 2011) Micheli in 1729 was the first mycologist to identify the *aspergillus* genera with its associated species. Further classification and reclassifications identified over 200 species within this genus (Amaike and Keller, 2011). Members within this genera are known to produce a wide range of mycotoxins (Table 2.1) (Amaike and Keller, 2011).

Table 2.1: List showing mycotoxins synthesized by species within the *Aspergillus* genera

Mycotoxin	Organism
Citrinin	A. carneus, A. terreus
Patulin	A. clavatus
Tryptoquivalene	A. clavatus
Aflatrem	A. flavus
Cyclopionic acid	A. flavus
Kojic acid	A. flavus
Nitropopionic acid	A. flavus
Aspenoxin	A. flavus
Aflam	A. flavus
Aspergillic acid	A. flavus
Sterigmatocystin	A. flavus, A. nidulans, A. versicolor,
Aflatoxin	A. flavus, A. parasiticus
Fumagilin	A. fumigatus
Fumigacin	A. fumigatus
Fumitremorgin A	A. fumigatus
Gliotoxin	A. fumigatus
Verruculogen	A. fumigatus
Viriditoxin	A. fumigatus
Malformin	A. niger
Oxalic acid	A. niger
Destruxin B	A. ochraceus
Ocratoxin	A. ochraceus
Penicillic acid	A. ochraceus
Citreoviridin	A. terreus
Austdiol	A. ustus
Austocystin	A. ustus
Austamide	A. ustus
Brevianamide	A. ustus

The information provided in this table was extracted and assembled from the written works of Varga *et al*., (2015).

A. flavus has a wide host range and it affects economically important crops such as maize, linseed, sunflower seeds, beans, tree nuts and cotton (Amaike and Keller, 2011). The consumption of contaminated feeds and crops leads to detrimental effects on animals and humans (Bennett and Klich, 2003). Owing to this, the research on identification and characterization and mycotoxin profiles of the *Aspergillus* species was considered novel and it gave rise to exciting information on the effects and regulation of their infection (Varga *et al*., 2015).

2.4 Classification of *A. flavus*

A. flavus belongs to the phylum *Ascomycota*, subphylum *Pezizomycotina*, family *Trichocomaceae*, order *Eurotiales,* class *Eurotiomycetes* and *Aspergillus* genus. Characterization of the members within the *Aspergillus* genera is complex owing to the intricate and ever evolving nature of this group (Rodrigues *et al*., 2009). To maintain a taxonomical system that is practical and reliable for industrial, economic and regulatory purposes, it is imperative to have a rigorous and stable identification methods (Norlia *et al*., 2018). Therefore the polyphasic approach that encompasses morphological, biochemical and molecular means should be adopted for the reliable identification of *Aspergillus* species (Rodrigues *et al*., 2009).

The presence of a conidiophore is a morphological feature that delineate *Aspergillus* genus is from other genera (Rodrigues *et al*., 2009). However, the species identification and differentiation within the *Aspergillus* genera is complex owing to close similarity in many features (Rodrigues *et al*., 2009). The features commonly used for morphological identification are: conidial and mycelial colour, colony reverse colour, colony diameter, exudates production, exudates colour and soluble pigments, presence of sclerotia and cleistothecia (Klich, 2002). The shape and size of vesicle, conidia and stipe morphology and presence of Hülle cells are the common micromorphological identification structures (Klich, 2002). Identification based on morphological structures are usually not straight forward however, several taxonomical keys and guides are available to guide the identification process (Klich, 2002).

Biochemically, the extrolites such aflatoxins, Cyclopiazonic acid (CPA), and *aspergillic* acid are mainly used for identification and differentiation (Samson *et al.*, 2006). For example, most of *A. parasiticus* and *A. nomius* strains produce aflatoxins but are deficient in CPA. *A. flavus* can or cannot produce either aflatoxin and/or CPA (Giorni *et al*., 2007; Razzaghi-Abyaneh *et al*., 2006). From biochemical perspective, closely related species may produce similar or different mycotoxins (Table 2.1).

Molecular methods employing techniques such as Polymerase chain reaction (PCR), sequencing of genomic regions and gene expression analysis have been widely used to characterize *Aspergillus* species (Rodrigues *et al*., 2011). To increase the reliability, multilocus data should perform better for species identification (Liu *et al*., 2017). The genomic regions that are usually targeted for the amplification and sequencing for identification are: ITS (internal transcribed spacer) region, b-tubulin gene (Hebert *et al*., 2003) and calmodulin gene (Liu *et al*., 2017). The exploration of the whole genome could provide additional important information for the identification and definition of the species (Liu *et al*., 2017).

The whole genome of *A. flavus* was sequenced and found to contain approximately 36.5 Mb of nucleotides and 13,485 functional genes (Payne *et al*., 2006). Similarly, A. parasiticus genome was sequenced and found to comprise of 39.4 Mb nucleotides and 8,645 functional genes (Linz *et al*., 2014). These two Aspergillus species are known to produce aflatoxin (Table 2.1). The 30 genes involved in aflatoxin biosynthesis was found in a cluster of around 70-80 kb away from the centromere on the third chromosomes in both species (Linz *et al*., 2014). A set of five genes aflR, aflS (aflJ), laeA and veA regulate the aflatoxin biosynthesis (Linz *et al.*, 2014). There are more than 20 different types of aflatoxin produced but the most important ones are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) (Yu *et al*., 2004). There are a number of variations that occur within the aflatoxins and this triggers the urge to understand and comprehend their biosynthesis (Giray *et al*., 2007; Kumar *et al*., 2017).

2.5 Aflatoxin biosynthesis

Aflatoxin biosynthesis pathway is well documented and consists of 27 orchestrated enzymatic steps. It commenced with the transformation of acetate to first stable aflatoxin precursor norsoloric acid (NOR) (Yu 2012). The polyketide synthase gene

(PksA) and synthase genes Fas-1 and Fas-2 catalyze this conversion (Mahanti *et al*., 1996). The three genes *aflD, aflE* and *aflF* that encode ketoreductase converts the 1′-keto group in NOR to Averantin (AVN). The *aflG* gene that encodes the P450 monooxygenase catalyzes the conversion of AVN to 5′-Hydroxyaverantin (HAVN) (Yu 2012). The alcohol dehydrogenase enzyme converts the HAVN to Averufin (AVF)(Yabe and Nakajima, 2004). A series of reduction reduce the AVF to VERB that is a common precursor for the synthesis of two different biosynthetic branches. One branch leads to the formation of the aflatoxin BI and aflatoxin G1 while the second branch leads to the formation of aflatoxin B2 and aflatoxin G2. The branches undergo a series of enzymatic action with intermediates to eventual aflatoxin formation. On the first branch, VERB is catalyzed by dehydroxygenase monooxygenase to form dihydrodemethylsterigmatocystin (DHDMST) which is catalyzed by O-methyltransferase A to form dihydrosterigmatocystin (DHST). The DHST is catalyzed by O-methyltransferase B to form dihydro-O-methylsterigmatocystin (DHOMST) which acted upon by oxidoreductase to form either aflatoxin B1 or aflatoxin G1(Fig 2.1) (Yu *et al*., 2004;Yu *et al*., 2002). The second branch begins with the conversion of VERB to Versicolorin A (VERA) through the enzymatic action of aflL gene. The VERA is catalyzed by dehydroxygenase monooxygenase to form demethylsterigmatocystin (DMST) which is catalyzed by O-methyltransferase A to form sterigmatocystin (ST). The DHST is catalyzed by O-methyltransferase B to form O-methylsterigmatocystin (OMST) which acted upon by oxidoreductase to form aflatoxin B2 and aflatoxin G2 (Fig 2.1).

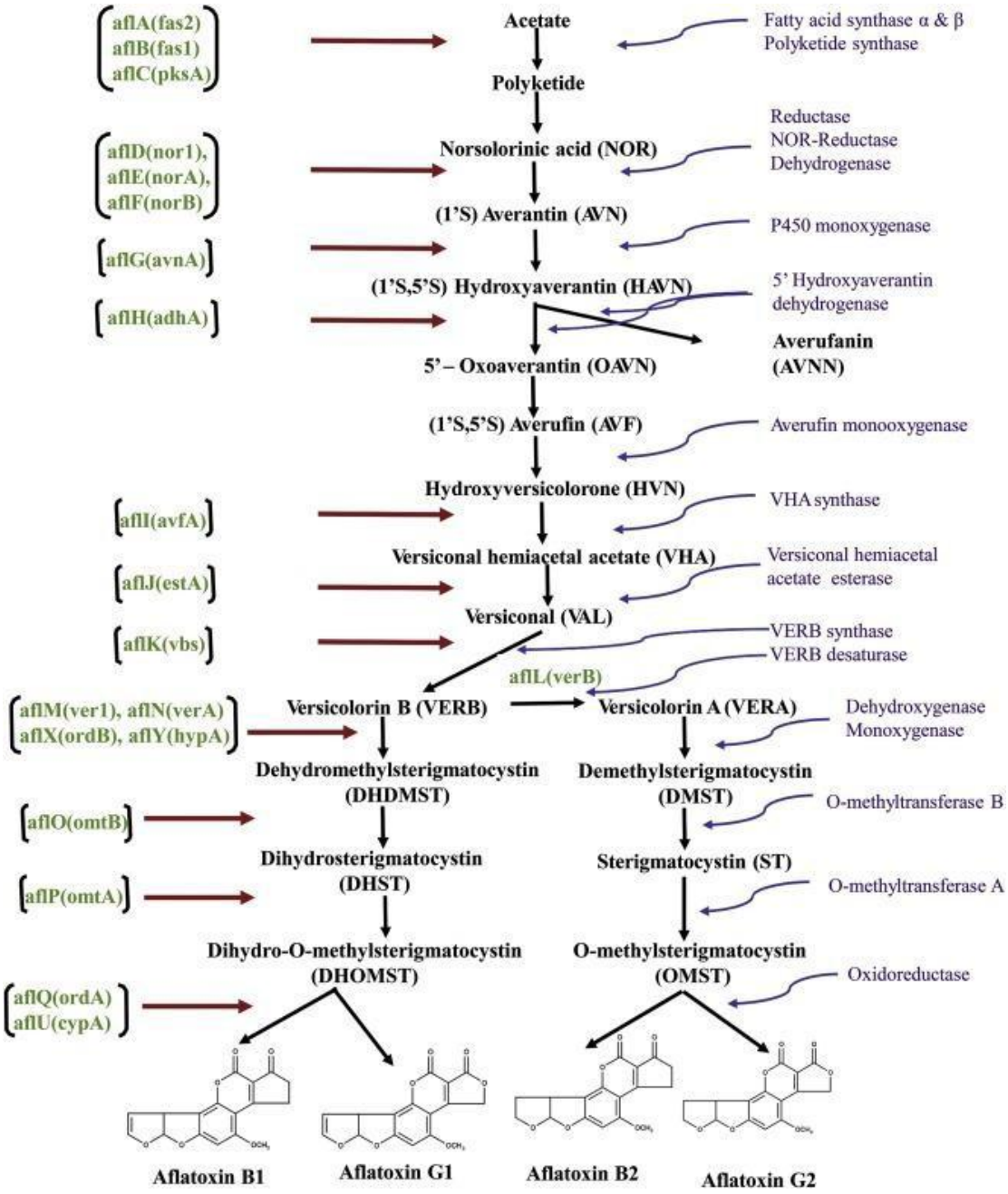

Figure 2.1: The chart of aflatoxin biosynthesis pathways leading to the formation of four types of aflatoxins. The genes in the pathways are indicated on the left side while the enzyme they encode are to the right of the chart. From the branch leading to four aflatoxins, the enzyme and genes act in both branches. The figure is based on Yabe and Nakajima, (2004)

2.6 Factors influencing aflatoxin production in groundnuts

A. flavus can infect the groundnut during the pre-harvest, harvest and postharvest stages in the food chain (Guchi, 2015). The high soil temperature and drought permits fungal spores to germinate and colonize the pod zone. The extending mycelia will

adhere to the groundnuts shells, penetrate the cell and seed coat and eventually reach the cotyledon (Guchi, 2015). According to Logrieco and Visconti, (2004), there is higher aflatoxin contamination in warm temperate zones (latitudes 26-35°) and high humidity areas.

Additionally, the rate of infection is higher when the crop experiences drought and temperature stress above 29 ^{0}C especially at the end of season (Cotty & Jaime-Garcia, 2007). Water stress reduces the plant capacity to produce phytoalexin thereby compromising its natural defense mechanism against invading fungus (Cotty & Jaime-Garcia, 2007).

Wilkinson *et al*., (2007) reported that the rate of infection is higher in acidic soils while alkaline media inhibits it. Light sandy soil enables the rapid penetration of *A. flavus*, particularly under late-season drought (Guchi, 2015). On the contrary, heavier soil has higher water-holding capacity and could reduce aflatoxin contamination level in groundnut (Torres et al., 2014). Cultural practices and insect damage that interfere with the pod integrity exacerbate *A. flavus* infection and aflatoxin contamination (Waliyar *et al*., 2008).

Late harvesting and damaging of the pods at harvest time influence *A. flavus* infection and aflatoxin contamination. The ideal harvesting time should be gauged based on the prevailing weather condition and maturity type of the groundnuts germplasm (Torres *et al*., 2014). The immature pods are predisposed more to *A. flavus* infection and aflatoxin contamination than mature ones (Sanders *et al*., 1985). This is because the immature pods will lose membrane water rapidly creating fissure that allows infection and also, they will lose the capacity to synthesize phytoalexins faster in comparison to the mature ones (Sanders *et al*., 1985). Mechanical damage during harvest provides avenues for the fungus entry at harvest in humid weather (Waliyar *et al*., 2015; Wild and Hall, 2000).

The level of aflatoxin contamination is reported to be higher at post-harvest compared to pre-harvest stages (Wild and Hall, 2000). The high humidity, temperature and moisture content during transportation and storage favors the *A. flavus* infection and aflatoxin contamination. (Waliyar *et al*., 2015). The *A. flavus* will grow optimally and colonize the stored kernels when the temperature is between 25 ^{0}C and 30 ^{0}C and

humidity levels of $0.99a_w$ in the stores (Giorni *et al*., 2009). Additionally, leaking stores and improper ventilation in the ware houses promote *A. flavus* infection and aflatoxin contamination (Waliyar *et al*., 2015).

2.7 Adverse effects associated with aflatoxins on health.

Approximately 5 billion peoples are exposed to aflatoxin contamination. The toxin accounts for 40 % prevalent disease affecting health (Strosnider *et al*., 2006). Depending on ingestion levels, susceptibility, age, gender and duration of exposure, the effect can vary from simple irritation to death (Mutegi *et al*., 2012). Aflatoxin doses greater than 6000 ppb can lead to acute toxicity characterized by vomiting, jaundice, oedema, acute hepatitis and occasionally death (Mutegi *et al*., 2012: Probst *et al*., 2007). Regular consumption of contaminated foods can lead to chronic diseases with aflatoxin-driven hepatocellular carcinoma (HCC) or liver cancer and bile duct hyperplasia being most severe and frequent (Strosnider *et al*., 2006; Liu and Wu, 2010). Recent studies have indicated that other organs such as pancreas, bone, viscera, kidney and the bladder cancers have developed as a result of aflatoxin contamination (Fouad *et al*., 2019). Other than cancer, the chronic exposure leads to other severe diseases such as teratogenicity, mutagenicity, immunosuppression, cytotoxicity and estrogenic effects in mammals (Klvana & Bren, 2019). It also interferes with the micronutrient's absorption, protein synthesis and metabolic enzyme activities leading to disorders such as kwashiorkor and stunted growth (Mutegi *et al*., 2012: Probst *et al*., 2007). Despite the chronic infection having slow disease progression, its effects globally are more severe and costly than the acute aflatoxicosis (Benkerroum 2020).

2.7 Mechanism of aflatoxin infection in humans.

Owing to aflatoxin health challenges, various investigation have been undertaken to elucidate the mechanism of carcinogenicity and toxicities (Benkerroum 2020). This is critical in aiding the design of curative and/or preventive methods to mitigate the toxins adverse effects (Benkerroum 2020). Many of the mechanism of aflatoxin toxicological effects have not been fully understood (Benkerroum 2020). Most investigation focused on mutagenic effects of AFBI molecule through its intermediate metabolite AFB1-exo-8,9 epoxide (AFBO) (Benkerroum 2019). AFBO is highly and reacts with cellular macromolecules, including nucleic acids, proteins, and

phospholipids, to induce various genetic, metabolic, signalling, and cell structure disruptions (Rushing and Selim 2017). AFB1 can also lead to the induction of oxidative stress (OS) thereby affecting the cell integrity and functioning (Benkerroum 2020). The toxicity mechanism of AFBI through the AFBO and OS species leads to genotoxicity, immunotoxicity and acute toxicity (Benkerroum 2020).

Genotoxicity occurs when the AFBI is ingested and absorbed in the duodenum and reaches the liver (Benkerroum 2020). Once in the liver, the AFBI is bioactivated by the catalytic action of the monooxygenases microsomal cytochrome enzymes (CYP450) (Fig 2.2). This enzyme catalyzes the oxidation of C8 = C9 double bonds found on the furan ring on the toxin to yield AFBI--exo and -endo 8,9 epoxide (AFBO) stereoisomers which is very unstable (Klvana & Bren, 2019). The AFBO is then released and binds the DNA upon alkylation reaction to the N^7 atom of guanine residue forming a stereospecific aflatoxin-DNA adduct, trans-8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB1 (AFB1-N^7-gua) (Smela *et al*., 2001) (Fig 2.2). The AFB1-N^7-gua adduct is a positive molecule which is highly unstable and detaches leaving apurinic DNA molecule (AP) (Benkerroum 2020; Smela *et al*., 2001). In an alkaline condition, the imidazole ring may be opened to release two stable isomers cis- and trans-AFB1-formamidopyrimidine (AFB1-FAPy) adducts (Benkerroum 2020; Smela *et al*., 2001). The AP, AFB1-N^7-gua, and AFB1-FAPy are the main molecules in AFB1 genotoxic and carcinogenic effects (Benkerroum 2020). On the other hand, the metabolism of the AFBI produces oxidative stress (OS) (Benkerroum 2020). The OS acts directly to the DNA to cause the Oxidative DNA damage (ODD) or indirectly through the formation of a by-products from lipid peroxidation (LPO) of membrane phospholipids (Klaunig *et al*., 2010). Additionally, the action of CYP450 in the liver on AFBI liberates large quantities of reactive oxygen species (ROS) (Klaunig *et al*., 2010; Benkerroum, 2020). These molecule attacks the nitrogen bases and deoxyribose moieties of the DNA to give rise to a large number of DNA adducts (Klaunig *et al*., 2010; Evans *et al*., 2004).

The AFBO also reacts with Immunocompetent cells in the body to affect proliferation and/or production of immune responses mediators and this suppresses the innate and adaptive immunity (Coppock *et al*., 2018) (Fig 2.2). The mechanism of acute aflatoxicosis is not well understood. However, various authors have suggested that the

interaction of large quantities of the AFBO and ROS-induced LPO with the proteins, phospholipids, and nucleic acids leads to rapid formation of various adducts which compromise the physiological and structural functioning of the macromolecules leading to fatality (Benkerroum 2020).

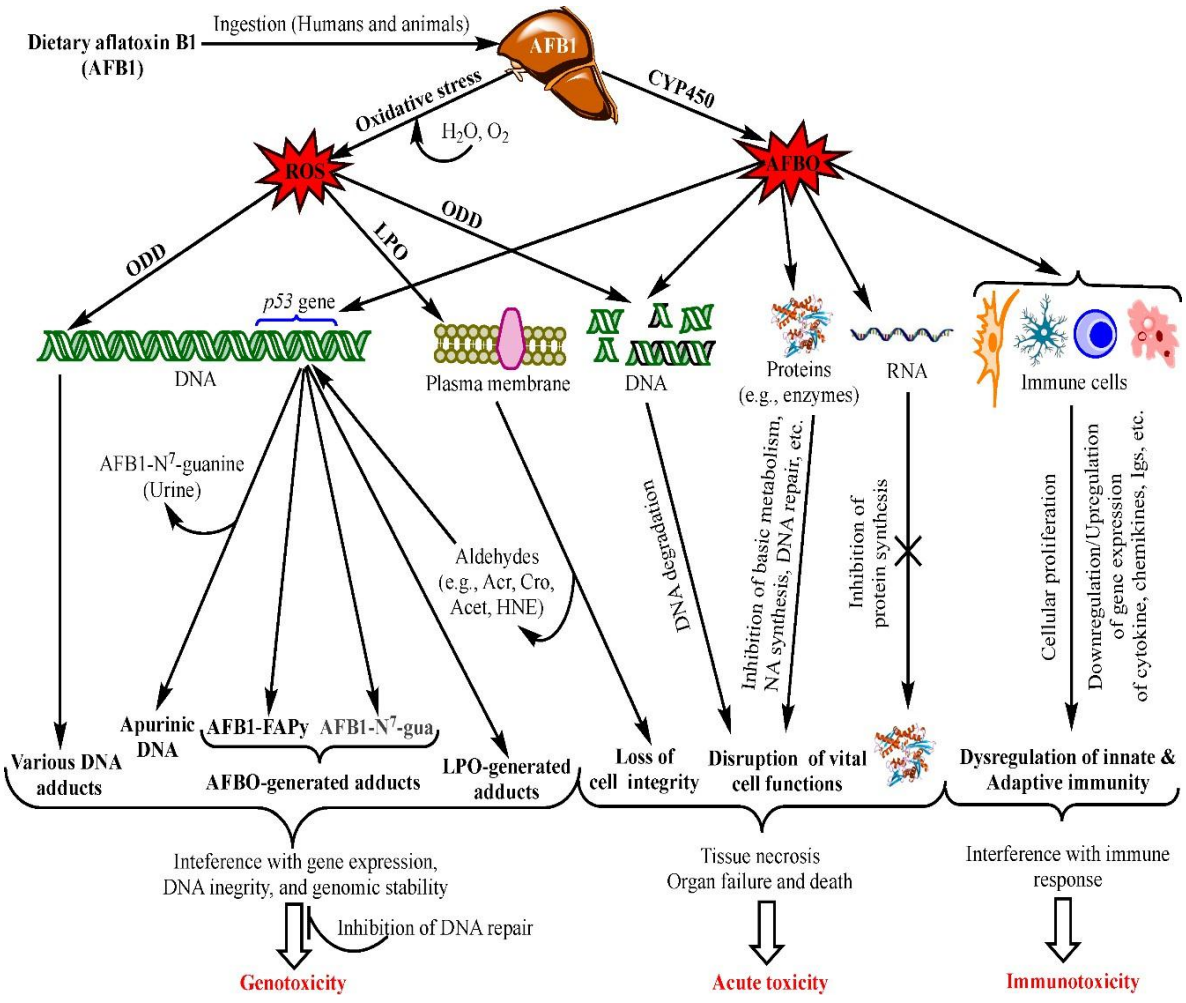

Figure 2.2. Aflatoxin BI mechanism of toxicity in mammalian cells through oxidative stress and AFBI exo-8,9 epoxide (AFBO). NA: Nucleic Acids; LPO: Lipid Peroxidation; ODD: Oxidative DNA Damage adapted from (Benkerroum 2020).

2.8 Tolerant levels in groundnuts allowed in various territories

Standard food safety parameters set by national regulatory bodies in different countries for the benefit of human health and permissible limits differ among countries (Sharma and Parisi, 2017). This may lead to trade loss due to high cost of meeting the standards and cost of testing, and eventual loss of admissibility into foreign markets (Sharma and Parisi, 2017). These standards are mainly geared

towards imported commodity and in most cases has little effect on the local produce (Wu, 2004). The strict European Union standards has negative effect on trade to the developing countries who are unable to meet the standard (Dimanche, 2001). According to ICRISAT, (2007), the establishment of these limits are influence by factors such as survey data, toxicological data, method of analysis, aflatoxin distributions and legislations.

The groundnut crop has different tolerant levels across countries and economic commissions. The EU has the strictest standards that specify 2 µg/kg Aflatoxin B1 and 4 µg/kg total aflatoxins (Wu, 2004*)*. The US allows a range from 0.10 to 0.25 µg/kg in infant foods (Lawley and Walker, 2013) and 20 µg/kg for adults (Kpodo and Bankole, 2008). In Kenya, the safe limit for groundnut and corn for total aflatoxin is 10 µg/kg while the allowable limit for aflatoxin B1 is 5µg/kg (Kenya Bureau of Standards, 2007).

2.9 Aflatoxin contamination determination

The method to use to determine the aflatoxin on food stuffs depends on cost effectiveness, precision of the results required, sampling strategy and the volume of samples to be tested (ICRISAT, 2007). The methodologies available for mycotoxin analysis are Thin Layer Chromatography (TLC), Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC), Liquid Chromatography/Mass Spectrometry (LC/MS), Enzyme-Linked Imunosorbent Assay (ELISA), lateral flow devices (LFDs), Fluorescence Polarization Immunoassay (FPIA), Infrared Spectroscopy, capillary electrophoresis, fibre-optic immunosensors, molecularly imprinted polymers and rapid tests (Mutegi *et al*., 2012; Pascale and Visconti, 2008). ELISA is the most preferred serological tests due to their simplicity, adaptability and sensitivity. It has flexibility in analyzing multiple samples thus ideal for screening purposes (ICRISAT, 2007). HPLC is highly sensitive, has good selectivity and is easily automated however, it is costly making it prohibitive for routine analysis (Pascale and Visconti, 2008).

2.10 Aflatoxin management in groundnuts

The host resistance has been fronted as the cost-effective way to exclude aflatoxin contamination in groundnuts. The use of biocontrol agents have been proposed and gave a better results in comparison to use of chemical that are expensive and affect

the ecosystem (Rajasekaran *et al*., 2009). According to Horn and Dorner, (2009) the biocontrol agents compete with the *A. flavus* in colonizing the groundnuts pods thereby reducing the infection by 77-98% in the field. Atoxigenic strains of *A. flavus* and *A. parasiticus* are some of the biocontrol agents used in groundnuts (Horn and Dorner, 2009; Dorner, 2008). Streptomyces spp. (*strain ASBV-1*) has been shown to reduce the viability of *A. parasiticus* spores by as much as 85 per cent (Zucchi *et al*., 2008). *Trichoderma harzianum* and *Trichoderma viride* have been showed to have a suppressive effect on the growth of *A. flavus* and reduce the aflatoxin contamination (Gachomo and Kotchoni, 2009). Cultural practices that ensured adequate moisture in the pod zone have also been employed to minimize the infection (Craufurd *et al*., 2006). Soil amendments such as lime application, manure and ploughing back crop residue in the soil at the time of sowing reduces the *A. flavus* seed infection and aflatoxin contamination (Waliyar *et al*., 2008). Early planting to avoid the terminal drought, good plant density, rouging dead or diseased plants, pests and diseases control, timely harvesting and excluding damaged and immature pods reduce aflatoxin contamination (Waliyar *et al*., 2008).

