

Molecular and Bio-analytical Characterisation as a means to understand Genetic diversity within Kenyan *Aspergillus flavus* strains

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Abstract

Toxigenic *Aspergillus* species produce mycotoxins that are carcinogenic, hepatotoxic and teratogenic immunosuppressing agents in both human and animals. Kenya frequently experiences outbreaks of aflatoxicosis with the worst occurring in 2004, which resulted in 125 deaths. This study sought to find possible reasons for frequent aflatoxicosis outbreaks in Kenya by isolating *Aspergillus flavus* strains from maize kernels sampled from different climatic regions of Kenya. Using diagonal transect random sampling, maize kernels were collected from Makueni, Homa Bay, Nandi, and Kisumu regions. The genetic diversity and variation among the isolates was examined by characterising the strains according to morphology, phenotype, vegetative compatible groups and molecular systematics. Selected atoxigenic and aflatoxigenic *A. flavus* isolates were also further analysed for aflatoxin production potential using quantitative real-time PCR and various bioanalytical techniques. The influence of the maize lines grown in Kisumu, Homa Bay, Nandi and Makueni region on *A. flavus* infection and aflatoxin production was also examined and served as the basis for an *in vitro* biocontrol assay. Out of 37 isolates identified, nitrate non-utilizing auxotroph's complementation test revealed 20 vegetative compatibility groups. These groups were further designated using the prefix "KVCG", where "K" represented Kenya and consequently assigned numbers 1 to 20 based on our findings. KVCG14 and KVCG15 had highest distribution frequency ($n = 13$; 10.8 %). The distribution of the L, S and S/L- morphotypes across the regions were 57 % ($n = 21$); 7 % ($n = 3$) and 36 % ($n = 13$) respectively. The phylogenetic analysis exhibited high diversity of *A. flavus* isolates from Makueni. ITS1 and ITS2 markers did not reveal significant information within intraspecies speciation of *A. flavus*. Furthermore, a unique isolate (KSM015) was identified that had characteristics of S-morphotype, but produced both aflatoxins B and G. Coconut agar medium (CAM) assay, TLC, HPLC and LCMS/MS analyses confirmed the presence or absence of aflatoxins in selected toxigenic and atoxigenic isolates. qPCR analysis revealed *afIP*, *afIS*, *afIR* and *afIO* transcripts as the most upregulated genes across the tested isolates whereas false detection of *afID* gene transcript was observed in both induced and uninduced *A. flavus* isolates. Diversity Index (H) analyses ranged from 0.11 (Nandi samples) to 0.32 (Kisumu samples). Heterokaryon compatibility ranged from 33 % (for the Makueni samples, $n = 3$) to 67 % (Nandi samples, $n = 6$). The KDV1 maize line was more sensitive to *A.*

flavus infection in comparison to GAF4. We also tested the biocontrol of atoxigenic isolates to inhibit toxin production by aflatoxigenic strains on infected maize kernels. It was shown that the atoxigenic strain (KSMO12) could inhibit the aflatoxigenic strain (KSM014) depending on the atoxigenic concentration during infection. To our knowledge, this is the first reported study for *A. flavus* genetic diversity, variation and distribution in Nandi, Homa Bay and Kisumu regions in comparison to and could assist researchers in the selection of biocontrol strategies to mitigate aflatoxin contamination, especially in Makueni and neighbouring regions.

Keywords: Aflatoxins, Morphotype, Genetic diversity, Heterokaryon compatibility, TLC, HPLC, Fluorescence.