Drying, control of storage pests and use of mechanical threshers are some of the management practices at the post-harvest stage (Waliyar *et al*., 2008). Groundnut processing methods such as roasting also lower aflatoxin levels (Ogunsanwo *et al.*, 2004). Sorting out infected and damaged kernels reduce the rate and magnitude of contamination by 40 to 80 per cent (Park, 2000). The integration of these factors and awareness raising campaigns could be most effective approach in reducing aflatoxin levels (Waliyar *et al*., 2008).

2.11 Development of host resistance

For many decades, groundnuts breeding programs had been geared towards developing varieties that meets desires and aspirations of growers, processors and consumers. The most common breeding targets include yield enhancement, stress factor resistance, oil content and resistance to *A. flavus* colonization (Janila *et al*., 2013; Liao *et al*., 2010). The main challenge encountered in breeding for aflatoxin resistance has been the scarcity of resistant germplasm with elite agronomic traits (Liao *et al*., 2010).

Over the years, several varieties of high yield potential and with varying degree of tolerance to *A. flavus* infection and aflatoxin contamination have been released worldwide (Janila *et al*., 2013). However, the performance of majority of the discovered varieties have been affected by large environmental influence (Utomo *et al*., 1990). Hence the selection and breeding of cultivars should be environment specific (Hamidou *et al*., 2014). To overcome this better resistant traits could be mined through molecular means and introgressed into novel varieties (Janila *et al*., 2013). Molecular studies on the groundnut's genome could therefore be used to discover these novel genes with potential resistance and to develop markers to be used in marker assisted selection.

2.12 Gene expression profiling in groundnuts against *A. flavus*

The genome studies on groundnuts have provided valuable information necessary for studies of genes, gene function and regulatory mechanisms of various physiological functions (Dinneny *et al*., 2008). The study of dynamics and composition of cell transcriptome provides a clue into the complexity of the gene regulatory network and key genetic players (Dinneny *et al*., 2008). DNA microarrays and high throughput RNA sequencing (RNA-Seq) are the most commonly used techniques for genome wide expression studies (Mantione *et al*., 2014). The RNA seq is progressively becoming popular as it directly reveals the sequence of transcripts. It is undergoing continuous improvements both in sequencing and data analysis (Mantione *et al*., 2014). The development of sequencing centers and large consortia focusing on specific crops such as Rice Genome Annotation Project, Arabidopsis Genomes Project and Maize Genome Sequencing Consortium among others have marked the increase in RNA seq adoption. These projects work with an objective of developing and standardizing protocols to facilitate aggregation and comparison of various data sets (Mantione *et al*., 2014). The RNA-Seq is applied in assembling the transcriptome with or without the reference genome information, gene expression analysis and discovery, identification of unknown exon junctions and alternative transcripts, measuring allele-specific expression and many more (Mantione *et al*., 2014). Conversely, microarrays can only derive information on targets that are represented by the microarray probes (Richard *et al*., 2014). It has a drawback in that they are sensitive to cross-hybridization, display poor signal resolution and had increased variation at low signal intensities (Richard *et al*., 2014). These drawbacks

notwithstanding, the results obtained in microarray platforms and RNA seq are similar (Wang *et al*., 2014). Moreover, various studies conducted by microarrays reflects the transcriptome composition with high fidelity and are rich source of valuable biological information (Zhu *et al*., 2012). Microarrays offer a great tool in searching for disease markers, alternative splicing and gene function prediction (Zhu *et al*., 2012), identification of transcriptionally active regions of the nuclear, mitochondrial and chloroplast genomes (Giegé *et al*., 2005) and many other applications. The microarray offers a cheaper alternative in genome exploration in terms of prices of the consumables and reagents, computations and human resource required for data analysis and storage in comparison to RNA seq (Goralski *et al*., 2016). Extraction of biological information from the RNA-Seq data requires a higher computational skills with precise knowledge of the study objectives (Goralski *et al*., *2016* Microarray provides relatively small datasets and easy data analysis in comparison to RNA seq and thus more preferred alternative for a variety of studies (Goralski *et al*., 2016). It is suitable in studies such as differential analysis of known genes in the conditions of study and in time-course studies, where a large number of samples are to be processed and compared in a repeatable manner (Goralski *et al*., 2016).

Several genes and pathways involved in resistance or tolerance to *A. flavus* have been identified through microarray analysis in groundnut (Krapovickas and Gregory, 1994). Gene expression analysis on a resistant groundnut cultivar GT-C20 against a susceptible cultivar Tifrunner using a fabricated microarray slides discovered 22 putative *Aspergillus* resistant genes in resistant cultivar GT-C20 in response to *Aspergillus* infection (Krapovickas and Gregory, 1994). The transcription factors like basic-domain leucine-zipper (bZIP), WRKY proteins, ethylene-responsive-element-binding factors (ERF), MYB, MYC, and NAC encoding for the pathways of jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) mediated signal transduction pathways have been documented by Chen *et al*., (2014) to control the expression of genes in groundnut under biotic and abiotic stresses. The Ethylene Responsive Transcriptional Factor (ERF) have been showed to take part in the regulation of genes responsible for cold, drought and biotic stresses expression (Wang *et al*., 2016). Lipoxygenase have been shown to influence defense mechanisms through the production of signal molecules such as jasmonic acid, methyl-JA, lipid peroxides,

toxic volatile and non-volatile fatty acid-derived secondary metabolites that directly counter the invading pathogens (Hammond-Kosack and Jones, 1996). The groundnut Lipoxygenase (PnLOX1) gene and their products (13S)-hydro-peroxy-(9Z,11E)-octadecadienoic (13-HPOD) and (9S)-hydroperoxy-(10E,12Z)-octadecadienoic acid (9-HPOD) have been implicated in the reduction of mycotoxin biosynthesis in mature groundnut kernels colonized with *Aspergillus spp*. (Hammond-Kosack and Jones, 1996).

Microarray have also been instrumental in identifying disease resistance genes (R genes) in plants that are critical in array of plant defense mechanism (Shan *et al*., 2011). Nucleotide binding site leucine rich repeat (NBS–LRR) have also been documented to confer resistance against pathogen attack (Shan *et al*., 2011). Investigating 1088 bp long PnAG1-2 gene through blast revealed that it has high homology to other resistant proteins, and it has been shown to have higher expression in *A. flavus* resistance varietyJ11 compared to susceptible JH1012 (Shan *et al*., 2011). Resistant gene, ARAhPR10 belonging to the PR10 family was reported to activate signal networks and is involved in host resistant to aflatoxin contamination as explored through the qRT-PCR analysis (Xie *et al*., 2013).

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CHAPTER 3. MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF TOXIGENIC *Aspergillus flavus* FROM GROUNDNUT KERNELS IN KENYA.

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Abstract

Pathogenesis of *Aspergillus flavus* on important agricultural products is a key concern on human health due to the synthesis and secretion of the hazardous secondary metabolite, aflatoxin. This study identified and further characterized aflatoxigenic *A. flavus* from groundnuts sampled from sundry shops in Kenya using integrated morphological and molecular approaches. The groundnuts were plated on potato dextrose agar for isolation and morphological observation of *A. flavus* based on macroscopic and microscopic features. Molecular characterization was done through amplification and comparison of the partial sequence of the ITS1-5.8S-ITS2 region. The expression analysis of *aflR, aflS, aflD, aflP* and *aflQ* genes in the aflatoxin biosynthesis pathways was conducted to confirm the positive identification of *A. flavus*. The gene expression also aided to delineate toxigenic isolates of *A. flavus* from atoxigenic ones. Morphologically, 18 isolates suspected to be *A. flavus* were identified. Out of these, 14 isolates successfully amplified the 500 bp ITS region of *A. flavus* or *Aspergillus oryzae* while 4 isolates were not amplified. All the remaining 14 isolates expressed at least one of the aflatoxigenic genes but only 5 had all the genes expressed. Partial sequencing revealed that isolate 5, 11, 12, 13 and 15 had 99.2%, 97.6%, 98.4%, 97.5% and 100% homology, respectively, to the *A. flavus*-isolate_LUOHE, ITS-5.8S-ITS2, obtained from the NCBI database. The five isolates were accurate identification of atoxigenic *A. flavus*. Precise identification of toxigenic strains of *A. flavus* will be useful in establishing control strategies of the fungus in food products.

Keywords: Aflatoxin; Aflatoxigenic genes; *Aspergillus flavus;* Isolates; Internal transcribed spacer; Primers.

3.1 Introduction

Aspergillus flavus is a facultative pathogen capable of existing in diverse ecological niches (Klueken *et al*., 2009). It survives optimally in the tropics at relatively high temperature of about 28 ^{0}C to 37 ^{0}C and high relative humidity of about 95 % (Yu, 2012). It derives its energy as a saprophyte on plant debris rich in carbohydrates (Abbas *et al*., 2009). Like other fungi, *A. flavus*, does synthesize and release a plethora of secondary metabolites such as aflatoxin B1 and aflatoxin B2, aspergillic acid, aflam, nitropopionic acid and kojic acid (Yu, 2004). These metabolites act as virulence factors during pathogenicity, as communication signals and for ecological adjustments to suit their existence (Payne *et al*., 2006).

Colonization of *A. flavus* and the succeeding aflatoxin contamination have been found in agriculturally important crops like maize, legumes, nuts and their products (Giorni *et al*., 2008). In Kenya, the outbreak of aflatoxin contamination in the years 2004 and 2005 focused most of the aflatoxin research efforts on maize (Probst *et al*., 2007; Okioma, 2008). This is because maize is a staple food and contributes majorly to the daily diet of most households in the country. However, the reported high incidences of *A. flavus* on groundnuts (*Arachis hypogea*) in Western Kenya (Mutegi *et al*., 2012) underscore the need for greater vigilance and surveillance of other foodstuffs other than maize. The current study is one such effort targeting groundnut which is a non-staple foodstuff that is a delicacy to many Kenyan communities especially in Western Kenya region.

Exposure to low dosage of aflatoxin overtime leads to chronic aflatoxicosis that leads to poor feed conversion and stunting growth in children, immune suppression and reduction in life expectancy (Probst *et al*., 2010). Exposure to high aflatoxin doses that are greater than 1000 parts per billion (ppb) leads to acute aflatoxicosis, within a relatively short time, which is characterized by liver damage, hepatitis and in some cases death (Williams *et al*., 2004). The International Agency for Research on Cancer (IARC), documented aflatoxin B1 as the most lethal and classified it as probable human carcinogen (IARC., 1993). However, only 40-50 % of *A. flavus* strains can produce the toxins (Davari *et al*., 2015) and hence there is need to distinguish the toxic from the non-toxic ones.

Morphological classification is one of the ancient means of distinguishing the species in *Aspergillus* section *Flavi* though it lacks precision owing to close resemblance of

these species (Norlia *et al*., 2018). It is, however, necessary in aiding the grouping of *Aspergillus* isolates into sections which allows easier scrutiny using advanced characterization methods such as molecular and biochemical tools (Zulkifli and Zakaria, 2017). Morphological classification employs the use of numerous available taxonomical keys for *Aspergillus* species identification at macroscopic and microscopic levels (Thathana *et al*., 2017). The keys delineated features such as conidia, conidiophore, mycelial colour, colony reverse colour, colony diameter, exudates production and the sclerotia and cleistothecia formation for macroscopic characterization (Klich, 2002; Cardwell and Cotty, 2002). The microscopic characterization depends on vesicles shape, size and seriation, stipe, hülle cells formation and cleistothecium (Klich, 2002; Cardwell and Cotty, 2002; Rodrigues *et al*., 2007). Morphological similarities both at interspecific and intraspecific levels within the *Aspergillus* genera hamper the use of the morphology-based taxonomical keys for distinguishing the various species (Rodrigues *et al*., 2007; Okuda *et al*., 2000) thus leading to inaccurate identification. In addition, the morphological methods are laborious, time consuming and require skilled microbiologists (Rodrigues *et al*., 2007). A complex mycobiota contains numerous compounds and possibly species that may hinder sensitivity and efficiency of detection of specific species. Molecular approaches involving amplification of the variable regions of the known DNA target followed by sequencing is an appropriate molecular approach for identification of closely related members of the *Aspergilli* (Zulkifli and Zakaria, 2017; Samson *et al*., 2014). However, morphological characterization is instrumental in categorizing the isolates into groups or sections that may thereafter be cultured for easier and specific diagnosis in pure culture by other methods. Molecular tools such as polymerase chain reaction (PCR) based methods that targets and amplifies the specific DNA regions for specific fungal species are better alternatives for sensitive and rapid diagnosis (Sweeney et al., 2000; Somashekar et al., 2004). The most commonly targeted genome regions for identification of *Aspergillus* species are the highly variable sequence of the internal transcribed spacer (ITS) regions and intergenic spacer (IGS) of the ribosomal DNA (rDNA) and nuclear genes, RNA polymerase II (*rbp2*) and β-tubulin (*benA*) (González-Salgado *et al*., 2008; Patino *et al*., 2005). To a lesser extent, two mitochondria genes; small rRNA subunit (*rns*) and cytochrome oxidase subunit 1 (*cox1*) are also used for the identification and phylogenetic studies (Krimitzas *et al*., 2013). The species *A. oryzae* is believed to be

the domesticated form of aflatoxigenic *A. flavus* and is known to have its aflatoxin biosynthetic genes silent (Nazir *et al*., 2014; Payne *et al*., 2006). Therefore, investigating the aflatoxin biosynthetic genes expression could provide an additional separation mechanism not only for *A. oryzae* from *A. flavus* but also to separate toxigenic strains of *A. flavus* from atoxigenic ones. The aflatoxin biosynthesis pathways consist of approximately 30 genes and 27 enzymatic steps (Davari *et al*., 2015; Ehrlich and Yu, 2010). They consist of structural and regulatory genes (Erami *et al*., 2007). The most essential structural genes that encode for the key enzymes in the aflatoxin production are *aflD, aflO, aflQ aflM* and *aflP* (Erami *et al*., 2007). According to Sweeney *et al*., (2000) the expression patterns of these structural genes are positively correlated to the aflatoxin production capacity. The *aflR* and *aflS* genes found in the middle of the gene clusters, regulate the expression of the structural genes (Chang, 2003). The *aflR* genes is well characterized and shown to encode the 47 kDa Sequence-specific Zinc Finger DNA binding proteins while the *aflS* is less characterized but its expression shows correlation with the aflatoxin production capacity (Chang, 2003).

This study sought to identify and characterize aflatoxigenic *A. flavus* from groundnuts using an integration of morphological and molecular approaches. Correct identification of the toxigenic *A. flavus* is important given that only 40-50% of *A. flavus* strains can produce the toxins (Davari *et al*., 2015) and are thus harmful to the consumer. In addition, groundnut host specific toxin producing *A. flavus* strains provide great tools for use in screening groundnut varieties for resistance/tolerance to *A. flavus*. We believe that this is the first report in Kenya to characterize *A. flavus* from groundnuts using a combination of morphological and molecular approaches.

3.2 Materials and methods

3.2.1 Groundnut sample collection

Groundnut samples were obtained from sundry shops in Bondo town in Bondo Sub-County, Siaya County, Kenya. Convenience sampling was used to obtain samples from various shops within the town. On each sack of seeds, the samples were picked from the bottom, middle and top. A composite sample was then obtained from the seeds drawn and 100 seeds from the composite was placed on a well labelled paper bag. The samples were kept at a room temperature in biology laboratory at Jaramogi

Oginga Odinga University of Science and Technology (JOOUST), before fungal isolation.

3.2.2 Isolation of Aspergillus flavus isolates

The seeds were surface sterilized in accordance with the method of Samson *et al*., (2010). Seeds from the sundry shops were thoroughly mixed after which approximately 100 seeds were randomly selected and washed in 350 ml of 0.5 % ethanol solution and rinsed with distilled water twice. Four kernels were randomly obtained and plated using sterile forceps onto PDA (20 g dextrose, 4 g Potato extract and 15 g Agar) growth media and incubated at 28 ^{0}C for 10 days (Fig. 3.1). Any visible A. flavus-like mycelial growth or spores characterized by greenish colouration was considered as the initial isolation criterion.

3.2.3 Morphological characterization of Aspergillus flavus isolates

Macroscopic features of the isolates including colony growth, colour, texture, conidia and reverse colour were observed after 10 days of inoculation (Klich, 2002**;** Samson *et al*., 2010). For microscopic assessment, the slide culture was prepared according Diba *et al*., (2007). The 18 x 18 mm cover slip was placed gently at an angle of 45^0 on inoculated culture agar media. Upon fungus sporulation, the cover slip was gently removed, placed on the microscope slide and a drop of lacto-fuchsine added and covered with a small cover slip. Another drop of lacto-fuchsine was placed on top of the small cover slip before completing the assembly with a 22 x 22 mm cover slip. The microscopic features such as conidiophores, vesicles, mutulae, philiades, conidia shape and texture were observed under a miotic BA 210 Basic biological light microscope using the immersion oil (100x) objective.

3.2.4 DNA extraction

The conidia of isolates representative of *A. flavus* was taken from a 10-day old culture on a PDA media and inoculated in a 150 ml potato dextrose broth (PDB) in 250 ml conical flask. The broth was incubated under agitation at 120 rpm for 72 hours at 30 ^{0}C. The Whatman filter paper No. 1 was used to harvest the mycelia through filtration. The harvested mycelial mats were freeze dried for 48 hours and kept at deep freezer at –80 ^{0}C. Extraction of genomic DNA was done following the method of Diniz et al., (2005) with slight modifications, including the use of 1.5 mm glass beads instead of fine sand and use of amalgamator instead of grinding in liquid nitrogen. One gram of fresh mycelia was put in pre-cooled motor and ground into a fine

powder. Lysis buffer (1.5 ml) was added to the mycelia powder and incubated at 69 ^{0}C for 20 minutes in a shaker. Using a temperature-controlled centrifuge (Neofuge 13r refrigerated centrifuge), the suspension was centrifuged at 13000 rpm for 15 minutes at 4 ^{0}C. The supernatant was then transferred into a new tube and 0.75 ml of 4 M sodium acetate added to precipitate polysaccharides and proteins at a pH of 5.2. The solution was mixed thoroughly by inversion and incubated in ice for 20 minutes. The resulting solution was centrifuged at 12000 rpm for 20 minutes and the resulting supernatant was transferred into a new tube where 0.175 ml isopropanol was added and mixed gently by inversion and placed in ice for 15 minutes. The DNA was pelletized by centrifugation at 13000 rpm for 15 minutes at 4 ^{0}C. The pellets were washed with 70 % ethanol and air dried by letting centrifuge tube containing the pallet to sit upside down on a paper towel to dry. After drying, 0.1 ml of TE buffer at a pH 8 was added and spinned briefly for 10 minutes. The DNA was stored in a refrigerator at -20 ^{0}C.

3.2.5 Amplification of internal transcribed spacer region

The primers FLA1 (5′-GTAGGGTTCCTAGCGAGCC-3′) and FLA2 (5′-GGAAAAAGATTGATTTGCGTTC-3′) designed on the basis of sequence alignments (González-Salgado *et al*., 2008) were used to amplify the partial sequence of ITS region of the rDNA. The PCR reaction was performed in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) using 2.5 µl of the DNA template, 1.5 µl of each primer set, 2.5 µl of reaction buffers, 1 µl of $MgCl_2$, 0.25 µl of dNTPs and 0.2 µl of Taq DNA polymerase. The PCR amplification parameters were set as follows: initial denaturation cycle of 5 minutes at 95 ^{0}C, 30 cycles of 30 seconds each at 95 ^{0}C for the subsequent denaturation, 30 cycles of 30 seconds each at 58 ^{0}C for annealing, 30 cycles of 45 seconds at 72 ^{0}C for extension and final cycle of 5 minutes at 72 ^{0}C for final extension. The PCR products were then held at 4 ^{0}C indefinitely and visualized in 1.2 % agarose ethidium bromide gels in TAE 1 × buffer (Tris–acetate 40 mM and EDTA 1.0 mM). The 100-bp DNA ladder (MBI Fermentas, Vilnius, Lithuania) was used as the molecular size marker. Electrophoresis was conducted at 80 V for 1 hour and the gel was observed under UV light. A single band of approximately 500 bp was expected when the amplified ITS was from *A. flavus.*

3.2.6 PCR purification and sequencing

The gel bands were cut with the aid of blue light and purified using the quick protocol for DNA gel purification by vacuum (wizard® SV Gel and PCR clean-up system). The sliced PCR product was placed into a micro-centrifuge tube and 10 µl of membrane binding solution added, vortexed and incubated at 65 ^{0}C in water bath until the gel dissolves. The solution was transferred into a minicolumn inserted into a micro-centrifuge tube, allowed to incubate at room temperature on the bench for 3 minute and centrifuged at 1600 rpm for 5 minutes. 700 µl of the membrane wash solution was added and centrifuged at 1600 rpm for 5 minutes and the flow through discarded. Two additional washing steps was done with 500 µl of membrane wash solution. The column assembly lid was opened to allow the residual ethanol to evaporate on the bench. Nuclease-free water was used to elute the PCR product on the miniculumn, incubated for 1 minute then centrifuged for 2 minutes. The purified PCR product was sequenced at Intertek Laboratory in Australia. The obtained sequences were aligned with the sequence of *A. flavus*-isolate_LUOHE (Accession no. MT645222.1) obtained from the NCBI GenBank using Clustal X 2.1 software (http://www.clustal.org/clustal2/).

3.2.7 Analysis of aflatoxin biosynthetic gene expression

1.1.1.1 Growth condition

The isolates were grown on a PDA at 20 ^{0}C until sporulation and inoculum of conidia suspension was harvested with 0.1 % Tween 80 (v/v). The isolates were each grown in aflatoxin inducing Yeast Extract Sucrose (YES) (yeast-extract-sucrose: 2 % yeast extract, 15 % sucrose) media and aflatoxin-repressing Yeast Extract Peptone (YEP) (yeast-extract peptone: 2 % yeast extract, 15 % peptone) media broth in 500 ml flask. The cultures were incubated in the dark for 7 days until enough mycelia growth was observed then harvested.

1.1.1.2 RNA extraction

The total RNA was extracted using RNeasy mini kit (QIAGEN, USA). The harvested mycelia were ground into a fine powder using a cooled pestle and mortar and 100 mg of tissue transferred into a 10 ml centrifuge tube. The cells were lysed through addition of 5 ml of lysis buffer. The mixture was then vortexed and incubated at 65 ^{0}C in a water bath for 20 minutes with gentle whirling and then allowed to cool for 20 minutes on the bench. Two (2) ml of chloroform was added and the mixture was

thoroughly vortexed and incubated in ice for 5 minutes before centrifuging at 8,000 rpm for 20 minutes at 4^0C. The clear supernatant was gently moved to a new RNase free tube and 1.5 ml of lithium chloride (LiCl) added, mixed by inversion and incubated at -80 ^{0}C for 2 hours. The solution was then centrifuged at 8,000 rpm for 20 minutes in a refrigerated centrifuge to pellet the RNA. The pellets were washed with 75 % ethanol and centrifuged at 10,000 rpm for 10 minutes at 4 ^{0}C. The pellets were then air dried and dissolved in 100 µL of RNase free water. The concentration and quality of RNA were assessed with NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA).

1.1.1.3 Reverse transcription PCR

Reverse transcriptase PCR (RT-PCR) analysis was used to detect expression of aflatoxin biosynthetic genes. This was done according to Super script TM Reverse transcriptase kit (Invitrogen, Carlsbad, CA). The reverse transcription was done in 20 µl nuclease free microcentrifuge tube containing 1 µl of primer pairs, 5 µl of isolated RNA, 1 µl of each dNTP mix at a neutral pH (dATP, dGTP dCTP and dTTP) and 13 µl of distilled water. The mix was then incubated at 65^0C for 5 minutes and incubated on ice for 5 minutes. Brief centrifugation was done to collect the mix at the bottom and 4 µl of the first strand buffer, 1 µl of RNase inhibitor, 1 µl of DTT and 1 µl of superscript were added and mixed through pipetting up and down. The incubation was then done for 45 minutes at 55 ^{0}C. The reaction was stopped by heating at 70 ^{0}C for 15 minutes in a water bath. The RT-PCR was done according to Rodrigues *et al*. (2009), 1 cycle of 4 minutes for initial denaturation at 94 ^{0}C; 30 cycles of 60 seconds for subsequent denaturation at 94 ^{0}C, 30 cycles of 1 minute for annealing at 55 – 60 ^{0}C; 30 cycles of 1 minute for extension at 72 ^{0}C and a final extension at 72 ^{0}C for 6 minutes.

1.1.1.4 Reverse transcription-PCR primers

The RT-PCR primers (Table 3.1) were designed using the primer 3 plus software and the expression of regulatory genes aflR and aflS and three structural genes; aflQ, aflP and aflD were analysed. This was done to distinguish isolates with the potential of aflatoxin production from non-producers. The tub1 gene encoding for β-tubulin was used as a housekeeping gene.

Gene	Primer Pair	Primer sequence (5'⟶3')	Amplicon size (bp)
aflR	Forward	CCGTCAGACAGCCACTGGACACGG	300
	Reverse	TGACCCACCTCTTCCCCCACG	
aflS	Forward	GAACGCTGATTGCCAATGCC	1256
	Reverse	CGGTCAGGATGTTACTAAGC	
aflD	Forward	ACCGCTACGCCGGCACTCTCGGAC	400
	Reverse	GTTGGCCGCCAGCTCTGACACTC	
aflP	Forward	GTGGACGGACCTAGTCCGACATCC	624
	Reverse	GTCGGCGCCACGCACTGGGTTGGG	
aflQ	Forward	TTAAGGCAGCGGAATACAAG	599
	Reverse	GACGCCCAAAGCCGAACACAAA	
tub1	Forward	GCTTTCTGGCAAACCATCTC	1198
	Reverse	GGTCGTTCATGTTGCTCTCA	

Table 3.1: RT-PCR targeted genes and primer sequences with their expected amplicon size.

3.3 Results

3.3.1 Macroscopic morphological features

Microbiota growth was observed on the PDA media (Fig. 3.1). Only the greenish coloured spores were needle picked and transferred onto new petri dishes with fresh PDA media for purification. Approximately more than 200 isolates based on the greenish coloration were picked and inoculated on a fresh media.

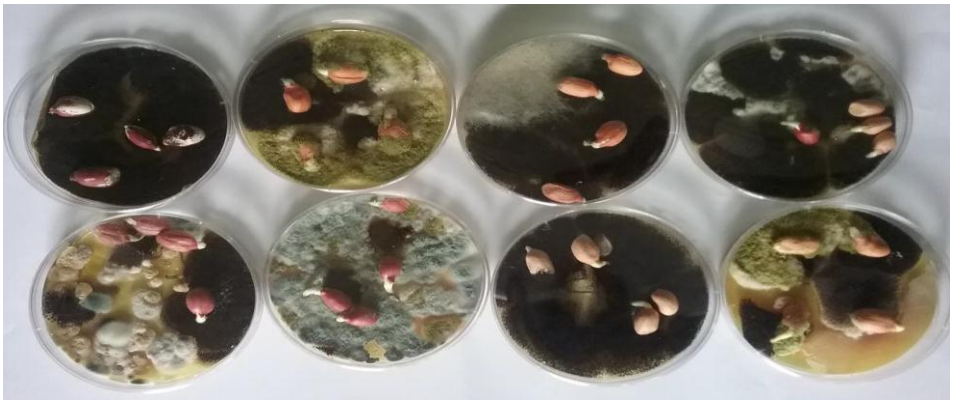

Figure 3.1: Complex microbiota growth on groundnut kernels incubated on PDA media.

Sporulation began after five days from the centre and progressed radially covering the surface of the colony. The conidia produced had yellowish to olive colour. As the sporulation spread outwards, it gave a characteristic white border encircling the sporulating mycelia (Fig. 3.2a). The white border was eventually covered as the entire

mycelia continued to sporulate and to produce more conidia by day 10. These colonies had clear exudates and cream colour on the reverse (Fig. 3.2b). The isolates representative of *A. flavus* had a greenish colony that spread radially from the point of inoculation. As the colony progressively grew, it become slightly raised as mycelia piled and the centre become floccose and rough (Fig. 3.2c). Eighteen uncontaminated isolates were obtained.

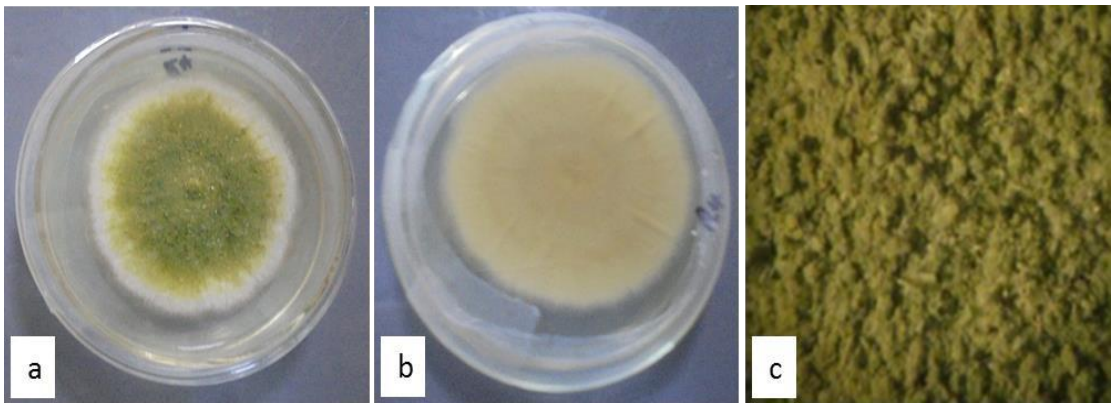

Figure 3.2: Macroscopic features of *Aspergillus flavus* isolate 5 colony showing the greenish conidia encircled with a white border (a); the reverse cream colour of the colony (b); and the colony texture (c).

3.3.2 Microscopic morphological features

The microscopic features of *A. flavus* showed that the colonies were biseriate with philiades radiating in all sides from metulae that were borne on sub-globose or globose vesicles of variable size. The metulae obscured the entire surface of the vesicles (Fig. 3.3a). The conidia had a globose shape ranging between 250 µm and 450 µm in diameter with thin walls and rough texture (Fig. 3.3b). The conidiophores had a rough texture and thick walls, were non-pigmented and unbranched (Fig. 3.3c).

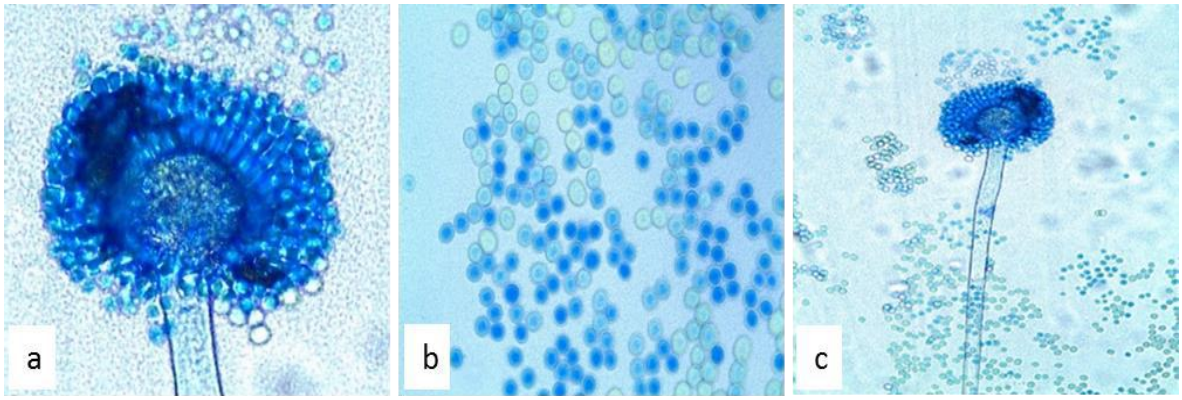

Figure 3.3: The microscopic characteristics of Aspergillus flavus isolate 5 under the basic biological light microscope showing the biseriate with philiades radiating from all sides (a); the globose conidia with varying sizes that are slightly roughened (b); and unbranched conidiophore which is non-septate, rough and hyaline (c).

3.3.3 Detection of *Aspergillus flavus* using PCR method.

A single band of approximately 500 bp characteristic of *A. flavus* amplified ITS was observed on 14 isolates out of the 18 suspected isolates (Fig. 3.4). This indicates that these isolates matched the DNA of either *A. flavus or A. oryzae*. There were no bands formed on isolate 3, 7, 14 and 16 showing that these isolates did not contain DNA from *A. flavus* or *A. oryzae.*

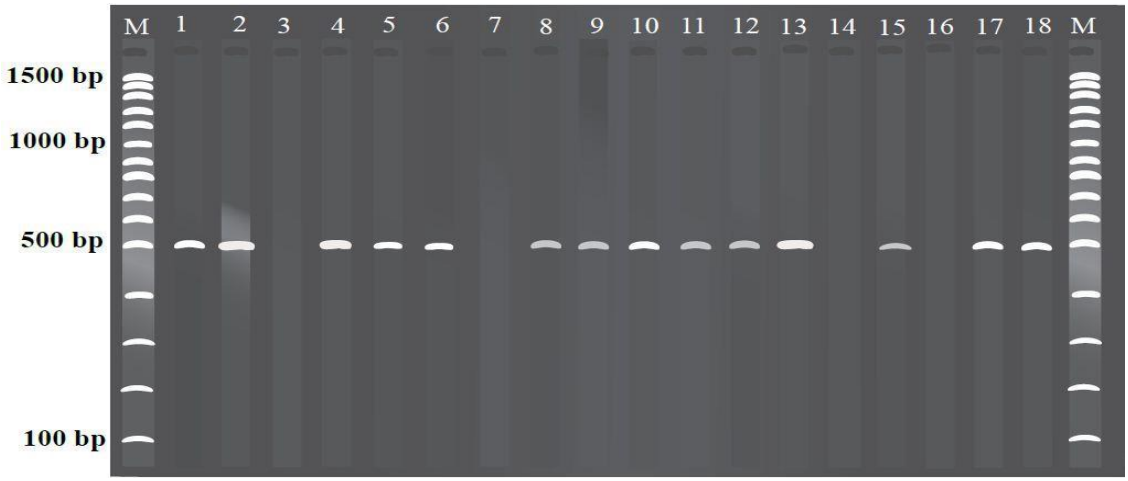

Figure 3.4: A gel image showing 500 bp marker of ITS1-5.8S-ITS2 region of *A. flavus* isolates from groundnuts amplified using primers FLA1/FLA2. Lane 1-18 represents isolates while M is a 100 bp DNA molecular ladder.

3.3.4 Aflatoxin gene profile.

The isolates varied greatly in their aflatoxin gene profiles (Table 3.2). At least one gene per isolates produced a detectable signal. Isolates 5, 11, 12, 13 and 15 had all the genes expressed, isolates 6 and 10 had 4 of their genes expressed, isolates 1, 4 and 18 had 3 of their genes expressed while isolate 17 had only 1 gene expressed (Table 3.2). The regulatory gene *aflR* was expressed by all the isolates (100% expression) while the other regulatory gene *aflS* was expressed by 44% of the isolates. The structural genes *aflQ*, *aflP* and *aflD*, were expressed by 56 %, 50 % and 44% of the isolates, respectively (Table 3.2).

Table 3.2: Gene expression in 14 *A. flavus* isolates indicating their aflatoxic capacity.

Isolate	Regulatory Genes		Structural Genes		
	aflR	aflS	aflD	aflP	aflQ
1	+	-	+	+	-
2	+	-	-	-	+
4	+	-	+	-	+
5	+	+	+	+	+
6	+	-	+	+	+
8	+	+	-	-	-
9	+	-	-	+	-
10	+	+	-	+	+
11	+	+	+	+	+
12	+	+	+	+	+
13	+	+	+	+	+
15	+	+	+	+	+
17	+	-	-	-	-
18	+	+	-	-	+

Key: + means expressed; – means not expressed

3.3.5 Sequence analysis

The sequence data was obtained from the 5 isolates that had all their aflatoxin genes expressed; isolate 5, isolate 11, isolate 12, isolate 13 and isolate 15. The sequences were blasted on the NCBI database and aligned with the *A. flavus*_isolate_LUOHE accession number MT645222.1 that had the highest similarity to the isolates. Isolate 12 had 98.4% similarity, isolate 5 had 99.2% similarity, isolate 11 had 97.5% similarity, isolate 13 had 97.5% and isolate 15 had 100% similarity to the *A. flavus*_isolate_LUOHE (Fig. 3.5). The sequences were submitted to the NCBI GenBank and the accession numbers allocated as follows: LC567154, LC567155, LC567156, LC567157 and LC567158 for isolates 12, 5, 11, 13 and 15 respectively.

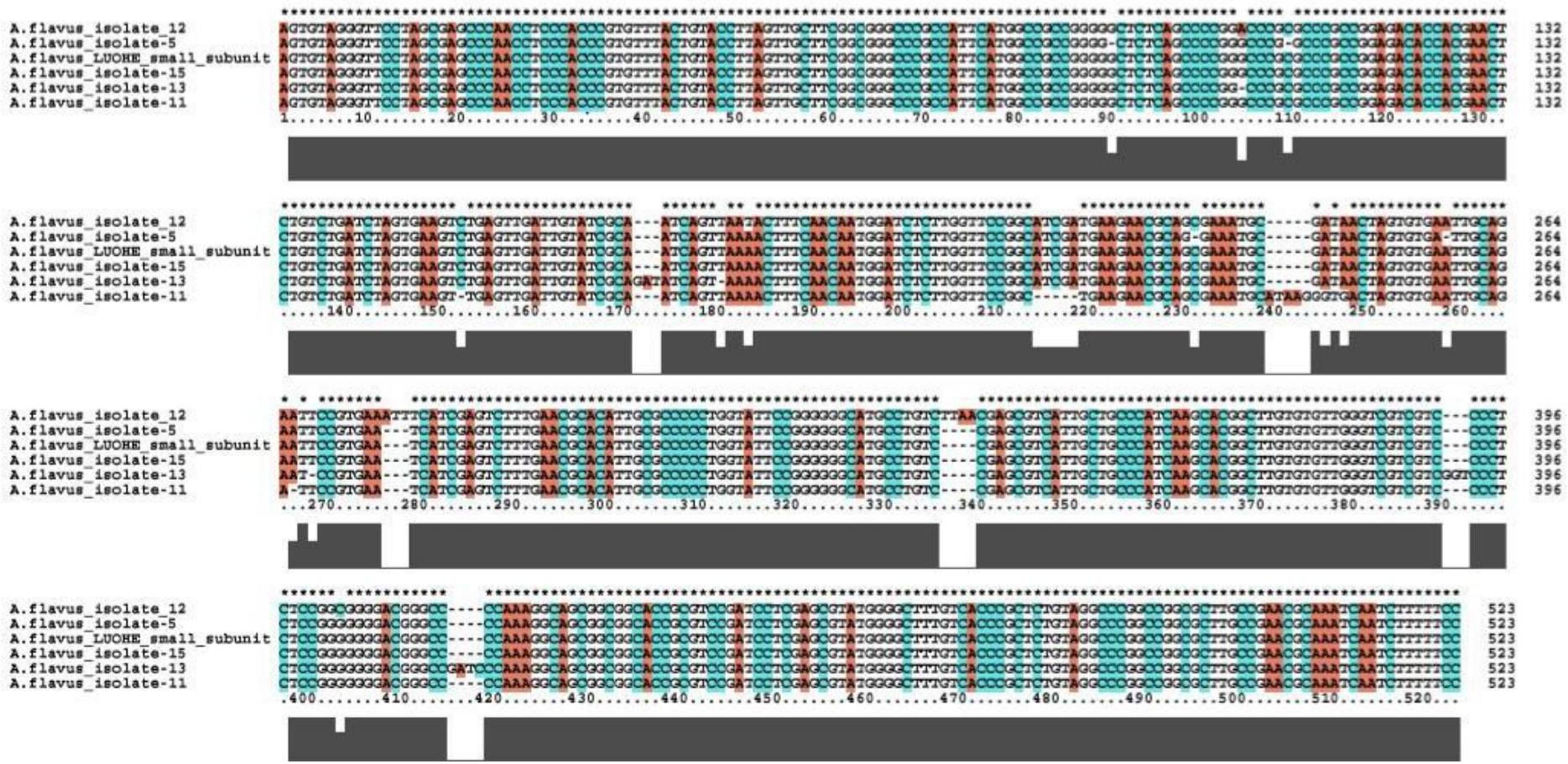

Figure 3.1: Nucleotide sequence alignment of *A. flavus* isolates with the *A. flavus-isolate*_LUOHE small subunits ITS1-5.8S-ITS regions (Gene bank accession no. MT645222.1). ***indicates the conserved bases among the nucleotide with gaps indicating the un-conserved regions.

3.4 Discussion

The aflatoxins production is a major source of food security threat. Majority of households in Kenya are exposed to acute or chronic aflatoxicosis depending on ingestion levels, susceptibility, age, gender and duration of exposure to aflatoxin (Lewis *et al*., 2005). Studies conducted by Mutegi *et al*., (2012) indicate that there is as high as 2277.1ppb aflatoxin contamination level in groundnuts from traders in Western and Nairobi provinces of Kenya. Acute aflatoxicosis in Kenya was reported in 2005 and 2004 growing season affecting 317 individuals of which 125 died (Lewis *et al*., 2005). Additionally, most infants are exposed to high level of aflatoxins in maize and sorghum-based diets and to aflatoxin MF1 (AFMI) through animal milk and breast milk (Kang'ethe *et al*., 2017). The AFM1 in urine, stunted growth and low weight gain in infants in Makueni County of Kenya indicates the high level of chronic aflatoxicosis (Kang 'ethe *et al*., 2017). This indicates that aflatoxin infection starts at early stages of development and progressed into adult hood. This emphasized the need to be more vigilant on aflatoxin causes, mitigation factors and control.

Identification of the causative agent is a critical step in disease control. Morphological characterization is the commonly adopted method for fungal isolation and characterization. It employs the use of culture media to aid the growth and establishment of the fungus for observation. Growth media such as malt extract agar (MEA), sabouraud dextrose agar (SDA), rose bengal chloramphenicol agar (RBCA) and czapeck dox agar (CZA) (Thathana *et al*., 2017) and potato dextrose agar (PDA) (Thathana *et al*., 2017; Diba *et al*., 2007) have previously been used. These media can provide adequate requirements for fungus colony establishment that allows the development of the macroscopic and microscopic features suitable for assessment (Thathana *et al*., 2017; Diba *et al*., 2007). This study employed the use of PDA which provided adequate growth and sporulation of the fungus allowing satisfactory evaluation.

Descriptive taxonomic keys were used as the initial fungal isolation criteria aiding the selection of presumptive *A. flavus* isolates. The colony growth started as a white mycelium that grew radially to cover the entire surface of the media (Fig 3.2). When sporulation began, a yellowish green or dark green colour of the conidia replaced the

white colony colour from the centre outwards, eventually covering the entire surface. This was consistent with Thathana *et al*., (2017) who found a white colour of the mycelia which produced an olive or dark green conidia delineated with a white ring. The colonies observed in this study had velvety to woolly texture often with floccose centre and cream colour on the reverse (Fig 3.2). Similar characteristics were reported by Thathana *et al*., (2017), Bastianelli and Le Bas, (2002) and Odhiambo *et al*., (2013). The isolates were confirmed to belong to *Aspergillus* genera by presence of conidiophores, a key feature of the *Aspergillus spp*. (Odhiambo *et al*., 2013).

The vesicles were found to be sub-globose to globose and varied in diameter with a biseriate sterigmata or philiades that radiated from all sides. This agreed with the preceding research results of Thathana *et al*., (2017), Rodrigues *et al*., (2007) and Diba *et al*., (2007). The metulae were borne on the vesicles in which the philiades arose while the globose, thin walled and slightly roughened conidia that differed in sizes were borne on the tip of the philiades (Fig 3.3). These features were typical of the descriptive taxonomic keys provided by Klich, (2002) and in harmony with *A. flavus* characteristics previously documented by Thathana *et al*., (2017), Rodrigues *et al*., (2007), Diba *et al*., (2007) and Odhiambo *et al*., (2013). Although morphology-based taxonomical keys are used as the initial isolation and identification criteria, they have various shortcomings and may not achieve accurate identification of the target fungal isolates (Rodrigues *et al*., 2007; Okuda *et al*., 2000). Therefore, further distinction among the groups/sections needs a comprehensive scrutiny using molecular means such as PCR based methods, gene expression and sequence analysis.

Using the FLA1/FLA2 primers, the ITS1-5.8S-ITS2 region of the ribosomal DNA was amplified in 14 out of the 18 isolates that were presumptive representatives of the *A. flavus* confirming that they were positive for either *A. flavus* or *A. oryzae* (Fig 3.4). This was in agreement with the protocol developed by González-Salgado *et al*., (2008). The primers are designed to align to a more variable region of the ITS and they specifically amplify the *A. flavus* target sequence. The other four isolates formed no bands suggesting that the genomic DNA were from other genera or members within the *Aspergilli* section Flavi that had high morphological resemblance to the *A. flavus* or *A.*

oryzae. Although *A. flavus* can be differentiated from other *aspergilli* by morphology and amplification of the ITS sequence of the rDNA, it is very difficult to distinguish it from *A. oryzae* as morphological characteristics of these two fungi are similar (González-Salgado *et al*., 2008). To overcome the problem, expression of aflatoxin biosynthetic genes at mRNA level can be employed since these genes are ascertained to be silent in *A. oryzae* (Nazir *et al*., 2014).

Scherm *et al*., (2005) reported strong connection between aflD, aflP and aflQ gene expression to aflatoxin production capacity. Furthermore, targeting aflD and aflP as documented by (Peterson, 2006) specifically detects aflatoxin producing strains and exclude those that have a common sterigmatocystin pathway. The regulatory gene aflR involvement in the aflatoxin production has been ascertained and thus its action provides adequate information on the aflatoxin production capacity as compared to the activity of aflS which is less characterized (Chang, 2003). The aflR gene is considered to increase the precision of discrimination and its expression was shown to have a good correlation with the aflatoxin synthesis in *A. flavus* strains (Scherm *et al*., 2005; Chen *et al*., 2002 and Restivo *et al*., 2007). Therefore, it is an accepted paradigm that the activation of this regulatory gene is necessary for the action of the structural genes.

In this study, one of the regulatory genes, *aflR* was activated in all isolates unlike *aflS* which was expressed by only 44% of the isolates (Table 3.2). This upholds the report of Chang, (2003) that the former gene provides more reliable information on the aflatoxin production capacity than the latter. The isolates 2, 9 and 18 had only one structural gene expressed out of the three that were targeted. The lack of the expression of some genes by some isolates even after expression of some or both of the regulatory genes could mean that they were weak aflatoxin producers and that the gene expression could not be detected. On the other hand, isolates 8 and 17 had none of the targeted structural genes expressed and may be considered atoxigenic strains of *A. flavus* or *A. oryzae*. These isolates were discarded since they were doubtful and it's better to have a false negative than false positive in the identification process. Isolates 5, 11, 12, 13 and 15 had all their genes expressed and thus they were considered to have the highest aflatoxin producing capacity.

The specific amplicons derived from isolate 5, 11, 12, 13 and 15 from the PCR were sequenced. The multiple sequence analysis of these isolates against *A. flavus* isolate_LUOHE ITS-5.8S-ITS2 as a reference was done with Clustal X 2.1. It revealed a complete sequence similarity to isolate 15 but had slight changes within the gene sequences of the other isolates (Fig. 3.1). As expected, when the genomic DNA is from the *A. flavus* or *A. oryzae*, the 5' and 3' end of the gene was conserved in all the sequences. The slight differences within the region did not change the amino acids produced by these isolates.

Conclusion.

Identification of fungi within the *Aspergillus* genera is a complicated venture that requires an integrated approach to attain a reliable identification and characterization of isolates capable of synthesizing aflatoxins. This study successfully cultured and isolated 5 toxigenic isolates of *A. flavus* and submitted them to the gene bank where they were allocated accession numbers LC567154, LC567155, LC567156, LC567157 and LC567158. Isolates 3, 7, 14 and 16 never formed a band and were considered not to be *A. flavus*. Isolate 1, 2, 4, 6, 8, 9, 10, 17 and 18 are *A. flavus* isolates but their capacity to produce aflatoxin was doubtful and thus were considered atoxigenic. The sensitivity of the methods used in the isolation led to the lower number of the isolates obtained. This may lead to underestimation of the risks imposed by the *A. flavus* in the sampled region but the detection of aflatoxigenic *A. flavus* by this study augmented the risk of aflatoxin contamination. Accurate identification of aflatoxigenic fungi is paramount to develop a mitigation measure against fungal infections and mycotoxin production. This study therefore recommends employment of similar fungal identification approach in continual random sampling and analysis of suspect food products for possible mycotoxins contamination.

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CHAPTER 4: INVESTIGATING TOLERANT LEVELS OF GROUNDNUT (*Arachis hypogea*) ACCESSIONS AGAINST *Aspergillus flavus* INFECTION AND AFLATOXIN CONTAMINATION

Submitted for publication: Robert Okayo, Darius Andika, Mathews Dida, George K'Otuto: investigating tolerant levels of groundnut (*Arachis hypogea*) accessions against *Aspergillus flavus* infection and aflatoxin contamination. Plant pathology and microbiology journal.

Abstract

A. flavus colonization and subsequent aflatoxin contamination is one of the abiotic factors that compromise the quality of groundnut and groundnut products in Kenya. This study was conducted to evaluate the pre-harvest resistance levels of Kenyan groundnuts accessions to *A. flavus* infestation and aflatoxin contamination. Thirty genotypes from the Kenya Agricultural and Livestock Research Organization gene bank, Muguga were evaluated in the green house and bioassay conducted in the laboratory. The accessions were grown on sterile media in pots and inoculated with aqueous conidia solution of aflatoxigenic *A. flavus*_JOOUST5. The pods were harvested upon maturity and subjected to bioassay in the laboratory. Result indicates a highly significant ($P<0.001$) difference of aflatoxin contamination, incidence and severity. The percentage incidence had a mean of 23.5% and a range between 7.5% to 48.5%. All except genotype 12 (GBK005111) had percentage incidence greater than 10%. The severity index had a mean of 0.25 and a range of 0.049 to 0.58. Genotype 12 (GBK005111) had the lowest mean rating for severity while genotype 1 (GBK000423) had the highest. Hierarchical clustering based on the incidence, severity and aflatoxin contamination grouped the genotypes into 3 with cluster 3 containing 7 promising genotypes that had lower levels of infection and contamination. Thus, these genotypes were considered tolerant and could be a good source of resistance in breeding program.

Key words: Accessions, Aflatoxin contamination, Incidence, Severity.

4.1 Introduction

Groundnut (*Arachis hypogea*) is a critical food security crop globally. It is ranked second as a food legume and the fourth edible oilseed (Janila *et al*., 2016). It contains 48-50% oil, 26-28% protein and is a good source of fibre, vitamins and minerals (Asibuo *et al*., 2008). The high protein content makes them ideal alternatives to fish and meat products which are more expensive for majority of households (Asibuo *et al*., 2008). Additionally, the seed cake and the haulms provide high quality protein feeds for livestock (Savage, 2011).

Groundnuts are mainly grown in the western part of Kenya in two cropping cycles per year (Anonymous, 2004). It is a critical crop to the small scale farmers not only as food source but also as a cash crop (Anonymous, 2004). However, it's quality and quantity production is affected by factors such as drought, low soil fertility, heat stress, floods, pest and diseases (Fountain *et al*., 2018). The aflatoxin contamination due to *A. flavus* and *A. parasiticus* is a major drawback to achieving quality production (Mutegi *et al*., 2012).

The groundnuts are vulnerable to the *A. flavus* colonization and aflatoxin contamination in the entire food chain (Shepard 2003). The post-harvest infection and subsequent contamination occurs during drying, transporting, processing or storage (Rahmianna *et al*., 2015). The rate and the severity of infection depends on inappropriate cultural practices such poor harvesting that compromise the integrity of the pod and the testa, storage in high humidity and temperature and harvesting immature pods (Xue *et al*., 2004; Rahmianna *et al*., 2015). In the fields, the A. flavus contaminated soils direct contact to the pods provides the first line of exposure to infection (Xue *et al*., 2004). Moreover, high levels of infection are attained in high temperature, high relative humidity, moisture stress and insect attack in pre-harvest stage (Cotty and Jaime-Garcia, 2007; Girdthai *et al*., 2010).

There are more than 20 different types of aflatoxin produced but the most important ones are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) (Yu *et al*., 2004; Amaike and Keller, 2011). The aflatoxin contamination affects

the nutrition of the poor people and accounts for 40% prevalent disease affecting health (Mutegi *et al*., 2012; Williams *et al*., 2004; Nagarajan, & Tilak, 1975). Depending on ingestion levels, susceptibility, age, gender and duration of exposure, the effect can vary from simple irritation to death (Mutegi *et al*., 2012). Exposure with aflatoxin doses greater than 6000 ppb can lead to acute toxicity characterized by vomiting, jaundice, oedema, acute hepatitis and occasionally death (Mutegi *et al*., 2012: Probst *et al*., 2007). Regular consumption of contaminated foods can lead to aflatoxin-driven hepatocellular carcinoma (HCC) or liver cancer (Liu and Wu, 2010), stunted growth in children, reproductive problems and immunosuppressive effects (Williams *et al.*, 2004). Aflatoxin is associated with the mutation in the P53 tumor-suppressor gene and activation of dominant oncogenes leading to hepatomas which accounts for 64% of cancers (Chen *et al*., 2013; Wang *et al*., 2001). Aflatoxin contamination and hepatitis B virus infection can form synergism thereby increasing the risk of primary hepatocellular carcinoma (Williams *et al*., 2004). These detrimental effects have led to the national regulatory bodies to establish food safety standards to safeguard their citizens. This leads to costly trade to meet these standards and may lead to loss of admissibility into foreign market (Craufurd *et al*., 2006).