List of abbreviations

A. flavus: *Aspergillus flavus*

A. parasiticus: *Aspergillus parasiticus*

ACN: Acetonitrile

AF36: *Aspergillus flavus* 36

AFB1: Aflatoxin B1

AFB2: Aflatoxin B2

AFG1: Aflatoxin G1

AFG2: Aflatoxin G2

Aflatox: Aflatoxigenic

afID (*nor 1*): Reductase

afIO (*omtB*): O-methyltransferase *B*

afIP (*omtA*): O-methyltransferase *A*

afIR: Transcription activator

afIS (*estA*): Esterase- transcription enhancer

AFM1: Aflatoxin M1

AFM2: Aflatoxin M2

ANOVA: Analysis of variance

ARISE: Africa Regional International Staff/Student Exchange

Atox: Atoxigenic

BLAST: Basic Local Alignment Search Tool

bp: Base pair

CAM: Coconut Agar Medium

cDNA: Complementary Deoxyribonucleic acid

ClO₃: Chlorate

Crn: Chlorate resistant nitrate-utilizing

Ct: Threshold cycle

CTAB: Cetyl trimethyl ammonium bromide

CYA: Czapek Yeast Extract Agar

CZA: Czapek-Dox Agar

DAD: Diode Array Detector

DCM: Dichloromethane

DCM: Dichloromethane

DDBJ: DNA Data Bank of Japan

DEPC: Diethyl pyrocarbonate

DNA: Deoxyribonucleic acid

E. coli: Escherichia coli

E: Efficiency

EC: European Commission

EDTA: Ethylene-diamine-tetra acetic acid

EF1 α : Elongation factor 1 alpha

ESI: Electrospray Ionisation

EtBr: Ethidium Bromide

EtOAc: Ethyl acetate

FA: Formic acid

FA: Formic acid

HB: Homa Bay

HPLC: High Pressure Liquid Chromatography

IAPO: International Academic Programmes Office

IITA: International Institute of Tropical Agriculture

Ind: Induced

INSD: International Nucleotide Sequence Databases

IPTG: Isopropyl β -D-1-thiogalactopyranoside

ITS 1: Internal Transcribed Spacer Region 1

ITS 2: Internal Transcribed Spacer Region 2

Kb: Kilo base pair

KSM: Kisumu

LA: Luria Agar

LB: Luria Broth

LCMS/MS: Liquid Chromatography coupled to tandem Mass Spectrometry

LOD: Limits of Detection

LOQ: Limits of Quantification

MC: Makueni county

MCL: Maximum Composite Likelihood

MEGA: Molecular Evolutionary Genetics Analysis

MeOH: Methanol

MEP: Membrane Protein

Min: Minute

ml: Millilitre

mm: Millimetres

Mm: Millimolar

MM: Minimal media

NaCl: Sodium chloride

NC: Nandi county

NCBI: National Centre for Biotechnology Information

ng: Nanogram

NH₄: Ammonium

nm: Nano metres

NO₃: Nitrate

Not 1: Nocardia otitidiscaviarum 1

NTC: No Template Control

OTA: Ochratoxin A

PCR: Polymerase Chain Reaction

PDA: Potato Dextrose Agar

PDAC: Potato Dextrose Agar Chlorate

pDNA: Plasmid DNA

PVP: Polyvinylpyrrolidone

R²: Linear regression

rDNA: Ribosomal Deoxyribonucleic acid

RED: Restriction Digestion Enzyme

RNA: Ribonucleic acid

rpm: Revolution per minute

RT: Room temperature

RT-qPCR: Real Time Polymerase Chain Reaction

S/L: Small/Large

Sig.: Significance

TAE: Tris–acetate

TLC: Thin Layer Chromatography

TMCT: Tukey's Multiple Comparison Test

TOF/Q-TOF: Time-of-flight/quadrupole Mass Spectrometer

UCT: University of Cape Town

Unind: Un-induced

UoN: University of Nairobi

USA: United States of America

USHEPiA: University Science, Humanities and Engineering Partnerships in Africa

UV: Ultra violet

Var: Variance

VCGs: Vegetative Compatibility Group

X-gal: X-Gal (5-Bromo-4-Chloro-3-Indolyl -D-Galactopyranoside)

YEP: Yeast Peptone Agar

YES: Yeast Extract Agar

β -Tub: β -Tubulin

μ l: Microliter

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Declaration

I, **Alfred Ochieng Mitema**, do hereby declare that, this is my original own work and/or where exceptionally indicated in the acknowledgment. None or portion of it has been presented for examination in this University or elsewhere in the world. I therefore give mandate to the University of Cape Town to use it purposely for academic work in portion or entirely.

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Date: 16.02.2018

Inclusion of Publications and Conferences

I, **Alfred Ochieng Mitema**, confirm that I have been granted permission by the Doctoral Degrees Board of the University of Cape Town to include the following publication(s) in my PhD thesis, and where co-authorships are involved, my co-authors are in agreement that I may include the publication(s):

Publication:

Mitema, A., Okoth, S., Rafudeen, M.S., Vegetative compatibility and phenotypic characterization as a means of determining genetic diversity of Aspergillus flavus isolates, Fungal Biology (2017), doi: 10.1016/j.funbio.2017.11.005.

Conferences:

- a. **Oral Presentation:** *Heterokaryon incompatibility and phenotypic characterisation of Aspergillus flavus isolates in low and high-risk zones in Kenya.*

Mitema, A., S., Rafudeen, S., Okoth, R., Iyer

MEEGID XIII 2016: 13th International Conference on Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases, Institute of Tropical Medicine, Antwerp, Belgium (10-13 May 2016)

- b. **Conference Abstract:** *Heterokaryon incompatibility/compatibility and phenotypic characterisation of Aspergillus flavus isolates in low and high-risk zones in Kenya*

Mitema A., Rafudeen S., Okoth S., Iyer R.

38th Mycotoxin Workshop, Berlin, Germany (02-04 May 2016)

- c. **Poster:** *Heterokaryon incompatibility and phenotypic characterisation of Aspergillus flavus isolates in low and high-risk zones in Kenya*

Mitema A., Okoth S., Iyer R., Hilgart A., Rafudeen S.

29th Fungal Genetics Conference, Asilomar Conference Centre, Pacific Grove, CA, USA. (14-19 March 2017)

Signature:  Date: 16.02.2018

Student Name: **Alfred Ochieng Mitema**, Student Number: **MTMALF001**

CHAPTER ONE

1.0 Introduction

Certain fungi of the *Aspergillus* genus produce secondary metabolites termed aflatoxins, which are a class of naturally-occurring mycotoxins (Klich, 2007). About 200 species of *Aspergillus* have been identified, of which 16 have been found to produce aflatoxins that act as carcinogenic, hepatotoxic, and teratogenic or immunosuppressing agents in both human and animals (Klich, 2007; Rotimi *et al.*, 2016). A number of *Aspergillus* species such as *Aspergillus flavus*, *Aspergillus bombycis*, *Aspergillus nomius* and *Aspergillus niger* produce aflatoxins with high carcinogenic activity (JECFA 2017; Bandyopadhyay *et al.*, 2005). Aflatoxin contamination has been detected in maize, beans, cottonseed, peanuts and other grain crops (Ventura *et al.*, 2004; Somashekar *et al.*, 2004). The contamination not only results in reduced crop value but can cause health problems in both humans and animals that consume contaminated crops and feeds (Wu & Khlangwiset, 2010).

Although aflatoxin-producing *Aspergillus* species are found worldwide, they are of greater concern in underdeveloped countries which lack appropriate infrastructure, management tools and resources required to prevent, control or monitor their impact on the wider community (Klich, 2007). Environmental conditions: unseasonal rains during harvesting, monsoons, increased temperatures and moisture promotes fungal pathogen proliferation and mycotoxin production (Wagacha & J.W. Muthomi 2008) (Bhat, 2003). Additionally, increased risks of mycotoxin production and fungal growth is facilitated by improper harvesting, poor storage facilities, inadequate transportation, and sub-optimal temperatures, processing and marketing practices. These environmental conditions and problems associated with the production of food and storage are common in most parts of sub-Saharan Africa, where to date, the largest poisoning of mycotoxin epidemic has been reported (Gieseke, 2004a; Lewis *et al.*, 2005).

1.1 Aflatoxins

Aflatoxins are categorized as the most important class of mycotoxins and can directly degrade human and animal health (Bandyopadhyay *et al.*, 2005). They are

synthesised by several *Aspergillus* species widely occurring saprophytic fungi, that grows on living and non-living substrates (Frisvad *et al.*, 2007) . The symptoms of aflatoxicosis and related diseases are dependent upon factors such as nutrition, age, species of *Aspergillus* present and concurrent exposure to other toxins. The liver is the primary target in animals and hence, aflatoxicosis is mainly a hepatic ailment (Iqbal *et al.*, 2010). Consumption of food contaminated with mycotoxins is correlated with an increased level of liver cancer in mammals (Ventura *et al.*, 2004; Magan *et al.*, 2003; Giorni *et al.*, 2007). In processed form, aflatoxins can enter the human food chain and be incorporated into animal feeds. Aflatoxins also can be found in meat, milk products, and eggs if the animals producing these products were fed contaminated materials (Kubelka *et al.*, 2003; Keller *et al.*, 2005).

Aflatoxins are furanocoumarins (Fig.1.1): aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁), aflatoxin G₂ (AFG₂), aflatoxin M₁ (AFM₁) and aflatoxin M₂ (AFM₂) (Reddy *et al.*, 2010). AFB₂ and AFG₂ are dihydroxy derivatives of AFB₁ and AFG₁, respectively, whereas, AFM₁ is a 4-hydroxy AFB₁ and AFM₂ is a 4-dihydroxy AFB₂. AFG₂ is not as toxic as AFB₁, which is classified as a class 1 carcinogen (IARC, 2002).

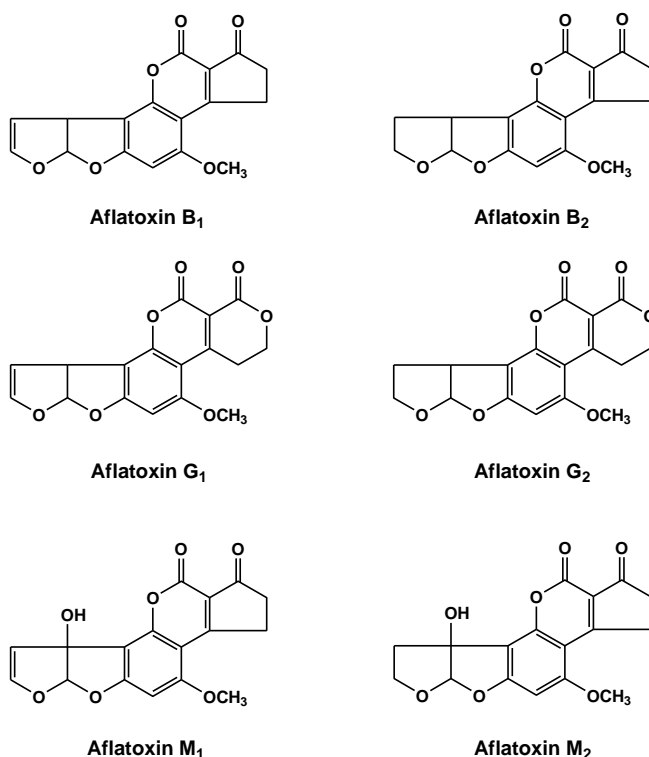


Figure 1.1 The four-major aflatoxin's (AFB₁, AFB₂, AFG₁, AFG₂), chemical structures and their derivatives, M₁ and M₂. The subscript 1 and 2, indicates respective compounds (major and minor).

The designation "B" and "G" indicates blue and green fluorescent light emitted respectively when exposed to ultraviolet radiation (Murphy *et al.*, 2006) (Fig.1.2).