Aflatoxin management practices that include: application of atoxigenic isolates (Kelley *et al.*, 2012), host genetic resistance, adoption of good agronomical practices and chemical control (Hell *et al*., 2000) have been put in place to mitigate its effects. However, there is a consensus that adoption of resistant or tolerant cultivars could exclude the toxin infiltration into the groundnuts thereby ensuring a safer produce (Mehan *et al*., 1986; Bisikwa *et al*., 2014). According to Bisikwa *et al.*, (2014), there exist a groundnut genotype that show less *A. flavus* colonization and aflatoxin contamination at pre- and post-harvest stages. They documented the existence of resistance at the pod and the kernel level against *A. flavus* colonization and resistance to aflatoxin synthesis at the kernels. The chemical composition and physical properties of the kernel (Xue *et al*., 2004) and the pod shell structure, thickness and hardiness determines its level of resistance (Zambettakis, 1975; Bisikwa *et al*., 2014).

The lack of known groundnut variety in Kenya that is tolerant or resistant to *A. flavus* infection justified the need to evaluate the accessions to access their tolerance levels. Thus, this study was conducted to evaluate the pre-harvest resistant or tolerant levels against *A. flavus* infection and aflatoxin contamination of Kenyan groundnut gene bank accessions.

4.2 Materials and Methods

4.2.1 Plant Materials

The plant material consisted of 30 genotypes sourced from the Kenya Agricultural and Livestock Research Organization gene bank, Muguga (Table 4.1). All these genotypes were collected within the country. The codes assigned will be successively used to refer to the genotypes.

Table 4.1: Groundnuts accessions and codes used in this study. The codes will be used in reference to the accessions for the subsequent results and discussions sections.

Code	Accession number	Code	Accession number
1	GBK000423	16	GBK005123
2	GBK043007	17	GBK005118
3	GBK000429	18	GBK005075
4	GBK043011	19	GBK036399
5	GBK036409	20	GBK032355
6	GBK005070	21	GBK005151
7	GBK005071	22	GBK005063
8	GBK005117	23	GBK005073
9	GBK036401	24	GBK005088
10	GBK005077	25	GBK005099
11	GBK043011	26	GBK005108
12	GBK005111	27	GBK005128
13	GBK005112	28	GBK005135
14	GBK005126	29	GBK028586
15	GBK005116	30	GBK036397

4.2.2 Growing media

Sterilized sand was used as a growing media. Pan and furnace were used to sterilize the sand. This was done to eliminate the soil dwelling microbes. The sand was evenly distributed in the pan to a depth of 4 inches, moistened and thoroughly mixed until consistency was met. The sand was then covered with a foil and a hole created to accommodate the thermometer. Sterilization was done for 30 minutes at a constant temperature of 82 ^{0}C. The intensity of the fire was adjusted to regulate the temperature. After 30 minutes, the pan was removed from the furnace uncovered and allowed to cool until room temperature was attained. The sand was then potted and transferred to the greenhouse.

4.2.3 Greenhouse screening

The greenhouse screening was done at Jaramogi Oginga Odinga University of Science and Technology horticultural farm. Thirty genotypes and 2 levels of treatment (Fungal inoculation and non-inoculation) were evaluated in 30 × 2 factorial design replicated 4 times. The planting was done in pots of 30 cm diameter and two plant per plot was allowed. The agronomical practices were optimally provided until the drought stress was imposed 40 days to harvesting. The plants were irrigated optimally for the first 60 days and thereafter intermittently done to allow for good vegetative and regenerative growth (Fig 4.1). Spraying against foliar disease and insect pest was done weekly. The foliar feed was provided twice a week. The digital soil thermometer was used to take the temperature within the podding zone at an interval of 3 days during drought stress.

4.2.4 Preparation of the inoculum

Pure inoculum isolates of toxigenic *A. flavus_JOOUST5*, (Okayo et al., 2020) was sub-cultured in PDA media on the petri-dish and allowed to incubate at 25 ^{0}C for 7 days. The spores were scrubbed gently from the petri dish, washed and dissolved in distilled water. The haemocytometer was used to estimate the concentration of the inoculum at a rate of 1 x 10^6 spores per litre. A 100 ml of an aqueous spore suspension was homogenously raked 10 cm beneath canopy of groundnuts at the beginning of flowering and later at mid-bloom, approximately 30 and 50 days after planting respectively. This ensured that there was enough distribution of inoculum in the pod zone of the plants.

Upon maturity (90-100 days after planting), the pods were hand harvested and packed in Khaki bags separately. Each genotype was dried separately for 3-4 days up-to a moisture content less 10%. The adhering soil and dust were washed clean by agitating in a distilled water then dried for further 12 hours. The unblemished pods were selected for shelling for *A. flavus* assay in the laboratory.

Figure 4.1. Groundnuts in the greenhouse under optimal growth (A and B) and drought stress (C and D). The plants were raised on pots and each pot had one plant. Watering was withheld to impose drought stress.

4.2.5 A. *flavus* assays

The mature intact and blameless pods were collected from treatment combination, shelled and pooled together. From each treatment combination, 40 seeds were obtained at random. The seeds were hydrated to around 30% moisture content and aseptically placed in PDA media in 4 petri-dishes and allowed to incubate at room temperature for 10 days. Observation and recording of the fungus colonization of the kernels commence on the 10 days and continued for 5 successive days. Each petri-dish was rated separately for fungal

incidences and severity of *A. flavus*. The formula below was used to tabulate the percentage incidence on the kernels.

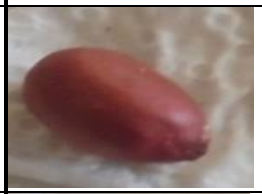
$$\text{Incidence (percentage)} = \frac{\text{Number of seeds showing pathogen colonization}}{\text{Total number of seeds}} X\ 100$$

The severity scale of A. flavus infection was determined as per the formula below as suggested by Tonapi *et al*, (2007). The scale has 0-5 ratings in which 0, non-infected seeds; 1, are kernels that have less than 20 % covered by the fungus; scale 2 are those kernels with fungus coverage that ranged from 20 %–40 %; scale 3 are those with colonization range between 40 %–60 %; 4 are those covered up to 80 % while above 80 % coverage were rated 5 (Table 4.2). Each kernel was rated individually and the severity index of infection per genotype was tabulated as below.

$$\text{Severity (\% Infection index)} = \frac{\sum_{i=1}^{n} (\text{Ni x } i)}{\text{Total of seeds x (n-1)}}$$

N*i* is the sum of kernels per scale level, *i* is the level and n is 6 the total number of scale levels from 0-5.

Table 4.2: The table showing the severity scale of the groundnuts using a modified Tonapi et al, (2007) scale.

Rating scale	Pictorial Presentation.	Description.
0		Clean. No fungal colonization.
1		surface cover is less than 20%
2		surface cover is between 20%–40%.
3		surface cover is between 40%–60%.
4		Surface cover is between 60%–80%
5		Surface cover is between 80%–100%

4.2.6 Aflatoxin contamination

The aflatoxin contamination was analysed using an indirect competitive enzyme-linked immunosorbent assay (ELISA). A 100 gms sub sample of kernels was drawn from each treatment combination then thoroughly mixed and ground with 25 ml of 70 % methanol into a fine powder using a kitchen blender. The homogenous sample was shaken for approximately in 30 minutes at 300 rpm in a conical flask. The filter paper was used for filtration and the extract diluted in phosphate buffer consisting of 500 $\mu l/l$ Tween-20. The resulting solution was subjected to ELISA using Waliyar *et al*., (2008) protocol.

The antibody aflatoxin bovine serum was diluted in a coating buffer at a rate of 100ng per millilitres. 100 μl solution was pipetted into ELISA well plates and allowed to incubate in a shaker at a revolution of 100 rpm for 4 hours at 30 ^{0}C. The serum was disposed, and the wash bottle used to wash the plates with PBS-tween (PBST). The washing was repeated three times and then the plates blotted by holding the frame in the reverse position and tap firmly on filter paper. A PBST solution was used to prepare 0.2 % Enzyme conjugate and 100 μl of the solution was pipetted into each well. The plates were then covered and incubated at 37 ^{0}C for 2-4 hours. Using the PBST solution and wash bottle, the plates were washed by holding the frame upside down and tap firmly on filter paper three times. One hundred microlitres of groundnut extract and 50 μl of the antiserum were added to each well. In the first 10 wells, a positive control of 100 μl of aflatoxin B1 concentration were added. The plates were covered, and incubation done at 37 ^{0}C for 1 hour to ensure a complete reaction between the antibody and the toxins. After the reaction, the plates were washed three times in PBST. A concentration of goat anti-rabbit 1gG antibody and PBST solution at a rate of 1:1000 was prepared and 150 μl of the solution added to each well. The plates were then incubated at 37 ^{0}C for 1 hour thereafter washed 3 times in PBST. A solution of p-nitro phenyl phosphate and diethanolamine buffer at a ratio of 9:1 was prepared adjusted to pH of 9.8 and added into the well. The plates were incubated in the bench for 1 hour. The plates were then mounted onto the ELISA plate reader and absorbent recorded at 450 nm.

The samples were then grouped into three categories depending on the aflatoxin contents as follows, afl $\leq$4ppb, 4ppb< afla$\geq$20ppb, afla > 20ppb. The first category are samples

that met the European union standards of less than 4ppb of the total aflatoxin. The second groups are those that fail to meet the EU standards but still admissible in Kenya as per the Kenya Bureau of Standards restrictions while the last group are those that can be condemned in both territories (Felicia, 2004).

4.2.7 Data Analyses

All analyses were done using R Studio statistical software version 3.6.3. The analysis of variance for incidence, severity and aflatoxin contamination of the tested genotypes were conducted using Ime4 package. The pastecs package was used to generate the average of incidence, aflatoxin contamination and severity. The correlation test function of the stats package was used to determine the relationship between the variables. The principal components analysis biplots was plotted using Factoextra package. The FactoMineR function was used to group genotypes into clusters based on principle components analysis and hierarchical clustering

4.3 Results

4.3.1 Soil temperature

The soil temperature data in Fig 4.2 reveal periodic temperature variation. The mean soil temperature was 31.9 ^{0}C and the lowest temperature was 28.92 ^{0}C and the highest was 33.29 ^{0}C.

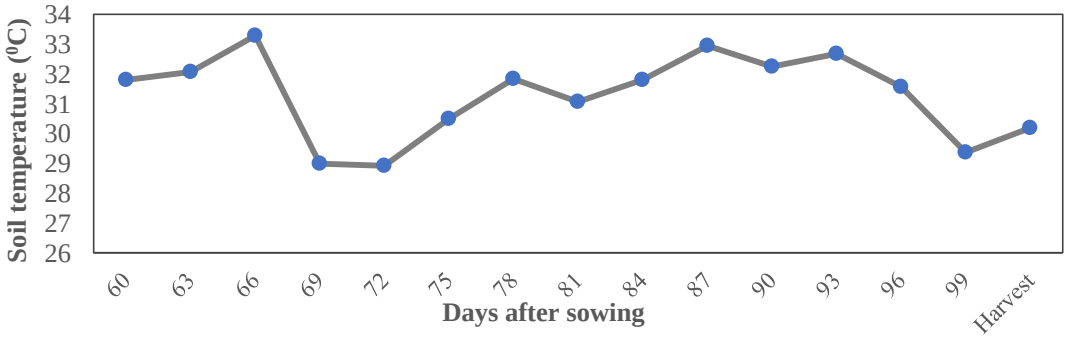


Figure 4.2: The average media temperature taken from 60 days after planting to harvesting. The data points are average of 10 pots on each recording day. The recording was taken in the mid-morning at an interval of 3 days and finalized at harvesting time.

4.3.2 Analysis of variance

There was high significant ($P < 0.001$) different in terms of aflatoxin contamination, incidence and severity between the genotypes and treatement. Genotype × Treatment had a significant ($P < 0.001$) difference for incidence and severity but had a significant ($P<0.01$) for aflatoxin contamination (Table 4.3). The relative magnitude of the main effects and their interactions for the traits reveal that the greater proportion of the source of variations was attributed to treatment, followed by genotype effect and their interaction respectively (Table 4.3).

Table 4.3: ANOVA table for incidence, severity, and aflatoxin contamination for 30 groundnuts genotypes. *** shows highly significance at $P<0.001$ and ** shows significance at $P<0.01$.

Source of Variation	Degrees of Freedom	Severity	Incidence	Aflatoxin contamination
Genotype	29	0.122***	846.724***	1255.046***
Treatment	1	9.841***	74166.596***	149768.48***
Genotype × Treatment	29	0.083***	594.848***	1171.31**

The incidence of infection ranged between 7.5 % to 48.5 % with an average value of 23.35 %. The severity ranged between 0.049 to 0.58 with a mean of 0.25. Genotype 12 and genotype 1 had the lowest and the highest incidence and severity scores respectively (Table 4.4). Only genotype 12 recorded an incidence less than less than 10 %, 10 genotypes exhibited incidence range between 10 % and 20 %, while 19 showed incidences above 20 % (Fig 4.4).

Genotype 12 and 8 had an aflatoxin concentration of 8.69 ppb and 8.468 ppb respectively. They were the only genotypes with aflatoxin less than 10 ppb. Genotype 22 had the highest aflatoxin concentration of 54.832 ppb. None of the genotypes had an aflatoxin concentration less than 4 ppb as recommended by the European union, 8 genotypes had an aflatoxin concentration below 20 ppb while 22 had aflatoxin concentration above 20 ppb (Fig 4.3)

Table 4.4: The mean values of aflatoxin concentration, percentage incidence and severity levels.

Code	*Genotype*	*Aflatoxin Concentration(ppb)*	*Severity*	*% Incidence*
1	GBK000423	50.058	0.585	48.500
2	GBK043007	23.942	0.233	21.500
3	GBK000429	20.135	0.130	12.500
4	GBK043011	29.771	0.194	20.500
5	GBK036409	16.130	0.136	12.500
6	GBK005070	11.157	0.091	11.500
7	GBK005071	15.448	0.146	15.500
8	GBK005117	8.468	0.145	12.500
9	GBK036401	17.217	0.224	23.000
10	GBK005077	19.612	0.361	35.000
11	GBK043011	21.652	0.280	26.000
12	GBK005111	8.690	0.049	7.500
13	GBK005112	23.685	0.260	26.500
14	GBK005126	28.354	0.220	19.500
15	GBK005116	21.918	0.153	14.000
16	GBK005123	23.314	0.223	20.500
17	GBK005118	26.724	0.189	20.000
18	GBK005075	27.818	0.474	43.500
19	GBK036399	37.553	0.100	11.000
20	GBK032355	34.493	0.225	23.000
21	GBK005151	29.861	0.369	34.500
22	GBK005063	54.832	0.189	17.000
23	GBK005073	38.480	0.318	26.500
24	GBK005088	20.919	0.233	22.000
25	GBK005099	38.561	0.294	25.000
26	GBK005108	46.600	0.338	30.500

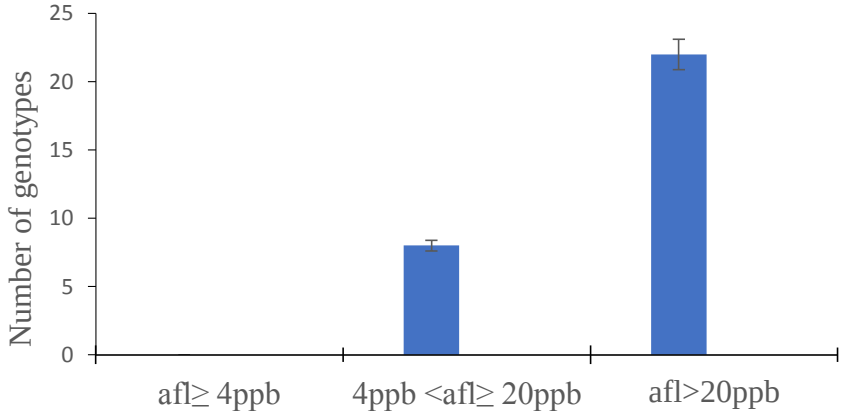


Figure 4.3: Number of groundnut genotypes grouped based on the aflatoxin concentration levels.

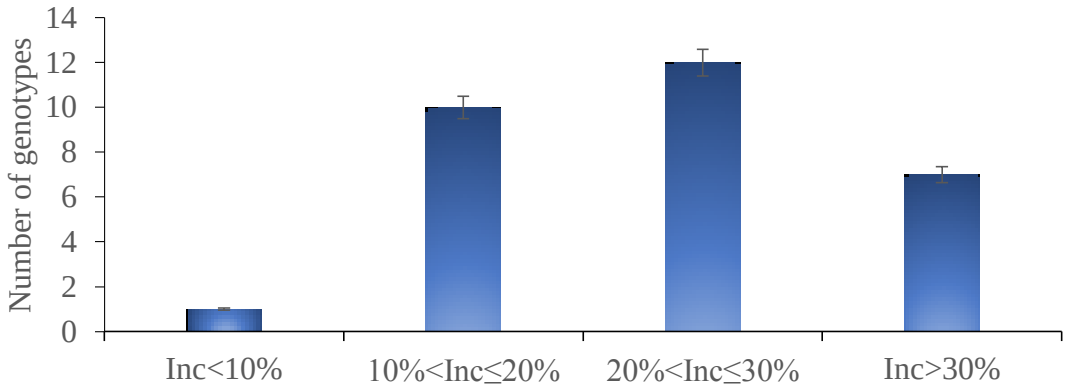


Figure 4.4: Number of groundnuts genotypes showing incidence intervals.

4.3.3 Correlation analysis

Our results revealed a postive correlation amongs the variables tested. A strong positve correlation coefficient of *r*=0.977 was observed between incidence and severity while a positive modorate correlations of *r*= 0.49 was detected between aflatoxin contamination to both incidence and severity (Table 4.5).

Table 4.5: Correlation analysis on incidence, severity and aflatoxin contamination data.

	Aflatoxin concentration	**Incidence**
Incidence	0.49	
Severity	0.48	0.98

4.3.4 Classification of genotypes according to mean performance with respect to incidence, severity and aflatoxin concentration

The biplot displays the overall performance and grouping of genotypes. The 79.6 % of the total variation amongst the genotypes was explained by the axes 1 while axes 2 explained 20.1% of the overall variability (Fig 4.5). The principal component analysis ranking of genotypes based on the variable traits mean revealed three clusters (Fig 4.5). The clusters 1, 2 and 3 grouped 8, 15 and 7 genotypes, respectively.

The aflatoxin contamination was significantly ($P<0.00$) associated to cluster 1. This cluster is characterized with genotypes having higher aflatoxin levels and are highly susceptible to *A. flavus* infection and contamination. The aflatoxin mean for the cluster is 65% greater than the overall mean (Fig 4.5, Table 4.6).

Cluster 2 was not significantly associated with any variable but consist of genotypes with lower mean for aflatoxin contamination but slightly higher mean for incidence and severity. The mean for the aflatoxin contamination, incidence and severity is 92 %, 108 % and 106 % of the overall mean, respectively (Table 4.6).

The incidence, severity and aflatoxin contamination were highly significantly ($P<0.001$) associated with cluster 3. The cluster consist of genotypes with lower percentage incidence, severity index and aflatoxin contamination. The average means for the traits is lower than the overall mean (Table 4.6).

Based on the results, 7 genotypes in cluster 3 were noted to be tolerant genotypes. GBK005117 and GBK005111 (genotypes 8 and 12, respectively) were considered the most tolerant with lower incidence, severity and aflatoxin contamination levels. Eight genotypes in cluster 1 were susceptible with genotype 30 and 1 being the most susceptible (Fig 4.5). Plate 1A represent genotype 12, a tolerant genotype while plate 1B represent genotype 30 a susceptible genotype.

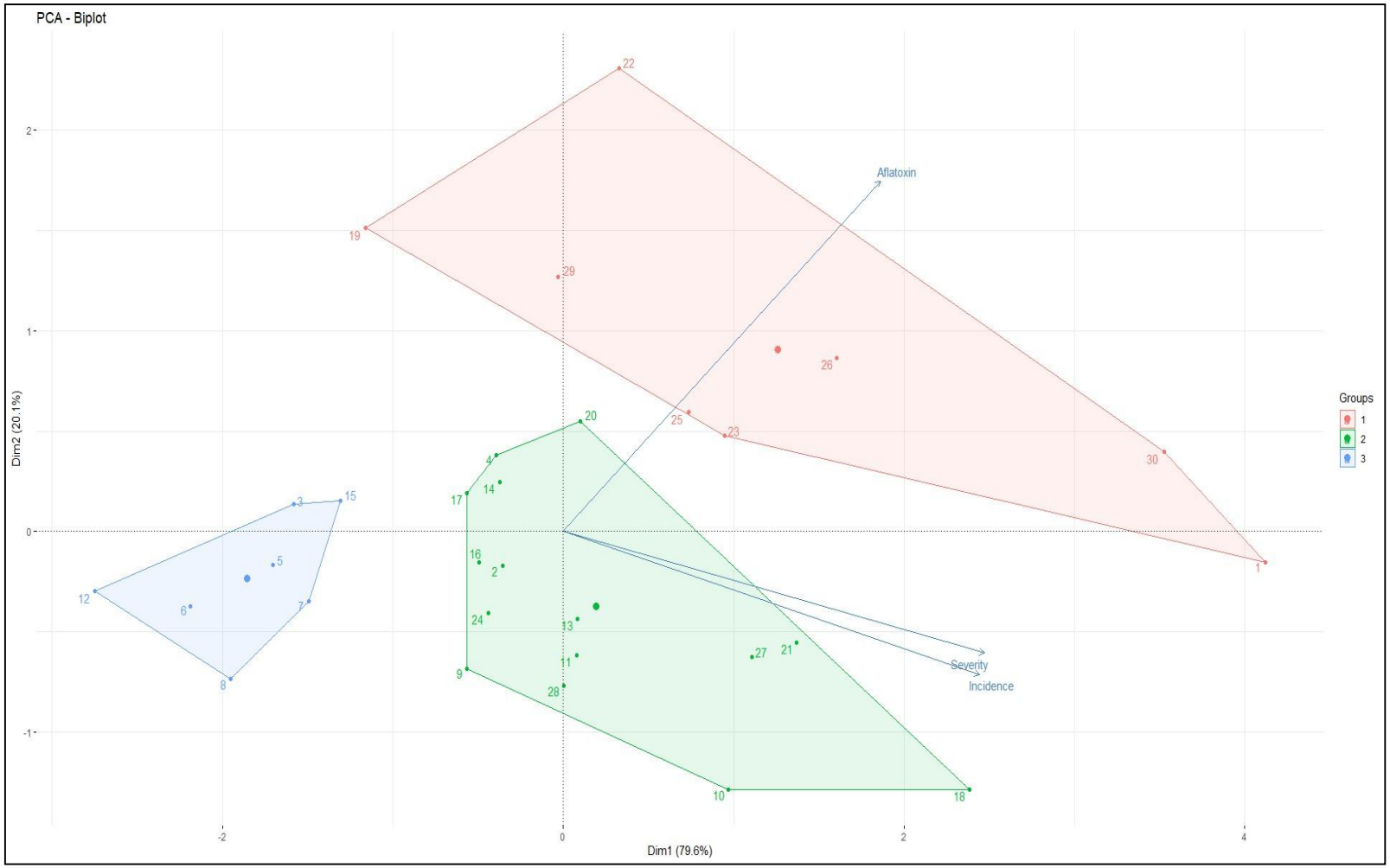


Figure 4.5: The mean performance of the genotypes with response to the incidence, severity and aflatoxin contamination as visualized by the PCA biplot.

Table 4.6: Statistics of each cluster based on the aflatoxin contamination, incidence and severity to fungal infection.

Trait	Overall mean	Cluster 1	p value 1	Overall mean	Cluster 2	p value 2	Overall mean	Cluster 3	p value 3
Aflatoxin contamination	27.89	46.11	5.32E-05	27.89	25.75	0.43	27.89	14.56	2.23E-04
Severity	0.25	0.35	0.15	0.25	0.26	0.65	0.25	0.12	3.38E-05
Incidence	23.35	30.09	0.21	23.35	25.25	0.49	23.35	12.29	7.14E-06

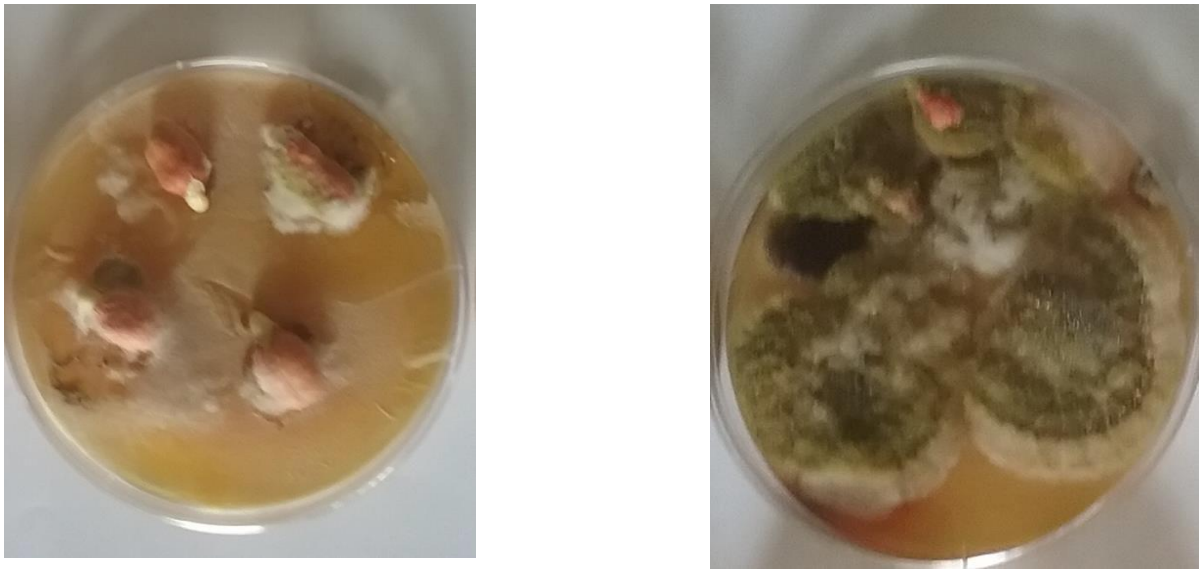


Plate 4.1: Heavily infested groundnuts on the right considered susceptible while on the left slightly infested genotype considered as tolerant after 7 days of inoculation.

4.4 Discussion

A. flavus is an osmophilic fungi that flourishes and dominate the groundnuts mycoflora during moisture and temperature stress (Kisyombe *et al*., 1985). The A. flavus infection and the risk of aflatoxin contamination is higher in terminal drought and high average soil temperature between 26 ^{0}C to 33 ^{0}C in the geocarphosphere (Rahmianna *et al*., 2015 ; Craufurd *et al*., 2006). The progressive drought stress for 40 days and average growth media temperature of approximately 30 ^{0}C provided conducive environment for infection in our study. These environmental conditions and high density of the inoculum supplied ensured a rigorous pre-harvest evaluation of the genotype's resistance.