Figure 1.2 Symptoms of *Aspergillus* ear rot (left) and fluorescence of infected kernels observed under ultra violet light 365 nm (Murphy *et al.*, 2006).

Aflatoxins are also classified based on their absolute chromatographic separations on thin-layer chromatography (Barros *et al.*, 2006; Iqbal & Paterson 2010). *A. flavus* strains usually produce AFB₁ and AFB₂ and *A. parasiticus* strains produce AFG₁, AFG₂ in addition to AFB₁ and AFB₂. AFB₁ occurs widely and is a genotoxin that is carcinogenic and teratogenic to mammals (McKean *et al.*, 2006). Aflatoxins M₁ and M₂ are metabolic products which are found in urine, milk and other products of mammals fed on mouldy aflatoxin contaminated grains (Klich *et al.*, 2003).

These mycotoxins have related chemical structures (Fig.1.1) and forms highly oxygenated group of naturally occurring compounds which are heterocyclic.

1.1.1 Conditions for *Aspergillus* growth and aflatoxin production

Aspergillus species are widespread saprophytes that grow in carbon rich environments such as soil, hay and on decaying vegetation, or that survive in a dormant state as sclerotia (Giorni *et al.*, 2007; Horn *et al.*, 2009). In these environments, *Aspergillus* is ecologically important for its role in nutrient recycling (Payne and Yu, 2010). The soil is the primary source of inoculum of *A. flavus* and *A.*

parasiticus. Different strains produce different aflatoxin amounts with production occurring typically at temperatures between 15 ° C and 35 ° C and at a water activity of 0.90 (Giorni *et al.*, 2007). Horn, (2003) observed that, *A. parasiticus* is active and grows at a lower temperature than does *A. flavus*. This difference is seen by the preference of the former to colonise ground and underground crops, which are in direct contact with the soil, while in other crops, such as maize and cotton, where aerial parts are harvested, it occurs as a secondary infection (Horn, 2003). *A. flavus* is isolated from a range of climatic zones but is more frequently found between latitudes 16° and 35° in warm climate zones and is not common above 45° latitudes (Klich, 2007).

1.1.2 Modes and factors influencing aflatoxin contamination in the food chain

As an opportunistic fungal pathogen, *A. flavus* is able to infect cereals (Leger *et al.*, 2000). *A. flavus* grows aggressively in many food crops and human exposure to aflatoxins is difficult to avoid during the various stages in the food chain (Pitt *et al.*, 2013). Insects predispose kernels to fungal infection through physical damage as they feed on kernels carrying spores from the surface to the interior (Scheidegger & Payne, 2003). The fungus forms many sclerotia in insect damaged kernels before harvest and are dispersed into the soil during harvesting season (Scheidegger & Payne, 2003). The sclerotia survive in the soil and produce conidiophores and conidia during the following season (Scheidegger & Payne, 2003). Once *A. flavus* is present in plant tissue it continues to grow, and mycelia spread superficially among the kernel and penetrate through to pericarp (Payne, 2003). Among the factors that promote fungal growth and contamination is storage of improperly dried maize, poor sanitary conditions, plant exposure to stress during growth, high humidity and poor aeration in the granary during storage (Azziz-baumgartner *et al.*, 2005).

Infection of stored products by aflatoxigenic fungi and subsequent contamination by mycotoxins are generally influenced by abiotic and biotic factors (Magan *et al.*, 2003; Dowd, 2003). Abiotic factors include environmental factors such as temperature, water availability and oxygen (Magan *et al.*, 2003). Biotic factors include variables such as the nature of the substrate, its inherent moisture content, rodent and insect infestation, all of which can contribute to increasing fungal population and the subsequent mycotoxin production (Dowd, 2003). Foreign materials and debris

can also have an impact on both the rate of fungal spoilage and the production of mycotoxins (Atukwase *et al.*, 2009).

1.1.3 Health and economic importance of aflatoxins

The danger of mycotoxins in the human diet resides in their inability to be detected biologically. Aflatoxin poisoning is responsible for many thousands of human deaths per annum (Reddy *et al.*, 2009). A large proportion of the world population is chronically exposed to aflatoxins as evidenced from the presence of aflatoxin M₁ in human breast milk in west Africa, Kenya, Sudan, Thailand and the United Arab Emirates (Bhat *et al.*, 2003; Rahimi *et al.*, 2010). In a longitudinal study by Turner *et al.* (2003) conducted in Benin, West Africa showed that over 98 % of human subjects tested were positive for aflatoxin markers. A related study in the same region by Gong *et al.* (2002) showed that 99 % of the children had aflatoxin in their blood samples with some of the highest aflatoxin levels reported for any population to date.

Aflatoxin poisoning occurs primarily through ingestion of contaminated food (Fung *et al.*, 2004) and can lead to a number of diseases in both humans and animals (Fig.1.3). The symptoms of severe aflatoxicosis include oedema, necrosis of the liver and profound lethargy (Williams *et al.*, 2004). Chronic sub-acute exposure to aflatoxin can result in stunted growth rate, lowered milk or egg production, immuno-suppression and liver damage (Wagacha & Muthomi, 2008). Acute poisoning has symptoms such as liver and kidney damage, DNA damage, and teratogenic cancer of unborn children (Rajasinghe *et al.*, 2009). However, symptoms of aflatoxin ingestion in humans vary with the overall health of the individuals, age and sex with males being more susceptible than females (Williams *et al.*, 2004).