All the treatment combinations registered *A. flavus* infection and aflatoxin contamination. However, variations were observed in the amount of aflatoxin accumulated, the percentage incidence and severity of *A. flavus*. The constitutive components in seed coat, phenolic compounds and induced defence mechanisms may have contributed to the variation in resistance. According to Liang *et al*., (2006) the different magnitude of infection is partly attributed to the composition of the pod and seed coats. They noted that the rate and magnitude of *A. flavus* colonization and haustoria penetration is related to the waxy and cuticle layers of the seed coat. Bisikwa *et al*., (2014) also observed that the resistant genotypes had more compressed structure of the testa, thicker wax on the surface of the seed coat and comparatively smaller hila.

Biochemical compounds present in the genotypes could be another plausible reason for the differential performance of these accessions. According to Schnur *et al*., (2021), the phenolic compounds such as tannins could hinder *A. flavus* establishment on seeds. The role of tannins in *A. flavus* inhibition was investigated by Commey *et al*., (2021). They performed a radial growth bioassay and concluded that the restricted growth of *A. flavus* demonstrate that both the groundnuts soluble and insoluble biochemical seed coat extracts possess antifungal activity.

Induced defence mechanism could also explain the observed variation in fungal infection. Studies have indicated that there is variation in induction of active oxygen and membrane lipid peroxidation during fungal infection. Pan *et al*., (2002) observed the systematic and differential changes in induced reactive oxygen species in resistance groundnut genotype in comparison to susceptible ones upon *A. flavus* infection. Reports have also indicated that groundnuts produce resveratrol and other stilbene-related

phytoalexins, a secondary antibiotic metabolite, in response to injury and pathogen attack (Commey *et al*., 2021). The resistant capacity of the groundnut to *A. flavus* depend on its capacity to rapidly accumulate stilbene phytoalexin (Commey *et al*., 2021). The pathogenic related proteins chitinase and β-1-3-glucanase have been reported to take part in plant defence mechanism through lysis action of the fungus cell walls and activation of other defence responses through oligosaccharide signal molecules synthesis (Shibuya and Minami, 2001).

The positive correlation between incidence, severity and aflatoxin contamination indicates that the aflatoxin production could be inferred from the fungus colonization and any one trait could be used as a selection criterion for the other. This is in line with the findings from Craufurd *et al*., (2006), that a strong positive relationship exist between *A. flavus* colonization and the magnitude of aflatoxin contamination. However, according to Rahmianna *et al*., (2015), the interaction between the environment, fungus and the groundnuts were the factors that determines the rate and magnitude of *A. flavus* infection and aflatoxin contamination. They conclude that the incidence and severity of *A. flavus* on the groundnuts does not automatically relate to the amount of aflatoxin pollution but suggest the possibly of infection relative to the fungus colonization (Rahmianna *et al*., 2015).

The incidence, severity and aflatoxin contamination are quantitative traits that are controlled by many recessive genes (Kelley *et al*., 2012). The selection of superior genotypes was based on low incidence, severity and aflatoxin contamination. Hierarchical classification grouped the genotypes into clusters based on average percentage incidence, severity index and aflatoxin contamination. The relative low infection and contamination levels found on the 7 genotypes on cluster 3 indicates that they are potential sources of resistance. A combined mean depicted genotype 12 as the most resistant genotype for both traits. It is the only genotype with incidence and aflatoxin contamination lower than 10%. Genotype 8 that had slightly lower aflatoxin content than genotype 12, but it had higher percentage incidence and severity levels. On the other hand, genotype 30 with higher aflatoxin contamination and percentage severity and incidence thus considered the most susceptible. The opposing genotypes could provide relevant check for the field evaluation of various genotypes. In addition, these

genotypes are suitable candidates for the evaluation of mechanism of resistance both at structural level, biochemical level and transcriptome level.

None of the genotypes met the stricter EU regulations of 4 ppb indicating that none of the produce from these genotypes could access the EU markets. About 36% of the samples were within Kenya regulatory limit indicating they were safe for human consumption locally.

4.5 Conclusion

The *A. flavus* colonization and subsequent pre-harvest aflatoxin pollution in groundnuts are complicated traits to screen and analyse owing to high genotype and environmental interactions. This study showed significant difference between the aflatoxin contamination, incidence and severity among the groundnut genotypes. Genotypes in cluster three with the lower mean of aflatoxin contamination, incidence and severity were considered tolerant to *A. flavus* and aflatoxin contamination in this study.

This study uncovers that GBK005111 (genotype 12) exhibited low values for incidence, severity and lower aflatoxin contamination accepted by the Kenya Standards. This genotype represents a relevant tool for the breeding program for resistance to *A. flavus* as a potential gene donor.

We recommended further research on genotypes in cluster three to determine their structural, biochemical and molecular sources of resistance. Additionally, further check could be done to evaluate their resistance and agronomical attributes in various environments. The two genotypes that differ for a resistance trait could be screened for polymorphic markers in QTL analysis.

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CHAPTER 5: IDENTIFICATION AND EXPRESSION PROFILING OF GENES DIFFERENTIALLY REGULATED IN RESPONSE TO *Aspergillus flavus* INFECTION IN TOLERANT AND SUSCEPTIBLE GROUNDNUT (*Arachis hypogaea* L.) VARIETIES.

Abstract.

Groundnut is one of the most susceptible host crops to colonization by *Aspergillus flavus* leading to aflatoxin contamination which are toxic and potential carcinogenic metabolites. In order to understand groundnuts defence machinery against *A. flavus*, we conducted a large-scale analysis of gene expression using microarray on two groundnut genotypes: resistance GBK005111 and susceptible GBK036397. We fabricated the microarray with 6,500 Express Sequence Tags selected from the NCBI dbEST database of the previous studies on groundnuts. A total of 163 genes were differentially expressed as determined by $\log_2$ ratio > 1.5 of the fold change out of which 63 genes were upregulated while 100 genes were down regulated. 57 genes differentially expressed had a putative function assigned to them while 106 genes were hypothetical. The upregulated genes with putative functions were grouped into 9 functional categories based on dominant biological function. Validation of the gene expression was done using RT-PCR and the results indicates consistency. Some of the identified genes were homologue to genes that were previously shown to confer resistance to fungal pathogen. The result obtain in this investigation, provides additional information for understanding the genetic basis of disease resistance and marker development.

Key words: Aflatoxin, EST, Groundnuts, Hypothetical, Microarray, Profiling, Transcriptomes, Upregulated.

5.1 Introduction.

Groundnuts (*Arachis hypogea*) is a critical crop in food security and trade in Kenya. The nuts are excellent source of proteins, carbohydrates, vitamins, minerals and bioactive compounds (Agong, 2006). Despite having relatively low protein contents in comparison to the animals sources, it contribute to 11 % of protein supply per year (Yang *et al*., 2017; Chen *et al*., 2014). This makes groundnuts a good source of human nutrition.

The *A. flavus* and *A. parasiticus* colonization and subsequent aflatoxin contamination affects the crop cultivation, trade and consumption globally (Torres *et al*., 2014). In addition to the groundnuts, these fungi affects other important crops such as corn, cotton, peanut and trees nuts (Torres *et al*., 2014). The consumption of aflatoxin contaminated foods can either led to acute or chronic toxicity in animals and human depending on the volume of ingestion (Abrar *et al*., 2013). The developing world have had more detrimental consequences of *A. flavus* infection and aflatoxin pollution owing to lack of appropriate mitigating technologies and inadequate enforcement of legislations to regulate aflatoxin contamination (Torres *et al*., 2014).

Several measures to curb the *A. flavus* colonization and aflatoxin contamination have been proposed. These measures have had various degree of success with none so far reported to exclude the aflatoxin completely in groundnuts (Torres *et al*., 2014). Cultural practices such as irrigation and early cropping to escape the terminal drought have been adopted but are unfeasible in arid and semi-arid areas (Torres *et al*., 2014). Chemical control through the use of insecticides and fungicides have also been applied at pre- and post-harvest conditions (Torres *et al*., 2014). The use of the atoxigenic strains of *A. flavus* to compete for the groundnut colonization with the toxigenic strains have shown great success in reducing *A. flavus* infection at a pre-harvest stage (Yin *et al*., 2008). Improvement of hygiene and storage conditions that don't favour the fungal growth reduce the nuts contamination at a post-harvest level.

The development of resistant genotypes to *A. flavus* have been considered a practical approach to exclude fungus infection (Holbrok and Stalker, 2003). This strategy does not require extra resources to the farmers, have limited maximum residue level problems, and is a better alternative for regions that lack an appropriate biocontrol measures (Garrido-Bazan *et al*., 2018). Conventional and molecular breeding initiatives to develop resistant varieties have been undertaken (Torres *et al*., 2014). Lack of sources of

resistance and linkage drag have slowed the conventional breeding (Torres *et al*., 2014). Despite these challenges, limited number of resistant cultivars have been identified through field screening and invitro analysis in the laboratory (Wang *et al*., 2010). However, the poor agronomical attributes of these cultivars have hindered their application into large scale production. The improvement in genomics has accelerated the molecular breeding and led to the discovery of novel genes, proteins and other regulators associated with groundnut resistance to A. flavus (Abrar *et al*., 2013). The progress in cultivated groundnut genomic study is relatively slow in comparison to other important crop owing to genome size (Luo *et al*., 2005). The large genome of about 2.8 Gb has complicated its assembly, analysis and annotation (Luo *et al*., 2005). It is an allotetraploid that resulted from a single hybridization incident of two diploid ancestors followed by polyploidization (Bertioli *et al*., 2016). It consist of AABB type genome; 2n=4x=40 with one pair of small chromosomal distinguish A from B sub genome (Bertioli *et al*., 2016). The molecular and cytogenetics evidence indicate that the *A. duranensis* and *A. ipaensis* are the donors of the A and B sub genomes respectively. The sub genomes have undergone relatively fewer mutation since polyploidization incident and thus sequencing the ancestors could assist in the exploration of the cultivated groundnut genomes (Bertioli *et al*., 2016).

The use of Express Sequence Tags (ESTs) have been fronted as an alternative to advance the understanding of the groundnuts genome at a manageable cost (Guo *et al*., 2011). The EST has been used in other system for genome wide gene expression analysis in various tissues, developmental stages and environmental conditions (Guo *et al*., 2008). The cDNA libraries constructed, have accelerated further molecular characterization of genes of interest and provided sequence information for microarray construction and genome annotation (Luo *et al*., 2005).

The first groundnut microarray was designed from the ESTs generated by Luo *et al*., (2005). Guo *et al*., (2009) working with the USDA research group generated 41,568 ESTs derived from groundnuts Tifrunners and a breeding line GT-C20. Shandong Academy of Agricultural Sciences in china conducted a study to establish the biotechnological platform for groundnut germplasm innovation. They reported 17,000 ESTs and used 5066 ESTs sequences to make a cDNA microarray (Bi *et al*., 2010).

In this study, microarray analysis was conducted to gain additional understanding of the molecular mechanisms of groundnut and *A. flavus* interaction. Even though there have been many attempts to understand groundnut resistance to *Aspergillus flavus* infection using microarray, little is known about the molecular mechanisms of resistance in Kenyans germplasm. The goal was to identify candidate genes that confer resistance to *A. flavus* infection due to up-expression in response to fungal infection using a resistant peanut line vs a susceptible line.

5.2 Materials and Methods.

5.2.1 Groundnuts accessions used.

The two groundnuts' genotypes GBK005111and GBK036397 used in this experiment were obtained from the screening experiment in our previous studies. GBK005111 is a resistance accession while GBK036397 showed susceptibility to the fungus invasion and aflatoxin contamination. All the seeds were surface cleaned in 70% ethanol for 1 minute and rinsed 4 times in distilled water. The seeds were planted in sterilized sand in plastic pots of approximately 30 cm diameter. The experiment was conducted in a greenhouse in Jaramogi Oginga Odinga University of Science and Technology horticultural farm.

Both the susceptible and resistance genotypes were subjected to drought stress 60 days after planting to harvesting and artificial inoculation with A. *flavus* at the onset of flowering. The spore suspension in distilled water was obtained from a sub-cultured *AF*-JOOUST5 isolate (Okayo *et al.*, 2020) in a potato dextrose agar media. At approximately 90 days after planting, the mature pods were collected, bulked per genotype and immediately frozen in liquid nitrogen before stored at -80 ^{0}C.

5.2.2 Extraction of Messenger RNA.

The total RNA was extracted using RNeasy mini kit (QIAGEN, USA). The groundnut samples were grounded separately from each biological replicate and 20g from each replicate were pooled together. From the pooled samples, 100 mg of the ground tissue were transferred into a 10 ml centrifuge tube. The cells were lysed through addition of 5 ml of lysis buffer and mixed by vortexing. The solution was then incubated at 65 ^{0}C in a water bath for 20 minutes with gentle whirling and then allowed to cool for 20 minutes on the bench. Two (2) ml of chloroform was added and the mixture was thoroughly vortexed and incubated in ice for 5 minutes before centrifuging at 8,000 rpm for 20 minutes at 4 ^{0}C. The clear supernatant was gently moved to a new RNase free tube and

1.5 ml of lithium chloride (LiCl) added, mixed then incubated at -80 ^{0}C for 2 hours. The RNA was then palletized through centrifugation at 8,000 rpm for 20 minutes and washed with 75 % ethanol. The pellets were air dried on the bench and then dissolved in 100 µL RNase free water. The RNA concentration and quality were assessed with NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). The RNA extract that had 1.8 $\leq$ 260/280 $\leq$2.1 and 2.0 $\leq$ 260/230 $\leq$ 2.3 was considered for cDNA synthesis. Approximately 5 µl of total RNA was used for the cDNA synthesis.

5.2.3 Purification of Poly(A)+ mRNA.

This separates the mRNA from the total RNA was done using RNeasy Plant Mini Kit (Qiagen), The salinized column provided with the kit was washed with 10 ml of sodium hydroxide and rinsed with clean water. A slurry solution of 0.5 gms of oligo (dT) cellulose dry powder in 1 ml of Sodium hydroxide was prepared and poured into the salinized column. The clean distilled water was then used to rinse the column. The pH of the column was adjusted using 10 to 20 ml of the loading buffer until the flow through had a pH of 7.5. The total RNA was warmed in a water bath at 70 ^{0}C for approximately 10 minutes and passed through column and washed with the poly A loading buffer and reuse the eluant for four times to ensure maximum poly A tail are adhered to the oligo dT. Two (2) ml sodium dodecyl sulfate was used to elute the RNA. The salt concentration of the eluted RNA was adjusted to 0.3 M sodium acetate and 0.5 ml of ethanol added. The RNA solution was then transferred to two silanized SW 55 tubes. Allow the solution to incubate overnight in -20 ^{0}C fridge. Centrifuge the solution at 20,000 rpm to accumulate the precipitate at the bottom. Discard the ethanol, air dry the pallets on the bench and resuspend the RNA pellets in 200 µl of RNAse free TE buffer.

5.2.4 Labelled cDNA Synthesis

Labelled star array kit (Qiagen) was used for the reverse transcription. The reverse transcription was done in the presence of labelled florescence dyes Cy3- or CY5-coupled aminoallyl-dUTP. The RT was conducted in a reaction tube comprising of 2µl of each dNTP mixture, 2 µl of poly (A+) RNA, 2 µl of oligo(dT)18-23 primer, 2 µl of each dNTP mixture, 4 µl of DTT, 2µl of Cy5-dUTP or 2µl of Cy3-dUTP, 2µl of RNase inhibitor, and 2µl of Superscript II reverse transcriptase. The RNA and primers were preheated at 65 ^{0}C for approximately 5 minutes and cool in ice before adding other reaction components. The reverse transcription reaction was completed in 1.5 hours at 42

^{0}C. To degrade the RNA, 5 µl of EDTA at pH 8.0 and 5 µl of NaOH and incubated in a water bath at 60 ^{0}C for 15 minutes. Twenty-five (25) µl of Tris-HCL at a pH of 8.0 was added to stop the reaction.

5.2.5 Oligo microarray design.

The microarray was designed and sourced from the Agilent. The microarray was designed using the agilent eArray platform. The express sequence tags spotted on the array were sourced from the NCBI database of the previous studies on groundnuts and other plants. A total of 6,500 ESTs were spotted and each EST was replicated 3 times in a different spot on the array. In addition, 600 agilent control and 475 random probes were incorporated. Each EST ranged between 50-70 mer. On each feature, 6 probes that match perfectly to the sequence of a specific region of the gene ware located.

5.2.6 Microarray Hybridization.

Heat the hybridization chamber to 65 ^{0}C for 2 hours prior to the hybridization. The hybridization mix was then prepared as follows: 1 µl of Cy3 for the resistant samples and 1 µl of Cy5 for the susceptible genotype sample, 10 µl of the blocking agent, 2.0 µl of the fragmentation buffer and topped up to 50 µl with RNase free water. Vortex the mixture slowly and incubate at 65 ^{0}C for 40 minutes. The reaction was stopped by adding 55 µl hybridization buffer and mixed. The contents were collected at the bottom and the air bubbles eliminated through centrifugation. Chill the tube in ice on a dark room and load the array in the loading chamber and hybridize the array in the oven for 65 ^{0}C for 17 hours.

5.2.7 Microarray washing.

The wash buffer, 2× SSC and 0.1% SDS, was used to wash the slides with gentle shaking for 15 minutes. The wash buffer was first preheated to 55 ^{0}C before washing. The slides were further washed in 0.5× SSC for 15 minutes and lastly in 0.05× SSC for additional 15 minutes at a room temperature. The slide was dried by spinning for 2 minutes in a centrifuge and immediately scanned.

5.2.8 Microarray data analysis.

The microarray slides were scanned using 42000AL GenePix scanner. The obtained image was then processed with GenePix Pro 6.1 software using morphological opening background method. The R/Bioconductor lamma package was used for data analysis to

tabulate the overall intensities. The ratios of the control genes were used to normalize the intensities of the Cy3 and Cy5. The loess within the array normalization method was used for data normalization. A gene with a positive value associated with it was considered expressed. The spot was considered differentially expressed when the absolute value of the Cy5/Cy3 log2 was more than 1 indicating that the disparity of gene expression was greater than double. The comparison was conducted between resistance and susceptible genotypes under drought and *A. flavus* infection.

5.2.9 Gene ontology annotation.

The gene sequences of the differentially expressed genes and predicted gene functions were analyzed using NCBI BLAST, search of the gene bank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Following the description of these genes in the public databases, we grouped these genes into different functional categories.

5.2.10 Real-time RT-PCR.

The real time PCR was used to validate the microarray data output. The total RNAs used in microarray hybridization was used for the RT-PCR. The SYBRR premix Ex Tag II kit was used based on manufacturer's instructions. The reverse transcription was done in 20 µl Nucleases free microcentrifuge tube containing 1 µl of primer pairs, 5 µl of isolated RNA, 1 µl of each dNTP mix at a neutral pH (dATP, dGTP dCTP and dTTP) and 13 µl of distilled water. The mix was then incubated at 65 ^{0}C for 5 minutes and incubated on ice for 5 minutes. 4 µl of the first strand buffer, 1 µl of DTT, 1 µl of RNase inhibitor and 1 µl of superscript were added and mixed through pipetting. The incubation was then done for 45 minutes at 55^{0}C. The reaction was termination by heating at 70 ^{0}C for 15 minutes in a water bath. The RT-PCR was done according to Rodrigues *et al*. (2009): 1 cycle of 4 minutes for initial denaturation at 94 °C; 30 cycles of 60 seconds for subsequent denaturation at 94°C, 30 cycles of 1 minute for annealing at 55 - 60°C; 30 cycles of 1 minute for extension at 72°C and a final extension at 72°C for 6 min. The primer sets used in the experiment (Table 5.1) was designed using primer express 3.0 plus tool. The house keeping gene, groundnut *actin*, was used as a reference to normalize the transcript abundance. The quantification of RNA levels was determined using the $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak, 2008).

Table 5.1: RT-PCR primer sequence of 10 upregulated genes used in RT-PCR.

Accession Number	Gene description	Primer sequence (5' → 3')	Amplicon size (bp)
P83595	Trypsin inhibitor	F: CGAGATACAGGGTTGGTTTGAG R: CCAAAAGCCTATTTCCCTCATC	128
AAF602701	Lipoxygenase-4 (L-4; VSP94)	F: TGGTGAAGAGTCACCAAAAGG R: TCCAATGTGGATTATCCCTCTC	141
gi|110810624	Late embryogenesis abundant protein B19.3 (LEA B19.3)	F: TAGTTCGGGTTGTAGTAGCAGGGT R: AAGGTTCCATCTTCTCGCCGATGT	99
gi|146771807	Glycinin	F: TATGATGATGACGATCGACGACCACG R: TGCATAGTGTTTCCTCCACTCCGT	82
NP_188086	Cytochrome P450 76B1	F: TGGGCTTACTCGAAATACCG R: GCATTATCACCCCAAAGTCC	123
P11670	Pathogenesis-related protein 1 (MSPR10-1)	F: AACACTCCATGGGGCCTTAC R: TGTAATGCAGGCACTCATCG	147
NC_037620.1	Endochitinase	F: TGCCAATACACTGCTGGAGG R: GTAAGGGCAGTTGCAGGGAT	146
NC_037622.1	12-oxophytodienoate reductase 2	F: CAGTCCCATGCTGCCTTGTA R: AGTTGTGGAAGGGTGTGTACC	112
NC_037620.1	Disease resistance response protein 206 Putative	F: GTAAAACCCTTGCGCTCGTC R: CGTCTTTGACGACCCCATCA	73
NC_037635.1	Defensin like proteins	F: CACGTTGCTGCCTCACTTTG R: ATTGCTGGCCCCTAGCATTC	96
DQ873525.1	Peanut Actin (control)	F: GTTCCACTATGTTCCCAGGCA R: CTTCCTCTCTGGTGGTGCTACA	85

5.3 Results

5.3.1 Overview and Classification of differentially Expressed genes.

Out of the 6500 EST used in the microarray design, only 4,213 were assigned putative functions. The rest 2,287 EST were considered hypothetical since they did not meet the criterion for annotation. To identify the deferentially expressed genes induced by *A. flavus* in GBK005111 (R) pods in relation to GBK036397 (S), transcripts levels with a fold change of $\log_2 \geq 1.5$ of the mean florescence intensity were up-regulated while a fold change of $\log_2 \leq -1.5$ were down regulated. A total of 163 genes were differentially expressed out of which 63 genes were upregulated while 100 genes were down regulated. 57 genes differentially expressed had a putative function assigned to them while 106 genes were hypothetical. There were 29 genes with putative function that were upregulated 28 genes with putative function were downregulated (Fig 5.1)

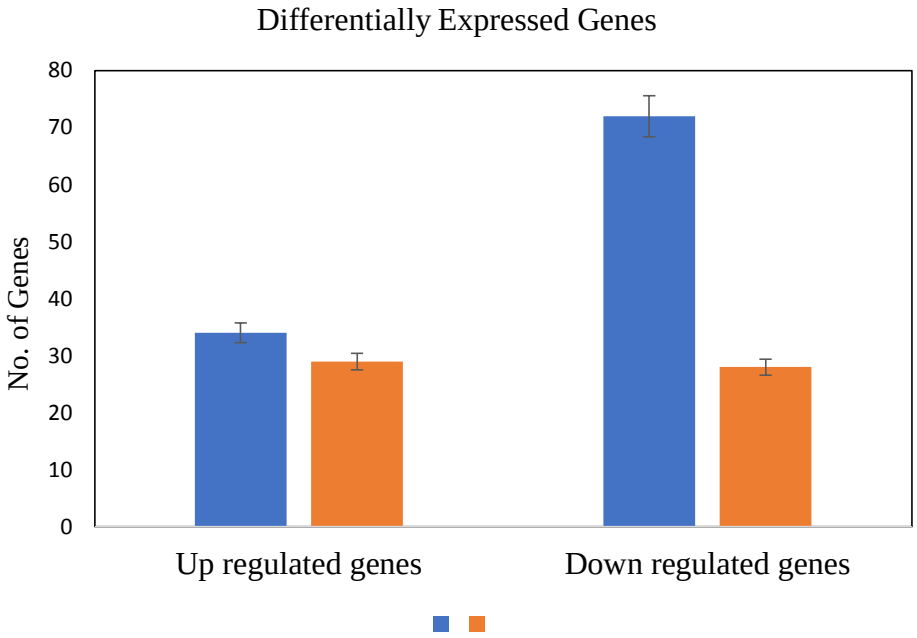

Figure 5.1: The number of differentially expressed genes in response to *A. flavus* infection from microarray screening of the gene expression.

5.3.2 Differentially expressed transcripts in the resistant genotype.

The upregulated genes were listed and grouped based on their putative functions. The genes either had a putative function or were hypothetical. Genes that act in multiple metabolic process or molecular function were classified according to their main role

(Table 5.2). The down regulated genes were identified and grouped into two categories. Transcripts with homology match with genes from the databases were grouped separately from the hypothetical ones (Table 5.3).

Table 5.2: Functional classification of upregulated genes in infected GBK005111 (R) mature pods

Putative function	Probe	Description of putative protein	$log_2 \geq 1.5$ (Microarray, R/S)
Cell wall biosynthesis and modification	LS43179	Endochitinase	3.5
Calcium binding proteins	LS35527	Calcineurine gene	2.3
	LS26059	Calmodulins	2.3
	LS36083	Calcium dependent Kinase	1.9
Lipid metabolism	LS21863	Acyl-CoA-binding protein (ACBP)	3.2
	LS39525	Lipoxygenase-4	3.1
	LS12366	12-oxophytodienoate reductase 2	1.9
	LS22177	lipid transfer protein	1.9
Protease inhibitor	LS20455	Trypsin inhibitor	2.3
	LS23970	Kunitz-type trypsin inhibitor 1 (KTI1) precursor	2.0
Proteolysis	LS21284	Serine carboxypeptidase 3 precursor	1.8
Mediators in hormone signaling pathways	LS35563	Ethylene-responsive transcription factor 5	3.2
	LS21217	1-aminocyclopropane-1-carboxylate oxidase homolog 1	2.1
	LS31592	Ethylene-responsive factor 1	2.0
	LS26855	Ethylene-responsive factor 6	2.5
	LS34462	Phospholipase	3.5
	LS17431	Plant defensin	2.5
	LS37967	Alcohol dehydrogenase	
Oxidative signaling	LS25239	Cytochrome P450	3.1
	LS22650	Oxalate oxidase	2.4
	LS38655	Peroxidase	4.4
	LS34246	Ascobase peroxidase	4.4
	LS37154	Glutathione S-transferase	1.8
Defense-related	LS36811	Pathogenesis-related protein 1	4.2
	LS14633	Defensin-like protein 1	3.6
	LS39893	Glycinin	2.8
	LS15582	Disease resistance response protein 206 putative	2.5
	LS12941	Brassinosteroid Insensitive1-associated receptor kinase 1 (BAK1) precursor	2.1
Stress responses	LS37495	Late embryogenesis abundant protein.	2.4

Unclear classification	LS32442	Hypothetical protein	9.4
	LS26863	Hypothetical protein	5.1
	LS38777	Hypothetical protein	4.3
	LS33908	Hypothetical protein	3.4
	LS11297	Hypothetical protein	3.4
	LS39325	Hypothetical protein	2.7
	LS11307	Hypothetical protein	2.6
	LS16427	Hypothetical protein	2.5
	LS33526	Hypothetical protein	2.5
	LS38249	Hypothetical protein	2.5
	LS12483	Hypothetical protein	2.4
	LS16350	Hypothetical protein	2.3
	LS20558	Hypothetical protein	2.3
	LS32382	Hypothetical protein	2.3
	LS35528	Hypothetical protein	2.3
	LS25863	Hypothetical protein	2.2
	LS33678	Hypothetical protein	2.2
	LS30071	Hypothetical protein	2.1
	LS32216	Hypothetical protein	2.1
	LS43301	Hypothetical protein	2.1
	LS34881	Hypothetical protein	2
	LS46379	Hypothetical protein	2
	LS13324	Hypothetical protein	1.9
	LS15388	Hypothetical protein	1.9
	LS17599	Hypothetical protein	1.9
	LS21222	Hypothetical protein	1.9
	LS21382	Hypothetical protein	1.9
	LS31143	Hypothetical protein	1.9
	LS32924	Hypothetical protein	1.9
	LS33198	Hypothetical protein	1.9
	LS38199	Hypothetical protein	1.9
	LS40338	Hypothetical protein	1.9
	LS44512	Hypothetical protein	1.9
	LS47212	Hypothetical protein	1.6

On the other hand, 29 genes (Table 5.3) were down regulated in challenged GBK005111 (R) pods.