A. flavus is considered to be the second leading causal agent of human invasive aspergillosis, infecting skin, oral mucosa, and subcutaneous tissue (Wu & Khlangwiset, 2010; Rokas *et al.*, 2007). Recently, *A. flavus* has been cited as a leading cause of mycotic keratitis, a fungal infection of the cornea (Tilak *et al.*, 2010). Most human infections arise through inhalation of fungal spores (Adhikari *et al.*, 2004), with

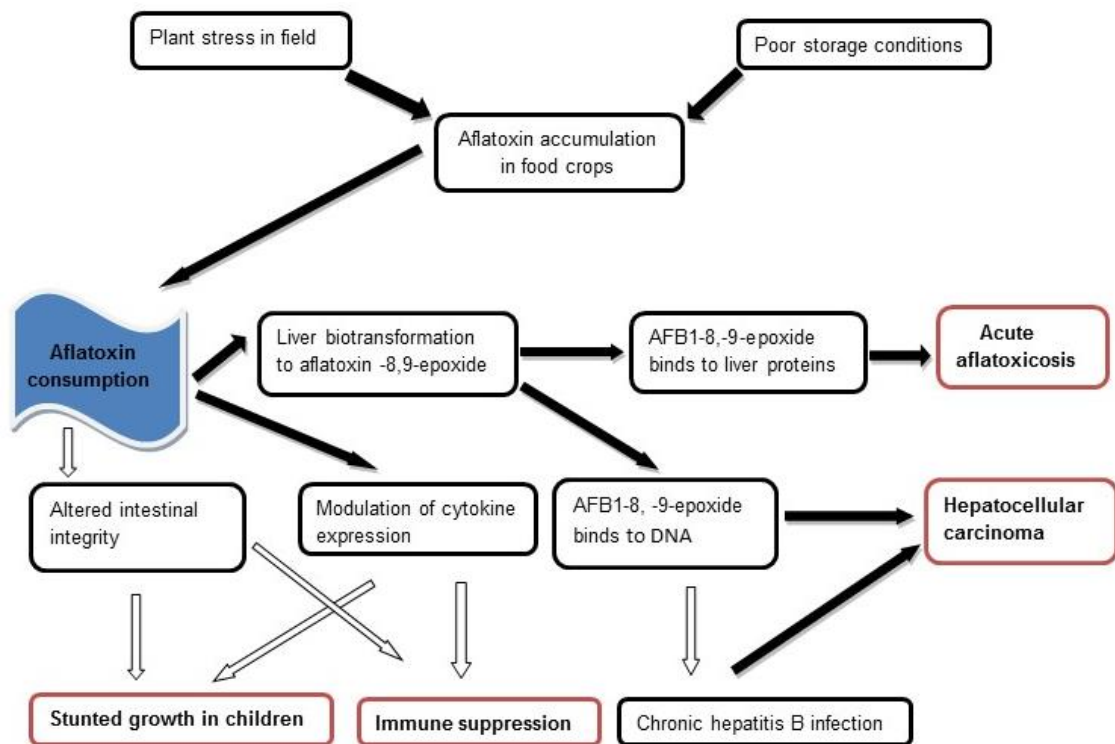


Figure 1.3 A schematic flow diagram showing aflatoxin disease pathways in human, which shows how aflatoxin accumulates in food crops and contributes to various adverse human health effects. The dark arrows show linkages established in agricultural and toxicological research, while the white arrows show relatively less established linkages. (Modified and adapted from Wu & Khlangwiset, 2010; Wu *et al.*, 2011).

farmers worldwide infected from breathing in spores from contaminated foods and feeds. Allergic responses may develop after repeated exposure to fungal spores, resulting in asthma, extrinsic alveolitis, or allergic broncho-pulmonary aspergillosis (Wu & Khlangwiset, 2010).

Distinct from aflatoxicosis are cancers arising from long-term aflatoxin B₁ exposure. AFB₁ is a potent hepatocarcinogen and induces tumours mainly in the liver, but also in the kidney, lung, and colon of humans and animals. Hepatocellular carcinoma (HCC), a primary liver cancer, is associated with AFB₁ consumption in Africa and Asian countries (Liu & Wu, 2010). Liu and Wu, (2010), showed that 4.6–28.2 % of all HCC in the world is associated with aflatoxin exposure and this association is strongest in developing countries where individuals also suffer from chronic hepatitis B infection. AFB₁-epoxides and AFB₁-exo-epoxides intercalate between the bases of DNA and integrate in codon 249 of the p53 gene, resulting in a mutation (AGG to AGT, R249S) (Bressac *et al.*, 1991). This mutation is considered the signature of aflatoxin exposure and is present in 75 % of HCC in developing

countries, whereas it is present in less than 3 % in developed countries (Gouas *et al.*, 2009).

Aflatoxins pose serious health threats in Kenya especially in the Eastern and Central Provinces where there have been reports of outbreaks of aflatoxin poisoning, especially among the rural communities (Ngindu *et al.*, 1982). The 1981 outbreak was a result of drought followed by heavy rains during harvest of maize from subsistence farming practices (Ngindu *et al.*, 1982). However, in 2004, concentrations of aflatoxin B1 were found in maize at levels 220 times greater than the limit suggested by Kenyan health authorities (Azziz-baumgartner *et al.*, 2005). In the same year, the worst outbreak of aflatoxin poisoning occurred where 317 cases and 125 deaths were reported (Azziz-baumgartner *et al.*, 2005; Probst *et al.*, 2007). This problem appears endemic to certain Kenyan regions as every year cases are reported in the affected regions (Lewis *et al.*, 2005).

Aflatoxin poisoning has also been reported in many parts of the world in domestic and non-domestic animals, and other non-human primates (Reddy *et al.*, 2009). Aflatoxin poisoning has led to serious economic losses through loss of livestock and poultry following consumption of contaminated feed or loss of income from local or international markets (Okoth, 2016). In 2010, Kenya had to withdraw about 2.3 million bags (estimated at \$69 million) from its strategic maize reserves due to aflatoxin contamination. This maize was not only unfit for human and livestock consumption but resulted in a loss of export revenue (Okoth, 2016).

1.1.4 Aflatoxin monitoring and legislation

Food safety requirements vary from country to country and can constitute an important barrier for international trade in food products as their main objective is to protect public health (Wagacha & Muthomi 2008; Broberg 2009; Okoth, 2016; JECFA, 2017). About 100 countries, of which 15 are African, have established regulations to protect consumers from harmful effects of mycotoxins (Wagacha & Muthomi, 2008; Okoth, 2016). Human foods are allowed to contain 4-30 µg/kg aflatoxin, depending on the country (Ward *et al.*, 2005). In the United States, the Food and Drug Administration uses 20 µg/kg as the maximum limit of aflatoxin allowed in food for human consumption, except for milk which has a 0.5 part per billion limit (JECFA, 2017). The European Union has enacted the strictest aflatoxin tolerance standard of 2 µg/kg

aflatoxin B₁ and 4 µg/kg total aflatoxin for human consumption with the exception of 0.5 µg/kg aflatoxin M₁ for milk and milk products (JECFA, 2017). During the worst aflatoxicosis outbreak in Kenya (2004), the levels of aflatoxin B₁ found in maize was 4400 µg/kg (Azziz-baumgartner *et al.*, 2005).

1.2 *Aspergillus* species systematics

The genus *Aspergillus* was first described by Mitchell (1729) and is composed of more than 180 accepted anamorphic species (Pitt 2000) with teleomorphs described in nine different genera. The genus is further sub-divided into sections *Flavi*, *Nigri*, *Fumigati*, *Candidi*, *Terrei*, *Clavati*, *Nidulantes*, *Versicolours*, *Restricti*, *Wentii*, *Usti*, *Cremeri*, *Ochraceorosei* and *Sparsi* with different distinctive colony colour formation on different media (Klich, 2002).

Although aflatoxins are produced by a number of *Aspergillus* species, these can be divided into three phylogenetically distinct sections, namely *Flavi*, *Ochraceorosei* and *Nidulantes*. The main aflatoxin producers in section *Flavi* are *A. flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii*, *A. parasclerotigenus* and *A. bombycis*. The *Ochraceorosei* aflatoxins producers include *A. ochraceoroseus* and *A. rambellii* while *Emericella astellata* and *E. venezuelensis* from section *Nidulantes* also can produce aflatoxins (Frisvad *et al.*, 2005).

1.2.1 *Aspergillus* species physiology

In the field, the fungus is present in soil as conidia or sclerotia and in plant tissues as mycelia (Fig.1.4) and sclerotia can survive in the soil under severe environmental conditions and produce conidia, leading to a population increase under hot dry conditions (Scheidegger & Payne, 2003). Sclerotia germinate as mycelia, which then form conidiophores. Air dispersal of conidia is associated with infection of above ground plant parts such as maize and tree nuts, whereas soil movement and rain splash dispersal may be more important for infection of peanuts and cotton seed (Boyd & Cotty, 2001). In the case of maize, the fungus colonises the silks and kernels of the plant (Scheidegger & Payne, 2003). Young maize kernels are susceptible at the late milk–early stages and the incidence of colonisation is higher on silks of mature maize ears than young ears (Scheidegger & Payne, 2003).