Table 5.3: List of down-regulated genes in infected GBK005111 (R) mature pods.

Probe	Description of putative protein	$log_2 \leq -1.5$ (Microarray, R/S)
LS37535	Ubiquitin-protein ligase 13	-2.2
LS11252	T-complex protein 1 subunit alpha	-2.2
LS46390	Nascent polypeptide-associated	-2.1
LS43798	complex	-2.1
LS42369	Gamma-secretase subunit	-2.1
LS41773	Signal recognition particle 68 kDa	-2.1
LS40311	protein	-2.1
LS40115	Argonaute	-2.1
LS33121	Ornithine aminotransferase	-2.1
LS33001	Bet1-like SNARE	-2.1
LS32658	phosphate translocator 2	-2.1
LS31549	Calnexin homolog precursor	-2.1
LS30523	Cyclin	-2.1
LS30205	Novel aldehyde dehydrogenase	-2.1
LS30005	Aspartic proteinase nepenthesin	-2.1
LS26699	Replication protein	-2.1
LS24352	nucleotide-binding protein	-2.1
LS23663	DNA-binding protein	-2.1
LS20938	FAM18-like protein	-2.1
LS19774	Glycine cleavage system H protein	-2.1
LS18645	Mitochondrial 60S ribosomal protein	-2.1
LS18070	L2	-2.1
LS16301	25.3 kDa vesicle transport protein	-2.1
LS12570	HVA22-like protein k	-2.1
LS10736	Glutaredoxin-C1	-2.1
LS10730	Delta (6) fatty acid desaturase	-2.1
LS35772	3-hydroxybutyryl-CoA dehydratase	
LS44955	Ferredoxin--NADP reductase	-2
LS11622	Jasmonate-ZIM-domain protein 8	-2
	Bifunctional dihydrofolate reductase-Thymidylate synthase	
	Alpha-glucanotransferase	
	Hypothetical protein	-2.5
LS43179	Hypothetical protein	-2.5
LS37721	Hypothetical protein	-2.5
LS37495	Hypothetical protein	-2.5
LS36811	Hypothetical protein	-2.5
LS35563	Hypothetical protein	-2.5
LS34969	Hypothetical protein	-2.5
LS34246	Hypothetical protein	-2.5
LS31592	Hypothetical protein	-2.5
LS26855	Hypothetical protein	-2.5
LS25239	Hypothetical protein	-2.5
LS22990	Hypothetical protein	-2.5
LS16163	Hypothetical protein	-2.5
LS15582	Hypothetical protein	-2.4

LS44225	Hypothetical protein	-2.4
LS41097	Hypothetical protein	-2.4
LS38655	Hypothetical protein	-2.4
LS38484	Hypothetical protein	-2.4
LS37832	Hypothetical protein	-2.4
LS36846	Hypothetical protein	-2.4
LS34387	Hypothetical protein	-2.4
LS34374	Hypothetical protein	-2.4
LS33254	Hypothetical protein	-2.4
LS26111	Hypothetical protein	-2.4
LS17338	Hypothetical protein	-2.4
LS16756	Hypothetical protein	-2.4
LS16032	Hypothetical protein	-2.4
LS12941	Hypothetical protein	-2.4
LS11815	Hypothetical protein	-2.3
LS44406	Hypothetical protein	-2.3
LS43558	Hypothetical protein	-2.3
LS42270	Hypothetical protein	-2.3
LS40648	Hypothetical protein	-2.3
LS39650	Hypothetical protein	-2.3
LS35985	Hypothetical protein	-2.3
LS35303	Hypothetical protein	-2.3
LS35187	Hypothetical protein	-2.3
LS32959	Hypothetical protein	-2.3
LS32421	Hypothetical protein	-2.3
LS32347	Hypothetical protein	-2.3
LS27203	Hypothetical protein	-2.3
LS25829	Hypothetical protein	-2.3
LS12698	Hypothetical protein	-2.3
LS11340	Hypothetical protein	-2.3
LS11270	Hypothetical protein	-2.2
LS43477	Hypothetical protein	-2.2
LS41936	Hypothetical protein	-2.2
LS39466	Hypothetical protein	-2.2
LS35465	Hypothetical protein	-2.2
LS32485	Hypothetical protein	-2.2
LS31933	Hypothetical protein	-2.2
LS31451	Hypothetical protein	-2.2
LS25835	Hypothetical protein	-2.2
LS20985	Hypothetical protein	-2.2
LS16748	Hypothetical protein	-2.2
LS15167	Hypothetical protein	-2.1
LS42665	Hypothetical protein	-2.1
LS32308	Hypothetical protein	-2.1
LS30713	Hypothetical protein	-2.1
LS30124	Hypothetical protein	-2.1
LS23261	Hypothetical protein	-2.1
LS19077	Hypothetical protein	-2
LS39722	Hypothetical protein	-2

LS39718	Hypothetical protein	-2
LS27255	Hypothetical protein	-2
LS27241	Hypothetical protein	-2
LS25255	Hypothetical protein	-2
LS22861	Hypothetical protein	-2
LS20945	Hypothetical protein	-2
LS15392	Hypothetical protein	-2
LS14127	Hypothetical protein	-2
LS13380	Hypothetical protein	-2

5.3.3 Validation of differential expressed genes.

The real time PCR gene analysis was conducted on 10 resistant genes to validate the microarray output. This allowed testing the accuracy of gene expression levels reported in microarray data and ruling out false positives (Bustin, 2000). The output of gene analysis through RT-PCR expression were in harmony with the microarray expression analysis. The RT-PCR determination of Pathogenesis-related protein 1 (MSPR10-1), Defensin-like protein, Disease resistance response protein, Glycinin, Endochitinase, Lipoxygenase-4, 12-oxophytodienoate reductase 2, Cytochrome P450 and Late embryogenesis abundant protein B19.3 mRNA showed a 4.2, 3.6, 2.5, 2.8, 3.5, 3.1, 1.9, 3.1, 2.4 and 2.3-fold increase respectively (Fig. 5.2). This compared favourably to the fold increase expression as determined by the microarray (Table 5.4).

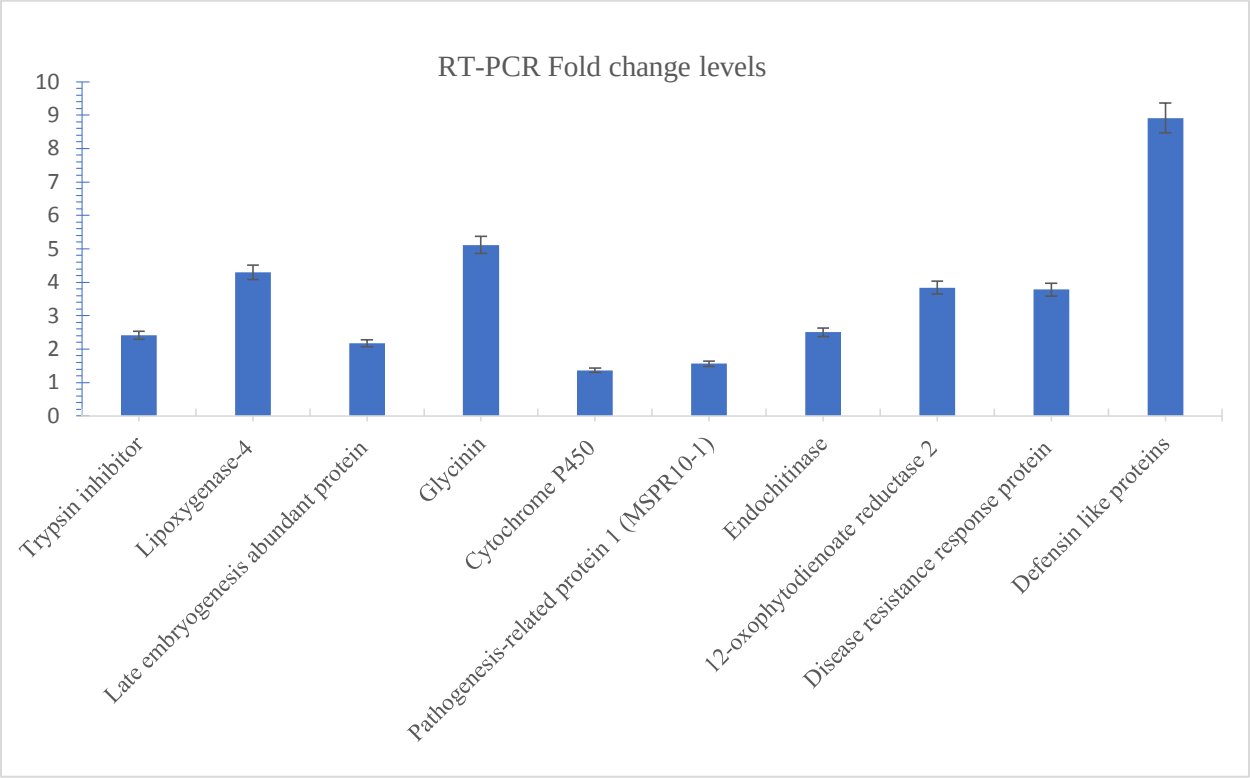

Figure 5.2: Comparative RT-PCR fold change analysis of selected defense upregulated genes under *A. flavus* infection and control conditions.

Table 5.4: Gene expression of microarray data and validation by RT-PCR for selected defense-related genes in response to *A. flavus* infection.

Putative function	**Probe**	**Description of putative protein**	**$log_2 \geq 1.5$ (Microarray, R/S)**	**ΔR/ΔS RT-PCR expression level**
Defense-related	LS36811	Pathogenesis-related protein	4.2	1.56
	LS43179	Defensin-like protein	3.6	8.94
	LS15582	Disease resistance response protein	2.5	3.78
	LS39893	Glycinin	2.8	5.12
Cell wall biosynthesis and modification	LS43179	Endochitinase.	3.5	2.51
Lipid metabolism	LS39525	Lipoxygenase-4	3.1	4.29
	LS12366	12-oxophytodienoate reductase 2	1.9	3.83
Oxidative signaling	LS25239	Cytochrome P450	3.1	1.37
Stress responses	LS37495	Late embryogenesis abundant protein B19.3	2.4	1.17
Protein inhibitors	LS20455	Trypsin inhibitor	2.3	2.41

5.4 Discussion

Microarray is a powerful tool for the analysis of the global gene expression profiles. It enables the gene function prediction at various physiological processes in response to environmental stresses (Clarke and Zhu, 2006). To add into the existing knowledge of the intricate molecular responses in groundnuts against *A. flavus* pathogen, microarray gene expression profiles of resistance (GBK005111) versus the susceptible (GBK036397) analysis was conducted and validated using a RT-PCR. The analysis identified several genes that ware differentially expressed (Table 5.2). The upregulated genes in the resistant genotype provides vital clues on molecular mechanism of resistance. There were 57 genes with putative functions that were altered in the *A. flavus* reaction with groundnuts. The upregulated genes were assigned functional categories based on the dominant biological function (Table 5.2)

We noted the upregulation of genes involved in the plant hormone signaling pathways. Plant hormone signalling pathways play a significant role in modulating defence responses to various biotic and abiotic challenges. Signalling pathways involve salicyclic acid (SA), jasmonic acid (JA), and ethylene (ET). They are traditionally known as 'stress' hormones and stimulate different mechanisms of plant defence. Four upregulated genes in the resistant genotype were found to act as mediators in ET signalling pathway. These included 1-aminocyclopropane-1-carboxylate oxidase and three transcription factors; Ethylene-Responsive Transcription Factor (ERF) 1, ERF5 and ERF6.

ET is a hormone that reprograms the plant physiology to adapt to biotic and abiotic stresses. It is synthesized through the 1-aminocyclopropane-1-carboxylic acid (ACC) pathway (Wang *et al*., 2002). The SAM-synthase converts the amino acid methionine to S-adenosyl-L-methionine and ACC-synthase cleaves the 5′-methylthioadenosine in SAM is to form, 1-aminocyclopropane-1-carboxylic acid (ACC). The third ET biosynthesis protein, ACC-oxidase (ACO), oxidizes ACC to generate ET, cyanide and carbon dioxide. Expression of ACO gene has been suggested to positively correlate with ethylene production (Ruduś *et al*., 2013). Consistent with the microarray data obtained in this study, previous reports have indicated ACO1 transcripts are significantly induced under biotic and abiotic stress (Pan and Lou, 2008).

The ERF is member of the APETALA2 and Ethylene-Responsive Element (AP2/ERF) superfamily. They consist of plant transcription factors that are involved in the regulation of plant immune systems (Gutterson and Reuber, 2004). Studies have showed that ET, JA, pathogens and abiotic stresses activate the expression of ERF genes (Lorenzo *et al*., 2003; Onate and Singh, 2002 and Fujimoto *et al*., 2000). The ERF genes have conserved DNA binding domain which interacts with GCC box (AGCCGCC) to form a complex (Ohme-takagi and Shinshi, 1995). The GCCGCC sequence in the GCC box is critical for ET responsiveness and expression of pathogenesis related genes which prevent disease progression (Hao *et al*., 1998; Sato *et al*., 1996). In Arabidopsis thaliana, both ERF1 and ERF5 function as a positive regulator of ET responses during defense and pathogen attack (Fujimoto *et al*., 2000). This was also evident in GBK005111 (R) pods where ERF1 transcripts were induced in response to *A. flavus* infection.

ERF6 was also upregulated in our study, but unlike ERF1, its activation does not depend on the ET (Meng *et al*., 2013). Upon pathogen attack, the accumulation of two pathogen responsive proteins, mitogen activated protein kinases 3 and 6 (MPK3 and MPK6) induce the expression of ERF6 (Meng *et al*., 2013). Meng *et al*. (2013), demonstrated ERF6 acts downstream of MPK3/MPK6 signalling cascade thereby activating expression of defence-related genes during pathogen attack.

The signalling hormones can work synergistically or antagonistically in response to attack. JA and ET work synergistically to overcome the invading pathogen while they have antagonistic action against SA induction (Bari and Jones, 2009). In our study there was an evidence upregulation of genes such as alcohol dehydrogenase and phospholipase that take part in ET biosynthesis. On the other hand, the gene that encodes a protein Jasmonate zim protein that antagonise the JA biosynthesis (Bari and Jones, 2009) was down-regulated. The JA responsive marker gene, plant defensin gene, that is linked to the necrotic pathogen resistance (Bari and Jones, 2009) was also seen to be upregulated in our study indicating that the JA and ET signalling pathway was preferred pathway for groundnut resistance to *A. flavus* infection and aflatoxin contamination.

Four defence related genes, pathogenic-related proteins 1 (PR1), defensin-like proteins 1, disease resistance response proteins 206 putative and brassinosteroid Insensitive1-associated receptor kinase 1 precurser were upregulated in our study. PR1 genes are ubiquitous across plant species and forms part of the basal and broad-spectrum defense (Durrant and Dong, 2004).

Plant defensins, also denoted as defence-related proteins, are basic cysteine-rich antimicrobial peptides. These proteins consist of 45 to 54 amino acid with eight conserved cysteine residues that form disulfide bridges (Halbach, 2002; Lay and Anderson, 2005). They have a well conserved tertiary structure composed of three anti-parallel β-sheets overlaid by one α-helix and stabilized by disulfide bridges to form a globular shape (Zhu *et al*., 2005). Defensin provides protective antimicrobial barriers in plant immune system. The characterized defensins display a constitutive expression pattern, with higher expression levels upon pathogen attack, injury and abiotic stress (de Beer and Vivier, 2011). This was evident in challenged GBK005111 (R) pods where expression level of Defensin-like protein 1 had a 3.6 and 8.94 increase in fold change in both microarray and RT-PCR experiments respectively.

Disease Resistance Response protein 206 putative (DRR206) is an inducible pathogenesis related gene involved in plant non-host resistance (Choi *et al*., 2004). This type of resistance confers plant immunity against all races of a pathogen and is typically broad-spectrum and more durable. DRR206 was first discovered in pea, *Pisum sativum*, as a defense gene that is strongly induced in response to broad range of fungal and bacterial pathogens and elicitors (Choi *et al*., 2004). Though the precise function of the defense gene remains elusive, previous reports indicate DRR206 shares a significant amino acid sequence homology (77%) with a dirigent protein involved in lignin/lignan biosynthesis (Choi *et al*., 2004; Wang *et al*., 1999). Lignan has antimicrobial properties while lignin is a major component of secondary cell walls that confers mechanical protection from penetration by fungal appressoria and cell wall degrading enzymes.

Glycinin is an 11S globulin that accumulates as the major storage protein in embryos or cotyledons of most dicotyledonous plants (Shewry, 1995). The hexameric protein is composed of five subunits consisting of an acid and a basic polypeptide chain linked by

single disulfide bond (Nielsen *et al*., 1989). According to Dhatwalia *et al*., (2009) basic proteins or peptides have antimicrobial properties. This is consistent with several studies which have demonstrated glycinin and its basic polypetide have antimicrobial activity including antioxidant properties and inhibition of spore germination (Mahgoub *et al*., 2016; Sitohy *et al*., 2012; Vasconcellos *et al*., 2014). The glycinin basic polypeptide chain is more hydrophobic than glycinin and has been shown to have a higher distinctive inhibitory action against pathogenic and spoilage bacteria. Though previous studies have focused more on the antimicrobial action of the seed storage protein against pathogenic bacteria, up-regulation of glycinin transcripts during *A. flavus* suggested a possible role in plant defense against fungus. In light with the current study, there is need to explore the application of glycinin as a potential antifungal protein for biocontrol of plant fungal diseases.

Brassinosteroid Insensitive1 (BRI1) - Associated Receptor Kinase1 (BAK1) plays essential roles in pathogen-associated molecular patterns (PAMP) - triggered immunity (PTI) and brassinosteroid regulated plant development (Boller and Felix, 2009). PTI is a basal defense mechanism induced upon recognition of elicitors such as damage associated molecular pattern (DAMPS) or PAMPs by pattern recognition receptor (PRR) (Boller and Felix, 2009). PRRs are typically plasma membrane- bound receptor-like kinases (RLKs) with extracellular domains allowing MAMP/DAMP perception (Böhm *et al*., 2014). In *A. thaliana* recognition of bacterial flagellin by Flagellin Sensing 2 (FLS2), a transmembrane PRR, leads to recruitment of BAK1 into the flagellin receptor complex (Boller and Felix, 2009; Chinchilla *et al*., 2009). The heteromeric complex initiates transphosphorylation events and induces pathogen-induced defense responses such as production of reactive oxygen species (ROS), activation of the mitogen-activated protein kinase 6 (MPK6) signaling and expression of defense- related genes (Boller and Felix, 2009; Chinchilla *et al*., 2009; Mithoe *et al*., 2016). Though Heese *et al*., (2007) demonstrated the biological significance of BAK1 in conferring resistance against fungal pathogens, the underlying mechanism of the gene in plant innate immunity is unclear.

Endo-chitinase gene was differentially activated in our study. The chitinase genes are known to be upregulated upon the plant perception of the virus, fungus and bacteria (Punja and Zhang, 1993). These genes affect the fungus development and virulence

through the hydrolytic action. They degrade N-acetylglucosamine polymer of the cell wall constituent of the fungal pathogens (Cleveland *et al*., 2004;Wang *et al*., 2012). The lysozyme activity of the plant endo-chitinases are highly effective in curbing the growth and establishment of the fungal pathogen (Slusarenko, 1987). According to Prasad and Bhatnagar-mathur, (2013), the over expression of endo-chitinase gene in tobacco, groundnuts and rice improved their resistance by 50%. The overexpression of the class 1 chitinase gene from potato in rice genome, improved its resistance to blister blight disease (Singh *et al*., 2015). Additionally Huang *et al*., (2013) reported an improved resistance in wheat crop against stripe rust disease upon over expression of rice chitinase gene RC24. The results in this study is in harmony with these findings and adds more information on the involvement of endo-chitinase enzyme in groundnut defence against *A. flavus*.

Genes involved in calcium binding proteins such as Calmodulins, Calcineurin and Calcium dependent kinases also had an elevated expression. This shows an increase in the calcium synthesis upon the fungal attack and it's a hint of its involvement in plant immunity. The elevation of the plant calcium level is the result of plant recognition of the pathogen elicitors and is an indication of the stimulation of innate immunity (Tena *et al*., 2011). It plays a part in plant immunity through stimulation and production of phytoalexin, pathogenic resistance genes and leads to programming cell death (Tena *et al*., 2011).

Fatty acid-metabolites and lipids are not only major structural and metabolic constituents of the cell but also function as signal transduction mediators in response to environmental stresses such as drought, temperature change, salinity and pathogen attack. Genes involved in lipid metabolism whose transcript levels were significantly up-regulated in GBK005111 (R) pods upon infection with *A. flavus* included Acyl-CoA-binding protein (ACBP), Lipoxygenase-4, 12-oxophytodienoate reductase 2 and lipid transfer protein. ACBP is a highly conserved ubiquitous proteins in eukaryotes (Burton *et al*., 2005). It has been elucidated that it regulate the intracellular acyl-CoA pool in lipid metabolism (Knudsen *et al*., 2000). Additional studies have suggested that it is also involved in gene

regulation, membrane biogenesis, protein sorting, vesicular trafficking and response to environmental factors (Xiao and Chye, 2011; Yurchenko and Weselake, 2011).

Lipoxygenases (LOXs) belongs to the family of non-heme iron-containing dioxygenases enzymes. It catalyzes the dioxygenation of polyunsaturated fatty acids in lipids having a cis,cis-1,4- pentadiene structure into unsaturated fatty acid hydroperoxides (Porta and Rocha-Sosa, 2002). In plants the derivatives of the LOX pathway are oxylipins which are synthesized through oxidation of linoleic and α- linolenic acids either at position 9 or 13 of their carbon chains (Feussner and Wasternack, 2002). The LOX has been showed in other studies to have antifungal activities in groundnuts, maize and soybean (Guillaume et al., 2011). In humans LOX have been reported to degrade the aflatoxin B1 through oxidative metabolism (Guillaume et al., 2011). The involvement of LOX in groundnut resistance to *A. flavus* was also evident in our study in which Lipoxygenase 4 (LOX-4) was upregulated to 3.1-fold on a resistant genotype.

Protease/proteinase inhibitors (PIs) are inhibitory polypeptides that are found mainly in plant seeds and tubers of legumes but are also induced in other plant tissues upon wounding (Garcia-Olmeda *et al*., 1987). During pathogen attack, many pathogenic fungi have been found to produce proteases which lead to disease development (Movahedi and Heale, 1990). To counteract the proteolytic activity of proteases released by the pathogenic microorganisms, plants synthesize inhibitory polypeptides that limit the synthesis of amino acids required for the pathogen growth and development (Ryan, 1990). In addition, increased synthesis of PIs has been strongly correlated with resistance to pathogen infection. This was also observed from the present gene expression studies with trypsin inhibitor and kunitz-type trypsin inhibitor 1 (KTI1) precursor being upregulated by 2.3- and 1.8-fold change respectively in the resistant cultivar. This was further confirmed through RT-PCR with the transcript levels of trypsin inhibitor being upregulated in response to *A. flavus* infection. Taken together, PIs act as defense proteins that provide a line of defense and resistance during pathogen attack. Kunitz-type protease inhibitors are present in many plants however, they have higher concentration in legumes, cereals and solanaceous species. Kunitz PIs have multiple functions including storage proteins, enzyme inhibitors and response to biotic and stress (Grosse-Holz and van der

Hoorn, 2016). Kunitz-type enzyme inhibitors are mostly active against serine proteinases, but may also inhibit other proteinases including cysteine, aspartate, trypsin, chymotrypsin and subtilisin (Heibges et al., 2003; Park et al., 2005). Kunitz-type trypsin inhibitors (KTIs), exhibit antifungal activity and are rapidly induced during mechanical wounding and herbivory attack. In tobacco a KTI gene, *NtKTI1*, conferred antifungal activity and resistance against Rhizoctonia solani (Huang et al., 2010) while in chick pea the KIT is induced in epicotyls and leaves upon mechanical wounding (Jiménez et al., 2008). Given (KTI1) was upregulated in challenged GBK005111 (R) pods, suggests the PI is involved defense response against *A. flavus.*

Late embryogenesis- abundant (LEA) proteins are hydrophilic and ubiquitous in higher plants seed embryos. The LEA proteins has been found to act as desiccant protectants that accumulate during post-abscission stage, a phase occurring at the end of embryo development (Espelund *et al*., 1992; Hughes and Galau, 1991). The LEA proteins are grouped depending on the amino acid sequence similarity (Wang *et al*., 2007). From the current study LEA B19.3 is classed in Group 1. Members of this group have been shown to have the highest sequence homology across plant species and a strongly conserved coding region (Stacy *et al*., 1995). In barley, LEAB19.3 gene contains an abscisic acid-responsive element and encodes for desiccation-protective protein (Espelund and Jakobsen, 1992). Notably, exposure of GBK005111 (R) cultivar to drought stress led to upregulation of LEA B19.3 transcripts which were detected in the microarray assays.

Serine carboxypeptidases (SCPs) are hydrolase proteins localized in vacuoles of higher plants and presumed to function in turnover and mobilization of intracellular proteins (Breddam, 1986). The proteolytic enzymes are exopeptidases that catalyse the release of amino acids from extracellular proteins and peptides by removing carboxy-terminal peptide bond. The catalytic site of SCPs contain a conserved amino acid triad, Ser-His-Asp, which act respectively, as a nucleophile, base and electrophile (Schaller, 2004). Different studies have revealed SCPs are involved in protein turnover for the mobilization of N-resources during seed germination and development, wound stress, senescence, programmed cell death and brassinosteroid signalling (Cercós *et al*., 2003; Li *et al*., 2001; Moura *et al*., 2001; Schaller, 2004). Serine carboxypeptidase 3 (CBP3) from

barley and wheat has been shown to have a high homology to yeast carboxypeptidase Y (Baulcombe *et al*., 1987; Sørensen *et al.*, 1989). CBP3 has been proposed to be involved in is programmed cell death and mobilization of storage proteins from the starchy endosperm (Domínguez *et al*., 2002).

Oxidative stress is the initial defence mechanism that the plant activates upon infection and is regarded as a symbol of pathogen perception (Cheong *et al*., 2002). The genes involved in reactive oxygen species synthesis such as ascorbate peroxidase, cytochrome P450, oxalate oxidase, peroxidase and S-transferase (Torres, 2010) showed differential expression. The reactive oxygen species are activated in an immunity triggered response and are involved in direct defence mechanism (Torres, 2010). The reactive oxygen species are signalling molecules and trigger the transcriptional factors production leading to the synthesis of antimicrobial metabolites and phytoalexins that hinder the growth and establishment of the fungus (Torres, 2010). In our study the activation of gene involved in reactive oxygen species synthesis indicates that the fungal attack on groundnuts is countered through the activation of reactive oxygen species.

Many of the upregulated and downregulated genes were regarded as hypothetical (Table 5.2 and Table 5.3). They have homology sequences to the genes whose role have not been elucidated. Some are differentially strong upregulated or strongly downregulated in groundnuts in response to *A. flavus* infection in groundnut. This indicate that they have a significant role in plant defence response and more so resistance against fungus within the *Aspergilli* family.

5.5 Conclusion.

Understanding the expression profile of genes, especially in response to the toxigenic strain infection, could provide clues to the molecular mechanisms of resistance, in addition to the physical barriers. Our transcript screening on resistant groundnuts in response to *A. flavus* infection identified genes that were highly expressed. The findings are in good line with previous investigations that identified similar transcripts reported in our study. The transcripts that were identified in the present study will enrich the groundnuts genomic resources in public databases.

The highly expressed genes could be valuable resources for follow on research to transfer genes into commercial groundnut cultivars through conventional breeding, marker assisted breeding, or through gene transfer by biotechnology. In addition, the genetic regulation may be employed to boost the expression levels of these genes in the commercial cultivars to reduce or prevent aflatoxin commination.

Due to lack of groundnut whole genome sequencing, the majority of the ESTs encode hypothetical proteins with unknown functions. Some of which were highly regulated, I would recommend functional annotation to provide an important resource of sequences and information for the groundnut community.

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CHAPTER 6: EXPLORING THE CHANGES IN RESISTANT GENES EXPRESSION IN GROUNDNUTS (*Arachis hypogea*) IN RESPONSE TO *Aspergillus flavus* EXPOSURE AT SEEDLING STAGE.

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Abstract

Aspergillus flavus infect groundnut seeds and produce secondary metabolites, aflatoxins. The aflatoxins are associated with various diseases in domestic animals and humans globally. Mitigating the aflatoxin contamination in crops through the development of cultivars tolerant to fungus colonization and aflatoxin contamination has been considered the most cost-effective measure to eliminate toxin problem. This research was conducted to ascertain that the resistance genes identified in the previous transcriptome analysis were involved in groundnut defence mechanisms to *A. flavus* infection. Eight genes were selected for additional scrutiny through the real time PCR on a groundnut seedling at an interval of 2 days within a 7-day period. The results indicate a network of gene expression patterns in a sequential order in both resistance and susceptible lines at a seedling stage. We conclude that the genes are involved in resistance and are expressed in a systemic manner when the gene action in crucial. Understanding the gene expression patterns in groundnuts is a critical information to breeders and other scientist interested in incorporating genetic resources of resistance against *A. flavus* into groundnut germplasm and/or commercial varieties via convention or molecular means.

Key words. Aflatoxin, *Aspergillus flavus*, Gene expression, Groundnuts, Real Time PCR, Seedling.

6.1 Introduction

Aspergillus flavus and *Aspergillus parasiticus* are known to synthesize large quantity of aflatoxin that compromise the quality of wide range of agricultural produce. This has been regarded as a major drawback in attaining food security and a major concern to human and animal health (Andrade and Caldas, 2015). Aflatoxin B1 on groundnut (*Arachis hypogaea* L.) has been documented as a causative agent of liver cancer (Nayak *et al*., 2017).

Maize (*Zea mays* L.) and groundnut are the most important source for human exposure to aflatoxin (Nayak *et al*., 2017). In groundnuts, the infection occurs at the farm, during harvesting, drying, storage and transportation (Waliyar *et al*., 2015). Globally, numerous initiatives have been undertaken to mitigate this problem. The development of resistant genotypes had been deemed a cost-effective and practical approach (Holbrook *et al*., 2010).

The progress in germplasm improvement in groundnuts have progressed slowly in comparison to crops such as maize because of dearth of resistant checks, linkage drags, paucity of information on plant microbe interaction and grater genotype by environmental interaction (Burow *et al*., 2008). Globally, limited breeding programs in groundnuts have realized varieties capable of resisting *A. flavus* colonization and aflatoxin contamination (Holbrook *et al*., 2009; Nigam *et al*., 2009).

Mixon and Rogers, (1973) conducted the initial screening of groundnuts against *A. flavus* infection. They characterized various resistant proteins in two groundnut lines upon A. flavus infection. The initial investigations focused more on the synthesis of novel proteins in the tolerant genotypes. Proteomics study by Szerszen and Pettit, (1990) in a tolerant versus susceptible groundnuts genotype discovered four important pathogenesis-related proteins 24 hours upon fungus infection. Four unique proteins were also identified in proteomic analysis of runner type groundnuts against Spanish bunch groundnuts under artificial inoculation with the *A. flavus* by Liang *et al*. (2006).

Currently, next generation sequencing, gene chip and comparative proteomics are applied in investigating the resistance level in plants. With these technologies, novel genes

markers and additional proteins essential in plant breeding have been discovered (Guo *et al*., 2008; Chen *et al*., 2009). Transcriptomics analysis have been done for late leaf spot (Luo *et al.*, 2007), *A. flavus* and bacterial wilt in groundnuts (Nayak *et al*., 2017).

Previously, we conducted gene expression profiling using peanut microarray. We discovered genes with *A. flavus*, and aflatoxin resistant properties based on their homology compared to the annotated entries and database searches. In order to provide addition information necessary for cultivar development, the expression analysis of the 8 resistant genes was conducted during fungal infection at a seedling stage. The main aim was to examine the changes and sequence of resistant gene activation in a seven-day period upon *A. flavus* infection.

6.2 Materials and Methods

6.2.1 Plant Genotypes and Fungal Isolate

The resistant GBK005111 and susceptible GBK036397 genotypes of groundnuts were used as a contrasting genotype. Toxigenic *A. flavus* strain, *A. flavus_JOOUST5* isolated and characterized in our previous study was used to inoculate these genotypes. It was sub-cultured on Potato Dextrose Agar until sporulation. The spores were washed over the surface of growth media with distilled water and a suspension for inoculation was prepared.

6.2.2 Seedling preparations

From each genotype line, 100 blameless seeds were picked and sterilized through immersion in 0.75% sodium hypochlorite for three minutes and rinsed 3 times in distilled for 5 minutes each. The seeds were then hydrated up to 20% water content by soaking in distilled water for 30 minutes. The hydrated seeds were then plated in petri-dishes lined with 6 sheets of sterile tissue papers moistened with sterile water. The plated seeds were incubated at 27 ^{0}C for 3 days in high relative humidity chambers for germination. Fifty seedlings of each genotype were immersed in sterile distilled water and were considered controls. The second set of 50 seedlings were immersed in a spore suspension and were considered infected samples. Both the sets were transferred back into their respective petri-dishes and incubated for additional 7 days.

6.2.3 RNA isolation

Disinfected scalpel was used to cut and collect the root and shoot tissues in Eppendorf tube on the 1, 3, 5- and 7-days post-inoculation. The samples were instantly frozen in liquid nitrogen and kept at -80 ^{0}C freezer. After collecting the samples from all the replicates, the sample per genotype, treatment and time points were pooled together and ground in precooled mortar and pestle. The total RNA was extracted from tissues using Total RNA isolation Reagent (Invitrogen, Carlsbad, CA, USA). One hundred milligram of the ground tissues was weighed and put in 10 ml tube and cells lysed with 5ml of the extraction buffer. The contents were vortexed fully to mix the contents and incubated at 65 ^{0}C for 40 minutes with gentle whirling in a water bath and allowed to cool on the bench. 2ml of chloroform was added, vortexed and incubated in ice for 5 minutes before centrifuging at 8,000 rpm for 30 minutes. The clear supernatant was gently moved to the new RNase free tube and 1.5ml of LiCl added mixed by inversion, incubated at -80 ^{0}C for 1.5 hours. The solution was then centrifuged at 8,000 rpm for 20 minutes in a refrigerated centrifuge to pallet the RNA. The pellets were washed with 0.75 ethanol solution and centrifuged at 8,000 rpm for 20 minutes. The RNA pellets were allowed to dry on the bench and dissolved in 100 µL RNase free water (Ambion inc., Life Technologies, Grand Island, NY, USA).

6.2.4 Reverse transcription PCR

The SYBRR premix Ex Tag II kit was used based on manufacturer instructions. The reverse transcription was done in 20 µl Nucleases free microcentrifuge tube containing I µl of primer pairs, 5 µl of isolated RNA, 1 µl of each dNTP mix at a neutral pH and 10 µl of distilled water. The mix was then incubated at in a water bath for 60 ^{0}C for 6 minutes and then transferred to ice for 5 minutes. Centrifugation was then done briefly to collect the mix at the bottom and 4ul of the strand buffer, 1 µl of dithiothreitol, 1ul of RNase inhibitor and superscript were added and pipetting up and down to mix them. The incubation was then done for 45 minutes at 55 ^{0}C and the reaction inactivated by warming the contents in a water bath at 70 ^{0}C for 15 minutes.

The RT-PCR was done according to the parameters of Rodrigues et al. (2009): 1 initial cycle for denaturation was set for 4 minutes at 94 ^{0}C. The subsequent 30 denaturation's

cycles were done for 1 minutes at 94 ^{0}C, annealing was done for 1min at 55 – 60 ^{0}C for 30 cycles each, the extension on each cycle was done at 72 ^{0}C for 1 minutes and the final extension at 72 ^{0}C for 6 min.

Primers were designed using the Primer Express 3.0 plus tool and presented in the Table 6.1. The expression analysis of the 8 resistant genes was conducted. The actin gene from groundnut kernels was used to quantify the gene expression using the $2^{-\Delta\Delta C_t}$ method (Claire et al. 2004).

Table 6.1: Outline of 8 genes and primer sets used in RT_PCR.

Accession No.	**Gene Analysed**	**Primer set (5' → 3')**	**Fragment length (bp)**
P83595	Trypsin inhibitor	F: CGAGATACAGGGTTGGTTTGAG R: CCAAAAGCCTATTTCCCTCATC	128
AAF602701	Lipoxygenase (LOX)	F: TGGTGAAGAGTCACCAAAAGG R: TCCAATGTGGATTATCCCTCTC	141
gi|146771807	Glycinin	F: TATGATGATGACGATCGACGACCACG R: TGCATAGTGTTTCCTCCACTCCGT	82
NP_188086	Cytochrome P450	F: TGGGCTTACTCGAAATACCG R: GCATTATCACCCCAAAGTCC	123
P11670	Pathogenesis-related protein 1	F: AACACTCCATGGGGCCTTAC R: TGTAATGCAGGCACTCATCG	147
NC_037620.1	Endochitinase	F: TGCCAATACACTGCTGGAGG R: GTAAGGGCAGTTGCAGGGAT	146
NC_037620.1	Disease resistance response protein 206_PUTATIVE	F: GTAAAACCCTTGCGCTCGTC R: CGTCTTTGACGACCCCATCA	73
NC_037635.1	Defensin like proteins	F: CACGTTGCTGCCTCACTTTG R: ATTGCTGGCCCCTAGCATTC	96
DQ8273525.1	Peanut Actin gene	F: GTTCCACTATGTTCCCAGGCA R: CTTCCTCTCTGGTGGTGCTACA	85

6.3 Results

6.3.1 Expression levels as detected by RT-PCR

The expression analysis as obtained by RT-PCR in this study were in harmony with the preceding transcriptomic study. All the 8 genes investigated were upregulated in at least two time points (Fig 6.1) and is consistence with their upregulation in the transcriptomic study (Table 6.2). The RT-PCR discovered varied trends in gene activation upon fungal infection in groundnuts seedling stage in a seven-day period.

Table 6.2: A comparison of the peak gene activation of defence related genes within 7 days at a seedling stage with microarray output conducted on mature groundnut crops previously upon fungal infection.

Gene Descriptions	RT-PCR Peak expression level	$\log_2 \geq 1.5$ (Microarray) (From the microarray study)
Lipoxygenase	4.14	3.1
Cytochrome P540	2.32	3.1
Endochitinase	5.23	3.5
Trypsin inhibitors	2.44	2.3
Pathogenic related proteins	7.38	4.2
Defensin like proteins	5.98	3.6
Disease resistance response proteins	8.22	2.5
Glycinin	1.23	2.8

6.3.2 Gene expression patterns

The results reveal a varied patterns of gene expression at a seedling stage after *A. flavus* inoculation (Fig 6.2). The peak values shown in the Table 6.2 identify the highest expression levels per gene. All genes had positive peaks that occurred at different time points, (Fig 6.1). The expression levels of genes varied from the time of inoculation to day 7 with some registering downregulation at some points. The level of expression correlates with the requirement of the gene action with the highest peak indicating the time in which the gene is most critical in the defense against the *A. flavus* infection.

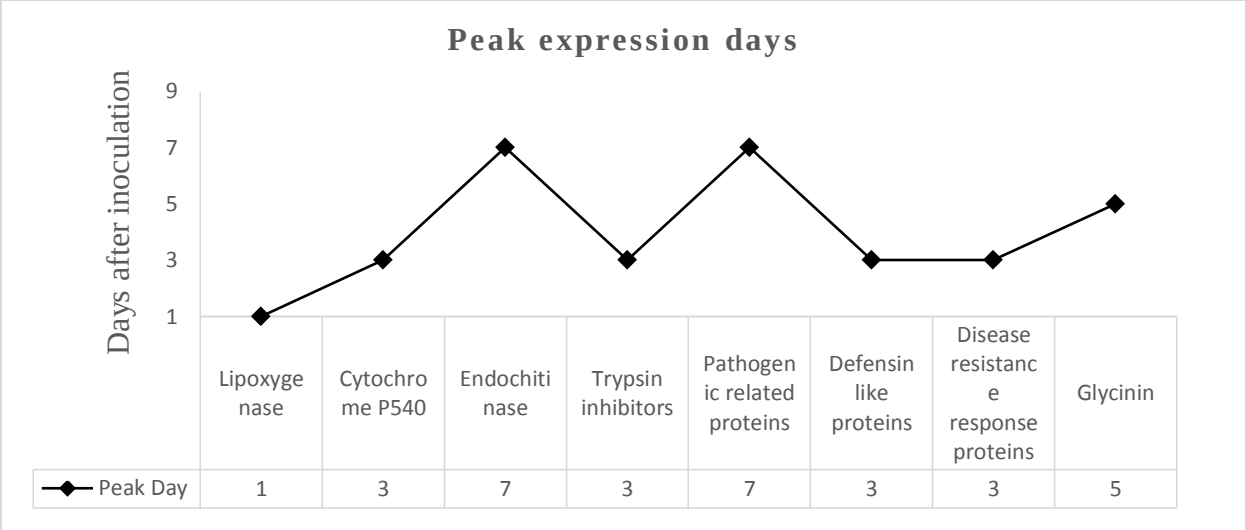

Figure 6.1: Gene expression peak times of the selected resistant genes in two contrasting groundnut genotypes (resistance and susceptible) challenged by *A, flavus* in a seven-day time period.

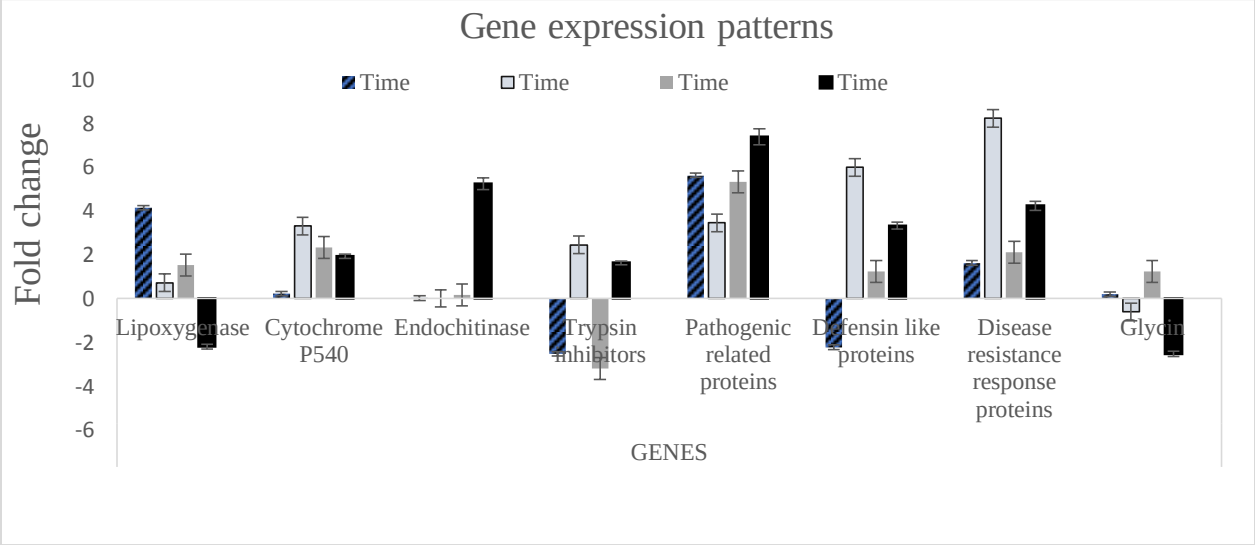

Figure 6.2: Gene expression patterns using RT-PCR in groundnuts at a seedling stage of resistant\susceptible genotype in response to *A, flavus* colonization in a seven-day time period. The pathogenic related proteins and disease response proteins had higher overall fold change while the glycinin had the lowest fold change.

Lipoxygenase (LOX) gene had its peak expression on day one after inoculation, expression levels plummeted at day three, then short up slightly at day five, and finally repressed at day seven. This suggests the involvement of LOX in the first line of defense during pathogenesis.

Cytochrome P540, Trypsin inhibitors, Defensin like proteins and Disease resistance response proteins had their peak expression levels on day three after inoculation. Even

though they had their peak expression on day three, they never had a pattern of expression. A common feature of these four genes is that they had the lowest expression level on day one. However, in Cytochrome P540, the expression levels fell steadily from day three today seven while Trypsin inhibitors, Defensing like proteins and Disease resistance response proteins had their expression falls to the lowest at day five but increases in day seven.

Unlike the other genes described earlier, Glycinin had its expression peak at day five after inoculation and had almost insignificant expression for the rest of the time points (Fig 6.2).

The slowest genes to show peak expression were Endochitinase and Pathogenic related proteins at day seven. The activity of the Endochitinase was silent during the early stages of pathogenesis up to day five. However, its expression short up to the highest up to fivefold at day seven after inoculation. Pathogenic related proteins had a slightly higher expression levels at day one, gradually fall to day three, and short up gradually up to the highest level at day seven (Fig 6.2).

6.4 Discussion

The study reveals that the resistance genes under investigation were upregulated in most of the time points (Fig 6.2). The maximum peak and deep expression levels of these genes occurred at different days. This is in agreement with phenomenon that genes are activated at different times upon perceiving or experience an attack (Liang *et al*., 2009). Our findings indicate that resistant genes under investigation acted in programmed manner in their resistance activity. The biological response to *A. flavus* infection in groundnuts is intricate. The genetic composition and the biochemical synthesis of the groundnut accessions facilitates the counter attack measure to invading fungi. This give rise to sequentially gene activation in both genotypes leading to a network of gene expression.

The Lipoxygenase (LOX) genes had its peak expression at day one. This indicates that the gene is critical in the first line of defense as an activator of signalling network. This agrees with the studies conducted by Liang *et al.*, (2009), in which they discovered a

rapid accumulation of LOX and elevated levels of membrane lipid peroxidation within 1-2 days upon fungal inoculation. LOX is non-heme-iron comprising of fatty acid dioxygenases that take part in lipid metabolisms. They are critical genes in oxidative degradation of lipids within the cells to confer resistance upon fungal attack. The lipid metabolites are believed to be essential soluble signal in plant counter attack to the invading fungal attack (Christensen and Kolomiets, 2011). LOX enzymatically converts polyunsaturated fatty acids into hydroperoxides. The hydroperoxides are eventually turned to oxylipins. The oxylipins takes part in hypersensitive response through the oxidative damage to the cell membranes (Christensen and Kolomiets, 2011). Oxylipins can also be processed into the traumatin, jasmonic acid and methyl jasmonates which are involved in the regulation of various physiological functions of the plants (Hwang and Hwang, 2010). The oxylipins produced through the catalytic action of 9-LOX and 13-LOX isoforms of LOX could mimic or interfere with the fungal oxylipins leading to cross kingdom communication (Gao *et al*., 2009). The study of LOX gene in *Capsicum annuum* by Hwang and Hwang, (2010), strengthened cross talk hypothesis when they revealed that *Xanthomonas campestris* thrived more on paper plants in which the LOX gene was knocked down.

Four genes, Cytochrome P540, Trypsin inhibitors, Defensin like proteins and Disease resistance response proteins genes had their peak expression on the third day after inoculation (Fig 6.2). These genes synthesize resistance molecules through oxidization of pre-cursers in response to fungal invasion. Cytochrome P540 comprise of largest and metabolically diverse protein families that take part in phytohormone homeostasis (Mizutani and Ohta, 2010). Cytochrome P450 regulates the production of Jasmonic acid (JA) in the cells for the plant defense (Park *et al*., 2002). Jasmonic acid offer plant protection through the suppression of the reproduction, development and production of secondary metabolites of plant pathogens (Mosblech *et al*., 2009). Cytochrome P450s are also involved in the Jasmonoyl-L-isoleucine (JA-IIe) turnover which activates the plant immune system (Koo *et al*., 2011).

Trypsin inhibitors, Defensin like proteins and Disease resistance response proteins genes function in various amino acids synthesis (Van Loon *et al*., 2006). Trypsin inhibitors

function as protein inhibitors and consist of amino acid with varying sequences and sizes. They have been found to attenuate the growth of *A. flavus* and inhibit the activity of alpha-amylase (Chen *et al*., 2007; Mellon *et al*., 2007). Plant defensins like proteins are basic antimicrobial peptides consisting of 45 to 54 amino acid with eight conserved cysteine residues that form three to four coupling of the thiol groups (Halbach, 2002; Lay and Anderson, 2005). It is documented that defensins have constitutive expression pattern with high upregulation upon pathogen invasion and wounding (de Beer and Vivier, 2011). They offer plant immunity by forming a protective barriers to pathogen invasion (de Beer and Vivier, 2011). Our results were in synchrony to these findings in which upregulation was observed in this gene after fungal inoculation.

Disease Resistance Response protein is an inducible proteins that provides a non-host resistance (Choi *et al*., 2004). This type of resistance confers plant immunity against all races of a pathogen and is typically broad-spectrum and more durable. This gene was first discovered in pea, Pisum sativum, as a defense gene in which it was highly upregulated upon bacteria and fungal pathogen and elicitors exposure (Choi *et al*., 2004). Though the precise function of the defense gene remains elusive, previous reports indicates that it shares a significant amino acid sequence homology (77%) with a proteins that regulates the production of lignin and lignan (Choi *et al*., 2004; Wang *et al*., 1999). Lignan has antimicrobial properties while lignin is a structural constituent of cell walls that confers mechanical protection from penetration by fungal appressoria and cell wall degrading enzymes. Its peak expression at the third day may indicate that it is required to reinforce the cell wall to hinder the penetration of the developing fungus.

Glycinin is an 11S globulin that accumulates as the major storage protein in embryos or cotyledons of most dicotyledonous plants (Shewry, 1995). The hexameric protein has a mass of approximately 350 kDa and is composed of five subunits $A_{1a}B_{1b}$, $A_{1b}B_{2}$, $A_{1}B_{1a}$, $A_{3}B_{4}$ and $A_{5A}4_{B3}$ (Nielsen *et al*., 1989). A single disulfide bond links the acid and basic polypeptide chain that forms the subunits (Nielsen *et al*., 1989). According to Dhatwalia *et al*., (2009) the basic peptides have been showed to reduce the mycelia growth and spore germination of *A. niger* and *Penicillium* species. It activation on the fifth day may

be due to response to the developing mycelium. The plant activates it to counter the developing mycelium.

The last group of genes to have their peak upregulation were pathogenic related (PR) proteins and its related protein genes, endochitinase. These genes are involved in the cell wall mediated resistant factors (Glazebrook, 2005; Park *et al*., 2004). The PR compounds coagulates on the cell wall of plant species during the pathogen invasion to hinder the penetration (Dixon and Harrison, 1990). These genes function in the cell wall strengthening through saccharides biosynthesis (Durrant and Dong, 2004).

This research gives essential information on the pattern of gene activation in groundnut upon fungal attack. The host provides mechanisms of resistance that involves several genes activation in a sequential manner (Fig 6.1). The order of gene expression determines the level of resistance and is critical in exclusion of fungal colonization.

6.5 Conclusion

The results obtained in this study were congruent to the microarray study and it establishes that the screening at seedling stage could provide quicker and reliable results. The study provides a clearer picture of the action of the resistance genes in conferring defence against invading fungus. The mechanism of resistance is provided by a combination of factors from the host plant involving several types of genes in a successive, orchestrated process. The importance of the host gene expression order is critical not only to determine the degree of the resistance against fungal invasion but also to take evasive measures to avert fungal colonization. Additionally, the identities of these genes are confirmed to be involved in defence and could provide important targets for groundnuts molecular marker screening and also provide foundation for functional genetic studies in the host.

6.6 Reference

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APPENDIX

Appendix 1. Individual sequences of isolates as stored in the NCBI gene bank.

>A. flavus_isolate_12

AGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTACCTTAG
TTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGAC
CCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGT
TGATTGTATCGCAATCAGTTAATACTTTCAACAATGGATCTCTTGGTTCCGGC
ATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCC
GTGAAATTTCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGG
GGCATGCCTGTCTTAACGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTG
TTGGGTCGTCGTCCCCTCTCCGGCGGGGACGGGCCCCAAAGGCAGCGGCGGC
ACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCG
GCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCC

>A. flavus_isolate_5

AGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTACCTTAG
TTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGCTCTCAGCCCCGGGCC
CGGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTG
ATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCAT
CGATGAAGAACGCAGGAAATGCGATAACTAGTGTGATTGCAGAATTCCGTGA
ATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGC
CTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCG
TCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGA
TCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTG
CCGAACGCAAATCAATCTTTTTCC

>A. flavus_isolate_11

AGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTACCTTAG
TTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGC
CCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTTGAGTT
GATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCT
GAAGAACGCAGCGAAATGCATAAGGGTGACTAGTGTGAATTGCAGATTCCGT
GAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCAT
GCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGT
CGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCC
GATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCT
TGCCGAACGCAAATCAATCTTTTTCC

>A. flavus_isolate_13

AGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTACCTTAG
TTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGCC
CGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTT
GATTGTATCGCAGATATCAGTAAAACTTTCAACAATGGATCTCTTGGTTCCGG
CATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATCC
GTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGC
ATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTC
GTCGTCGGTCCCTCTCCGGGGGGGACGGGCCGATCCCAAAGGCAGCGGCGGC
ACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCG
GCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCC

>A. flavus_isolate_15

AGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTACCTTAG
TTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGC
CCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGT
TGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGC
ATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCC
GTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGC
ATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTC
GTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGT
CCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGC
GCTTGCCGAACGCAAATCAATCTTTTTCC

Appendix 2. Microarray based gene expression profile data providing level of up regulation of genes in resistant and susceptible genotype challenged by *Aspergillus flavus* at a pre-harvest stage.