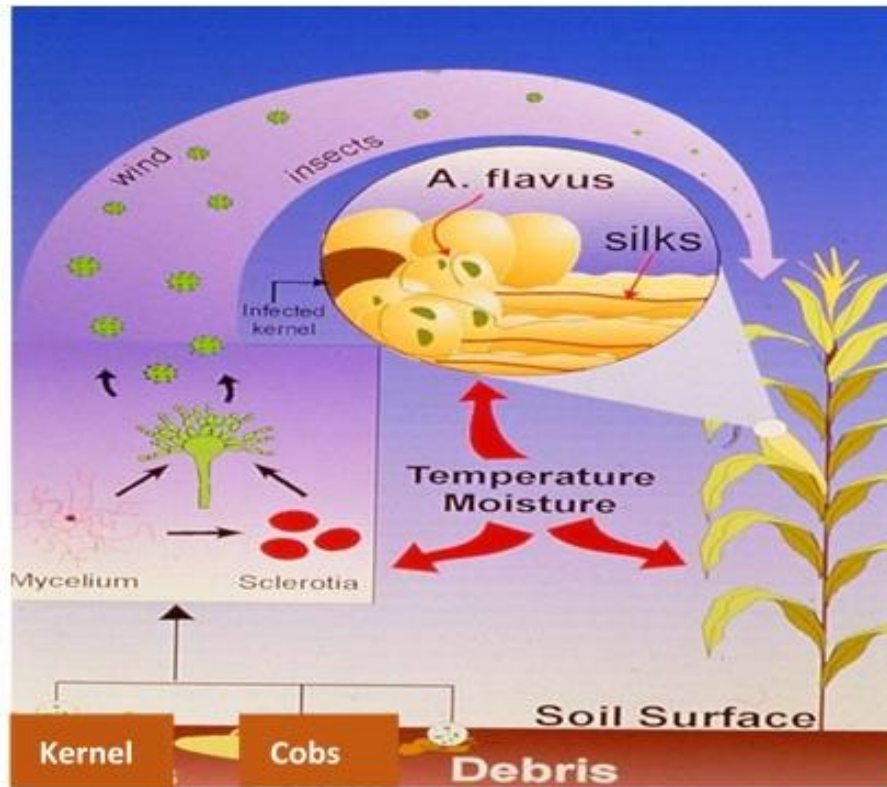


Figure 1.4 The life cycle of *Aspergillus flavus* fungus on maize. The fungus overwinters in dead decaying organic matter, maize cobs and maize kernels either as mycelium or as sclerotia. When conditions are favourable the sclerotia germinates to produce hyphae or conidia, which are dispersed in the soil and air. Maize are then infected via spores carried to the maize ears by wind or insects. The spores germinate and infect maize kernels under favourable conditions (Scheidegger & Payne, 2003).

Colonisation is enhanced not only by favourable environmental conditions but also by insect and bird damage, which provide entry sites for the fungus (Kanaania *et al.*, 2008).

1.2.2 Morphological Identification

The defining characteristic of the genus *Aspergilli* is the aspergillum-like spore bearing structure (Fig.1.5). During mycelial differentiation, certain cells enlarge, develop a heavy cell wall and form 'T' or 'L' shaped 'foot cells' that produce a single conidiophore perpendicular to the long axis of the cell (Klich, 2007). The erect hyphal branch developing from the foot cell is the conidiophore, which enlarges at its apex to form a rounded, elliptical or club shaped vesicle. The fertile area of the vesicle gives rise to a layer of cells called phialides that produce long chains of mitotic spores called conidia or conidiophores.

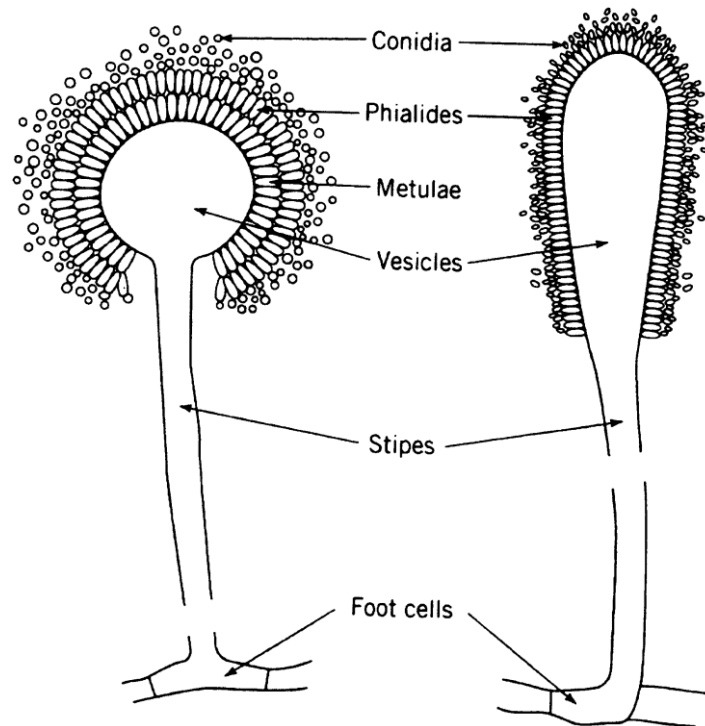


Figure 1.5 Micro-morphological features (conidia, phialides, metulae, vesicles shape, stipes and foot cell) used to identify *Aspergillus* section *Flavi* (Klich, 2007).

The size and arrangement of the conidial heads as well as the colour of the spores they bear are important identifying characteristics (Figs.1.5 and 1.6). Species in the *A. niger* group have black spores, those in the *A. ochraceus* group are yellow to brown, while *A. fumigatus*, *A. nidulans*, *A. parasticus* and *A. flavus* have green spores (Varga *et al.*, 2011) (Fig.1.6).