Probe Name	Description	Processed Signal (Control)	Processed Signal (Treatment)	Fold change	log2 Fold Change	Regulation	Corrected P-value	Flag
LS11297	Hypothetical protein	76.00236202	820.5524605	10.79640736	3.432479412	UP	0.046840527	detected
LS11307	Hypothetical protein	47.49951111	294.911334	6.208723566	2.634296699	UP	0.048064692	detected
LS12366	12-oxophytodienoate reductase 2	25.88994069	99.8787609	3.857821155	1.947786263	UP	0.043657318	detected
LS12483	Hypothetical protein	157.4841418	804.3476543	5.107483492	2.352612636	UP	0.044810144	detected
LS12941	Brassinosteroid Insensitive1-associated receptor kinase 1 (BAK1) precursor	160.3425904	705.6456527	4.400862248	2.137786215	UP	0.045051959	detected
LS13324	Hypothetical protein	243.3737111	932.5129398	3.831609155	1.937950406	UP	0.040155397	detected
LS14633	Defensin-like protein 1	36.96452739	442.227754	11.96357117	3.580576198	UP	0.047109667	detected
LS15388	Hypothetical protein	34.51591583	129.0411142	3.73859743	1.902497131	UP	0.046840527	detected
LS15582	Disease resistance response protein 206 putative	114.6039829	639.3532637	5.57880492	2.479956104	UP	0.043657318	detected
LS16350	Hypothetical protein	200.4733746	995.8590005	4.967537471	2.31253085	UP	0.034738375	detected
LS16427	Hypothetical protein	280.9248563	1625.782636	5.787250932	2.5328782	UP	0.043657318	detected
LS17431	Plant defensin	200.9280429	1100.453659	5.476854516	2.453347558	UP	0.033117998	detected
LS17599	Hypothetical protein	172.8526819	630.5142784	3.647697401	1.866986054	UP	0.039441948	detected
LS20455	Trypsin inhibitor	315.6194409	1561.554054	4.947585134	2.306724532	UP	0.040628438	detected
LS20558	Hypothetical protein	168.4257902	814.6598037	4.836906527	2.274084658	UP	0.032376809	detected
LS21217	1-aminocyclopropane-1-carboxylate oxidase homolog 1	156.9182712	695.3335033	4.431182538	2.147691758	UP	0.035073783	detected
LS21222	Hypothetical protein	148.1594416	545.0707547	3.678947144	1.879292949	UP	0.044119389	detected
LS21284	Serine carboxypeptidase 3 precursor	321.8706774	1125.416815	3.496487547	1.805906367	UP	0.038864157	detected
LS21382	Hypothetical protein	246.575791	906.9620729	3.678228383	1.87901106	UP	0.030544029	detected
LS21863	Acyl-CoA-binding protein (ACBP)	153.9311386	1403.788501	9.119587586	3.188968583	UP	0.036504602	detected

LS22177	**lipid transfer protein**	**558.742579**	**2098.980495**	**3.756614537**	**1.90943309**	**UP**	**0.029952455**	**detected**
LS22650	**Oxalate oxidase**	**358.4805103**	**1889.791178**	**5.271670631**	**2.398260235**	**UP**	**0.029355174**	**detected**
LS23970	**Kunitz-type trypsin inhibitor 1 (KTI1) precursor**	**105.518766**	**422.2643422**	**4.001793787**	**2.000646827**	**UP**	**0.029207077**	**detected**
LS25239	**Cytochrome P450**	**47.51626267**	**408.5919811**	**8.59899239**	**3.104167618**	**UP**	**0.032410846**	**detected**
LS25863	**Hypothetical protein**	**307.5428682**	**1417.183962**	**4.608085924**	**2.204167618**	**UP**	**0.033267205**	**detected**
LS26059	**Calmodulins**	**239.8091868**	**1186.319352**	**4.946930383**	**2.306533597**	**UP**	**0.038299687**	**detected**
LS26855	**Ethylene-responsive factor 6**	**269.4991181**	**1116.620283**	**4.14331702**	**2.05078621**	**UP**	**0.028352669**	**detected**
LS26863	**Hypothetical protein**	**20.97036761**	**719.9831719**	**34.3333596**	**5.10153913**	**UP**	**0.046027663**	**detected**
LS30071	**Hypothetical protein**	**279.2915998**	**1265.798381**	**4.53217491**	**2.18020354**	**UP**	**0.032745781**	**detected**
LS31143	**Hypothetical protein**	**311.8514658**	**1192.387175**	**3.823574059**	**1.934921818**	**UP**	**0.030405364**	**detected**
LS31592	**Ethylene-responsive factor 1**	**227.1548944**	**1229.095041**	**5.410823501**	**2.435848182**	**UP**	**0.036504602**	**detected**
LS32216	**Hypothetical protein**	**121.121146**	**532.8857088**	**4.399609205**	**2.137375382**	**UP**	**0.045849237**	**detected**
LS32382	**Hypothetical protein**	**70.17929858**	**355.2953213**	**5.062679857**	**2.339901257**	**UP**	**0.041835646**	**detected**
LS32442	**Hypothetical protein**	**11.20068142**	**114.6104029**	**10.23244913**	**3.35507959**	**UP**	**0.025051433**	**detected**
LS32924	**Hypothetical protein**	**299.9430091**	**1088.668345**	**3.629583995**	**1.859804203**	**UP**	**0.024575985**	**detected**
LS33198	**Hypothetical protein**	**338.9416266**	**1270.987698**	**3.749871949**	**1.906841331**	**UP**	**0.033575916**	**detected**
LS33526	**Hypothetical protein**	**146.9720092**	**836.8208185**	**5.693742794**	**2.509377323**	**UP**	**0.02505962**	**detected**
LS33678	**Hypothetical protein**	**125.7130305**	**580.4333956**	**4.617129927**	**2.206996331**	**UP**	**0.032782689**	**detected**
LS33908	**Hypothetical protein**	**26.03114789**	**269.6710224**	**10.3595517**	**3.372889668**	**UP**	**0.040706461**	**detected**
LS34246	**Ascobase peroxidase**	**40.54694947**	**842.6499235**	**20.78207941**	**4.377268109**	**UP**	**0.024093929**	**detected**
LS34462	**Phospholipase**	**209.2380113**	**837.107598**	**4.000743425**	**2.000268109**	**UP**	**0.023278319**	**detected**
LS34881	**Hypothetical protein**	**435.9932555**	**1693.613335**	**3.884494344**	**1.957726811**	**UP**	**0.026853684**	**detected**
LS35527	**Calcineurine gene**	**284.7991314**	**1408.207993**	**4.944565617**	**2.305843785**	**UP**	**0.028264465**	**detected**
LS35528	**Hypothetical protein**	**93.5856994**	**456.6111126**	**4.879069297**	**2.286605974**	**UP**	**0.042977099**	**detected**
LS35563	**Ethylene-responsive transcription factor 5**	**137.7048592**	**1256.510263**	**9.12466176**	**3.189771081**	**UP**	**0.028617155**	**detected**

LS36083	**Calcium dependent Kinase**	**282.7165213**	**1051.664075**	**3.719853618**	**1.89524585**	**UP**	**0.022140981**	**detected**
LS36811	**Pathogenesis-related protein 1**	**39.01353682**	**720.0024605**	**18.45519579**	**4.205955138**	**UP**	**0.023207**	**detected**
LS37154	**Glutathione S-transferase**	**168.7491853**	**586.2228496**	**3.473929954**	**1.796568665**	**UP**	**0.022403562**	**detected**
LS37495	**Late embryogenesis abundant protein.**	**86.57807826**	**460.0412636**	**5.313599849**	**2.409689587**	**UP**	**0.039932694**	**detected**
LS37967	**Alcohol dehydrogenase**	**95.64518016**	**1090.200837**	**11.39838762**	**3.510757854**	**UP**	**0.038299687**	**detected**
LS38199	**Hypothetical protein**	**96.73344119**	**365.0702193**	**3.773981519**	**1.916087358**	**UP**	**0.025315646**	**detected**
LS38249	**Hypothetical protein**	**191.9156751**	**1101.050994**	**5.73716031**	**2.520336831**	**UP**	**0.02785189**	**detected**
LS38655	**Peroxidase**	**18.74946924**	**403.8221571**	**21.53779139**	**4.42879841**	**UP**	**0.033774886**	**detected**
LS38777	**Hypothetical protein**	**32.28670331**	**648.4168154**	**20.08309146**	**4.32790946**	**UP**	**0.026201271**	**detected**
LS39325	**Hypothetical protein**	**8.161598752**	**54.013067**	**6.617951781**	**2.72638478**	**UP**	**0.023514244**	**detected**
LS39525	**Lipoxygenase-4**	**69.37182287**	**611.0134753**	**8.807804812**	**3.138782498**	**UP**	**0.020569855**	**detected**
LS39893	**Glycinin**	**32.1467072**	**230.4000964**	**7.167144522**	**2.841398446**	**UP**	**0.040037249**	**detected**
LS40338	**Hypothetical protein**	**353.8340233**	**1324.350073**	**3.74285678**	**1.904139845**	**UP**	**0.035535222**	**detected**
LS43179	**Endochitinase**	**26.36371486**	**308.0664839**	**11.68524563**	**3.546616156**	**UP**	**0.044222479**	**detected**
LS43301	**Hypothetical protein**	**273.3062103**	**1211.789839**	**4.43381743**	**2.148549366**	**UP**	**0.019629596**	**detected**
LS44512	**Hypothetical protein**	**208.461069**	**752.2320721**	**3.608501461**	**1.851399839**	**UP**	**0.019396704**	**detected**
LS46379	**Hypothetical protein**	**263.9191172**	**1056.083567**	**4.001542511**	**2.000556236**	**UP**	**0.018650092**	**detected**
LS47212	**Hypothetical protein**	**343.7766508**	**1051.253568**	**3.057955116**	**1.612567231**	**UP**	**0.032745781**	**detected**

Appendix 3. Microarray based gene expression profile data providing level of up regulation of genes in resistant and susceptible genotype challenged by Aspergillus flavus at a pre-harvest stage

Probe Name	Description	Processed Signal (Control)	Processed Signal (Treatment)	Fold Change	log_2 Fold Change	Regulation	Corrected P-value	Flag
LS43179	Hypothetical protein	94.0606684	17.01090932	0.180850398	-2.467131322	DOWN	0.013260434	detected
LS10730	Jasmonate-ZIM-domain protein 8	103.8307526	23.98140827	0.230966334	-2.114245518	DOWN	0.010320749	detected
LS10736	Ferredoxin--NADP reductase	105.7847694	24.21260577	0.228885556	-2.127301671	DOWN	0.009843731	detected
LS11252	T-complex protein 1 subunit alpha	264.0601336	56.76282128	0.214961723	-2.217848305	DOWN	0.000256472	detected
LS11270	Hypothetical protein	570.8407778	124.648692	0.218359824	-2.195220653	DOWN	0.000107996	detected
LS11340	Hypothetical protein	117.5088705	23.80801331	0.202606094	-2.303250524	DOWN	0.019147258	detected
LS11622	Alpha-glucanotransferase	94.0606684	16.50414359	0.17546275	-2.510763306	DOWN	0.013260434	detected
LS11815	Hypothetical protein	479.0019862	99.22262939	0.207144505	-2.271290544	DOWN	0.00011186	detected
LS12570	3-hydroxybutyryl-CoA dehydratase	107.7387863	24.95320429	0.231608366	-2.110240726	DOWN	0.00938132	detected
LS12698	Hypothetical protein	232.7958642	47.03186692	0.202030509	-2.307354922	DOWN	0.003828488	detected
LS12941	Hypothetical protein	185.89946	35.69290577	0.192001127	-2.380813318	DOWN	0.004445815	detected
LS13380	Hypothetical protein	12.92283895	3.230491703	0.249983128	-2.000097368	DOWN	0.004960969	detected
LS14127	Hypothetical protein	12.92283895	3.230578213	0.249989822	-2.000058734	DOWN	0.004474818	detected
LS15167	Hypothetical protein	211.3016789	49.2392154	0.233028037	-2.101424552	DOWN	0.005718154	detected
LS15392	Hypothetical protein	166.3592915	41.26716887	0.248060499	-2.011236075	DOWN	0.013628465	detected
LS15582	Hypothetical protein	219.1177463	40.56563026	0.185131652	-2.433376524	DOWN	0.002203993	detected
LS16032	Hypothetical protein	187.8534768	35.83234012	0.190746217	-2.390273647	DOWN	0.00427638	detected
LS16163	Hypothetical protein	58.8883652	10.4552266	0.177543163	-2.493758293	DOWN	0.032091849	detected
LS16301	Delta (6) fatty acid desaturase	109.6928031	25.38701006	0.231437335	-2.111306477	DOWN	0.008963285	detected
LS16748	Hypothetical protein	103.8307526	22.3754451	0.215499209	-2.214245518	DOWN	0.025934947	detected
LS16756	Hypothetical protein	344.1748241	64.25722687	0.186699382	-2.421210945	DOWN	0.000478001	detected
LS17338	Hypothetical protein	195.6695442	36.49241575	0.186500234	-2.422750655	DOWN	0.00364742	detected
LS18070	Glutaredoxin-C1	113.6008368	26.34493649	0.231907944	-2.108375855	DOWN	0.008179475	detected
LS18645	HVA22-like protein k	344.1748241	78.59091073	0.228345902	-2.130707195	DOWN	0.000185642	detected

LS19077	**Hypothetical protein**	**92.1066516**	**23.01655669**	**0.249890277**	**-2.000633326**	**DOWN**	**0.033698453**	**detected**
LS19774	**25.3 kDa vesicle transport protein**	**346.128841**	**80.1616498**	**0.231594829**	**-2.110325052**	**DOWN**	**0.000180007**	**detected**
LS20938	**Mitochondrial 60S ribosomal protein L2**	**119.4628873**	**27.64345048**	**0.23139781**	**-2.111552885**	**DOWN**	**0.007098191**	**detected**
LS20945	**Hypothetical protein**	**557.1626599**	**139.1748017**	**0.249792048**	**-2.001200547**	**DOWN**	**0.000211983**	**detected**
LS20985	**Hypothetical protein**	**430.1515652**	**95.42076117**	**0.221830557**	**-2.172469988**	**DOWN**	**0.000450625**	**detected**
LS22861	**Hypothetical protein**	**271.876201**	**67.90929047**	**0.249780195**	**-2.001269005**	**DOWN**	**0.00352891**	**detected**
LS22990	**Hypothetical protein**	**58.8883652**	**10.14278844**	**0.172237562**	**-2.537528293**	**DOWN**	**0.032091849**	**detected**
LS23261	**Hypothetical protein**	**92.1066516**	**22.03768673**	**0.239262706**	**-2.063332552**	**DOWN**	**0.033698453**	**detected**
LS23663	**Glycine cleavage system H protein**	**129.2329715**	**30.09997438**	**0.232912499**	**-2.102140031**	**DOWN**	**0.005634055**	**detected**
LS24352	**FAM18-like protein**	**140.9570726**	**31.94904165**	**0.226657954**	**-2.141411306**	**DOWN**	**0.00427638**	**detected**
LS25239	**Hypothetical protein**	**62.7963989**	**11.44134812**	**0.18219752**	**-2.456424776**	**DOWN**	**0.02894315**	**detected**
LS25255	**Hypothetical protein**	**277.7382515**	**69.36928573**	**0.249764969**	**-2.001356951**	**DOWN**	**0.003159197**	**detected**
LS25829	**Hypothetical protein**	**353.9449084**	**72.73665395**	**0.205502755**	**-2.282770357**	**DOWN**	**0.000826409**	**detected**
LS25835	**Hypothetical protein**	**105.7847694**	**23.46206269**	**0.221790555**	**-2.172730167**	**DOWN**	**0.024796715**	**detected**
LS26111	**Hypothetical protein**	**351.9908915**	**65.60471643**	**0.186381858**	**-2.423666655**	**DOWN**	**0.000414258**	**detected**
LS26699	**DNA-binding protein**	**142.9110894**	**30.83977729**	**0.215796951**	**-2.212253617**	**DOWN**	**0.004094361**	**detected**
LS26855	**Hypothetical protein**	**410.6113968**	**72.15415781**	**0.175723709**	**-2.508619235**	**DOWN**	**0.000150875**	**detected**
LS27203	**Hypothetical protein**	**240.6119315**	**49.40710472**	**0.20533938**	**-2.283917764**	**DOWN**	**0.003271709**	**detected**
LS27241	**Hypothetical protein**	**84.2905842**	**21.70003369**	**0.257443152**	**-1.957674199**	**DOWN**	**0.040724353**	**detected**
LS27255	**Hypothetical protein**	**283.6003021**	**70.82192544**	**0.249724436**	**-2.001591097**	**DOWN**	**0.002847615**	**detected**
LS30005	**nucleotide-binding protein**	**156.5892073**	**38.31727562**	**0.24469934**	**-2.030917887**	**DOWN**	**0.002936247**	**detected**
LS30124	**Hypothetical protein**	**199.5775779**	**46.02027736**	**0.230588415**	**-2.116608063**	**DOWN**	**0.007125454**	**detected**
LS30205	**Replication protein**	**166.3592915**	**38.73392275**	**0.232832939**	**-2.102632924**	**DOWN**	**0.002305843**	**detected**
LS30523	**Aspartic proteinase nepenthesin**	**166.3592915**	**38.73392275**	**0.232832939**	**-2.102632924**	**DOWN**	**0.002305843**	**detected**
LS30713	**Hypothetical protein**	**203.4856115**	**47.24188327**	**0.232163262**	**-2.106788402**	**DOWN**	**0.006623374**	**detected**
LS31451	**Hypothetical protein**	**105.7847694**	**23.46206269**	**0.221790555**	**-2.172730167**	**DOWN**	**0.024796715**	**detected**
LS31549	**Novel aldehyde dehydrogenase**	**172.2213421**	**39.81734806**	**0.231198686**	**-2.112794894**	**DOWN**	**0.002023211**	**detected**
LS31592	**Hypothetical protein**	**68.6584494**	**12.50939918**	**0.18219752**	**-2.456424776**	**DOWN**	**0.024980859**	**detected**
LS31933	**Hypothetical protein**	**217.1637294**	**47.02111835**	**0.216523811**	**-2.207402407**	**DOWN**	**0.005130801**	**detected**
LS32308	**Hypothetical protein**	**422.3354978**	**98.03388241**	**0.232123236**	**-2.107037149**	**DOWN**	**0.000510417**	**detected**

LS32347	**Hypothetical protein**	**359.8069589**	**73.84875865**	**0.205245499**	**-2.284577514**	**DOWN**	**0.000750042**	**detected**
LS32421	**Hypothetical protein**	**125.3249379**	**25.71173046**	**0.205160528**	**-2.285174904**	**DOWN**	**0.016163354**	**detected**
LS32485	**Hypothetical protein**	**107.7387863**	**23.87402376**	**0.221591727**	**-2.174024073**	**DOWN**	**0.023716554**	**detected**
LS32658	**Cyclin**	**187.8534768**	**43.20691621**	**0.230003282**	**-2.120273647**	**DOWN**	**0.00139889**	**detected**
LS32959	**Hypothetical protein**	**133.1410052**	**27.22658525**	**0.204494365**	**-2.289867009**	**DOWN**	**0.013595711**	**detected**
LS33001	**Calnexin homolog precursor**	**193.7155273**	**44.81677667**	**0.231353559**	**-2.111828802**	**DOWN**	**0.001220109**	**detected**
LS33121	**phosphate translocator 2**	**209.3476621**	**48.48004684**	**0.231576729**	**-2.110437809**	**DOWN**	**0.000846473**	**detected**
LS33254	**Hypothetical protein**	**45.2102473**	**8.246397501**	**0.18240107**	**-2.454813899**	**DOWN**	**0.046769899**	**detected**
LS34246	**Hypothetical protein**	**252.3360326**	**45.47991986**	**0.180235535**	**-2.472044615**	**DOWN**	**0.001104502**	**detected**
LS34374	**Hypothetical protein**	**47.1642642**	**8.891018378**	**0.188511758**	**-2.407273581**	**DOWN**	**0.04433103**	**detected**
LS34387	**Hypothetical protein**	**49.118281**	**9.235207556**	**0.188019763**	**-2.411043781**	**DOWN**	**0.041869021**	**detected**
LS34969	**Hypothetical protein**	**72.5664831**	**12.93991998**	**0.178318136**	**-2.487474656**	**DOWN**	**0.022593137**	**detected**
LS35187	**Hypothetical protein**	**383.255161**	**76.97307782**	**0.20084029**	**-2.315879379**	**DOWN**	**0.000510417**	**detected**
LS35303	**Hypothetical protein**	**271.876201**	**55.35120209**	**0.203589729**	**-2.296263315**	**DOWN**	**0.001808411**	**detected**
LS35465	**Hypothetical protein**	**439.9216494**	**97.41363965**	**0.221434066**	**-2.175050906**	**DOWN**	**0.000388908**	**detected**
LS35563	**Hypothetical protein**	**262.1061168**	**46.00416511**	**0.175517327**	**-2.510314632**	**DOWN**	**0.000906244**	**detected**
LS35772	**Bifunctional dihydrofolate reductase-Thymidylate synthase**	**99.9227189**	**24.9672933**	**0.249866032**	**-2.000773306**	**DOWN**	**0.011421284**	**detected**
LS35985	**Hypothetical protein**	**148.77314**	**30.23512098**	**0.203229703**	**-2.298816824**	**DOWN**	**0.009694172**	**detected**
LS36811	**Hypothetical protein**	**76.4745168**	**13.35269341**	**0.174603175**	**-2.517848305**	**DOWN**	**0.020393683**	**detected**
LS36846	**Hypothetical protein**	**49.118281**	**9.368060268**	**0.190724514**	**-2.390437809**	**DOWN**	**0.041869021**	**detected**
LS37495	**Hypothetical protein**	**283.6003021**	**50.09580776**	**0.176642293**	**-2.501097287**	**DOWN**	**0.000595115**	**detected**
LS37535	**Ubiquitin-protein ligase 13**	**295.3244031**	**62.80603294**	**0.212667942**	**-2.233325522**	**DOWN**	**0.000135247**	**detected**
LS37721	**Hypothetical protein**	**291.4163694**	**52.1646103**	**0.179003707**	**-2.481938633**	**DOWN**	**0.000510417**	**detected**
LS37832	**Hypothetical protein**	**49.118281**	**9.120698938**	**0.18568848**	**-2.429043781**	**DOWN**	**0.041869021**	**detected**
LS38484	**Hypothetical protein**	**211.3016789**	**40.05616511**	**0.189568608**	**-2.399208018**	**DOWN**	**0.002588858**	**detected**
LS38655	**Hypothetical protein**	**213.2556957**	**39.55115553**	**0.185463537**	**-2.430792524**	**DOWN**	**0.002486948**	**detected**
LS39466	**Hypothetical protein**	**113.6008368**	**23.42524231**	**0.2062066**	**-2.277837586**	**DOWN**	**0.02077572**	**detected**
LS39650	**Hypothetical protein**	**408.6573799**	**83.66690448**	**0.204736066**	**-2.288162824**	**DOWN**	**0.000339108**	**detected**
LS39718	**Hypothetical protein**	**391.0712284**	**97.35085646**	**0.248933824**	**-2.006165823**	**DOWN**	**0.000826409**	**detected**
LS39722	**Hypothetical protein**	**393.0252452**	**101.0143157**	**0.257017372**	**-1.960062218**	**DOWN**	**0.000801222**	**detected**
LS40115	**Bet1-like SNARE**	**217.1637294**	**49.77799194**	**0.229218719**	**-2.125203227**	**DOWN**	**0.00070992**	**detected**
LS40311	**Ornithine**	**221.0717631**	**50.08279876**	**0.226545435**	**-2.142127674**	**DOWN**	**0.000654295**	**detected**

	aminotransferase							
LS40648	**Hypothetical protein**	**287.5083357**	**57.56465393**	**0.200219078**	**-2.320348645**	**DOWN**	**0.001340742**	**detected**
LS41097	**Hypothetical protein**	**51.0722979**	**9.472048993**	**0.185463537**	**-2.430792524**	**DOWN**	**0.03971941**	**detected**
LS41773	**Argonaute**	**223.02578**	**50.57415323**	**0.22676371**	**-2.140738319**	**DOWN**	**0.000626077**	**detected**
LS41936	**Hypothetical protein**	**226.9338136**	**50.13939302**	**0.220942804**	**-2.178255153**	**DOWN**	**0.004265671**	**detected**
LS42270	**Hypothetical protein**	**168.3133084**	**32.04990783**	**0.190418144**	**-2.392757141**	**DOWN**	**0.006431146**	**detected**
LS42369	**Signal recognition particle 68 kDa protein**	**238.6579147**	**55.02818312**	**0.230573468**	**-2.116701581**	**DOWN**	**0.000445855**	**detected**
LS42665	**Hypothetical protein**	**529.8064241**	**126.1305547**	**0.238069131**	**-2.070547531**	**DOWN**	**0.000184197**	**detected**
LS43477	**Hypothetical protein**	**228.8878305**	**50.54422228**	**0.220825293**	**-2.179022668**	**DOWN**	**0.004115038**	**detected**
LS43558	**Hypothetical protein**	**176.1293758**	**34.96727801**	**0.198531777**	**-2.332558149**	**DOWN**	**0.005452766**	**detected**
LS43798	**Gamma-secretase subunit**	**244.5199652**	**55.4504788**	**0.226772807**	**-2.140680444**	**DOWN**	**0.000391214**	**detected**
LS44225	**Hypothetical protein**	**51.0722979**	**9.472048993**	**0.185463537**	**-2.430792524**	**DOWN**	**0.03971941**	**detected**
LS44406	**Hypothetical protein**	**326.5886726**	**68.406083**	**0.209456386**	**-2.255278226**	**DOWN**	**0.000652436**	**detected**
LS44955	**Thymidylate synthase**	**303.1404705**	**75.22130104**	**0.248140081**	**-2.010773306**	**DOWN**	**0.000407208**	**detected**
LS46390	**Nascent polypeptide-associated complex**	**248.4279989**	**56.50224204**	**0.227439106**	**-2.13644776**	**DOWN**	**0.000361542**	**detected**