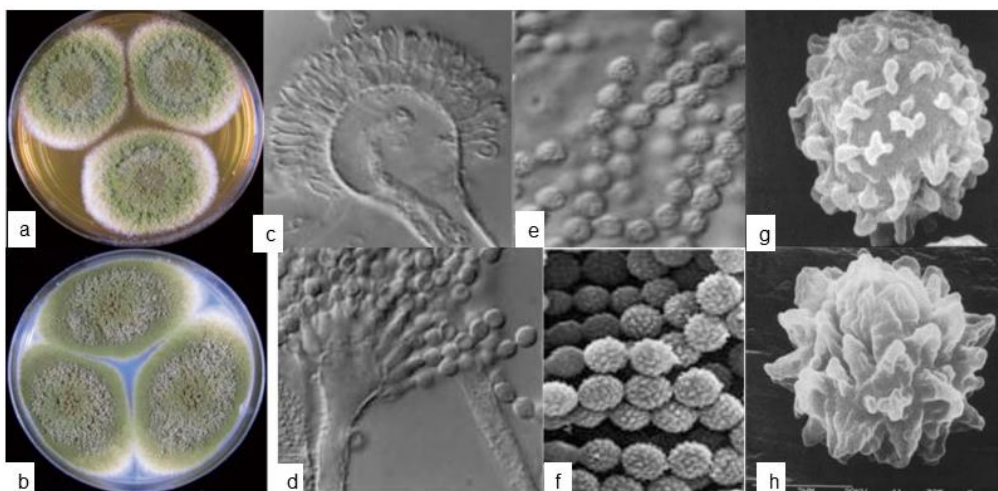


Figure 1.6 Visualisation of *Aspergillus* section *Flavi* growth. **a.** *Aspergillus flavus*, and **b.** *Aspergillus parasiticus* on Czapek yeast extract agar incubated at 25 ° C for 7 days (Varga *et al.*, 2011), **c-d.** conidiospore and vesicle shape, **e.** biseriate conidia, **f.** uniseriate conidia

(adapted from de Hoog *et al.*, 2000) and **g-h**. conidia ornamentation (Rodrigues *et al.*, 2007). Scale bars = 10 μ m.

The macro-morphological features used in species identification are the colour of the colony, the growth rate, exudates and thermo-tolerance (Klich, 2007; Samson *et al.*, 2007). Since *Aspergillus* species have varying morphological and growth responses to different nutrients, it is important to standardise growth conditions and media. Species identification depends upon pure cultures grown on known media (Klich, 2007). Strain variation is quite extensive within species and a variety of subtle effects such as air exchange, light and volume of the medium can affect morphology (Klich, 2007). Contemporary taxonomists usually grow strains on several media, at different temperatures, to identify a particular species (Klich, 2002; Varga *et al.*, 2011).

In addition to conidiophores, other morphological structures useful for identification include sclerotia, cleistothecia and Hulle cells (Samson *et al.*, 2007). Both sclerotia and cleistothecia are closed and usually round structures about the size of a poppy seed, that may be so abundant as to dominate a colony surfaced. Sclerotia are rounded masses of mycelium with an outer melanised rind that microscopically resemble cleistothecia but do not contain sexual spores. They are believed to serve as resting structures that allow strains to survive adverse growth conditions (Samson *et al.*, 2007). Hulle cells are thickened, often globose, cells that are associated with cleistothecia. Cleistothecia are the sexual reproductive structures and contain the meiotic ascospores in asci (Samson *et al.*, 2007).

1.2.3 Molecular systematics of *Aspergillus flavus*

Single copy conserved genes have been used as a target for taxonomic studies within *A. flavus*. Universal β -tubulin, calmodulin and topoisomerase II genes have been used to differentiate fungal species, but only with distantly related species, since variability is generally low (Kanbe *et al.*, 2002). The most widely used DNA target regions for discriminating *Aspergillus* species are the ones in the rDNA complex, mainly the internal transcribed spacer regions 1 and 2 (ITS 1 and ITS 2) and the variable regions at the 5' end of the 28S rRNA gene (D1-D2 region) (Hinrikson *et al.*, 2005).

Nuclear regions/genes like the RNA polymerase 11 largest sub unit gene (*rpb1*) and the ITS region of the rRNA repeat proved to be excellent tools for discriminating

species within *Aspergillus* (Peterson, 2008). The molecular identification of *Aspergillus* species is currently based on sequences from β -tubulin, calmodulin, actin, and ITS (Balajee *et al.*, 2007; Samson *et al.*, 2011; Varga *et al.*, 2011).

1.2.4 Vegetative compatibility groups in *Aspergillus flavus*

The formation of heterokaryons between different strains is an important and common component of the life cycle of many filamentous fungi. Lineages that are capable of fusing (anastomosis) and forming stable and functional heterokaryons are known as sexually or vegetatively compatible. Even though *A. flavus* seemingly lacks host specificity (Leger *et al.* 2000); distributions of different *A. flavus* lineages suggest that they may be adapted to specialised niches and exhibit competitive advantages in specific soils, hosts, regions, and seasons (Bock *et al.*, 2004; Jaime-Garcia, 2006). Generally, variation in toxin production levels in *A. flavus* is also associated with vegetative compatibility group (VCG). Isolates in the same VCG have compatible *het* loci and can form stable hyphal fusions (Horn *et al.*, 2009). Identification of atoxigenic VCGs has been important in developing biological control agents (Donner *et al.*, 2010).

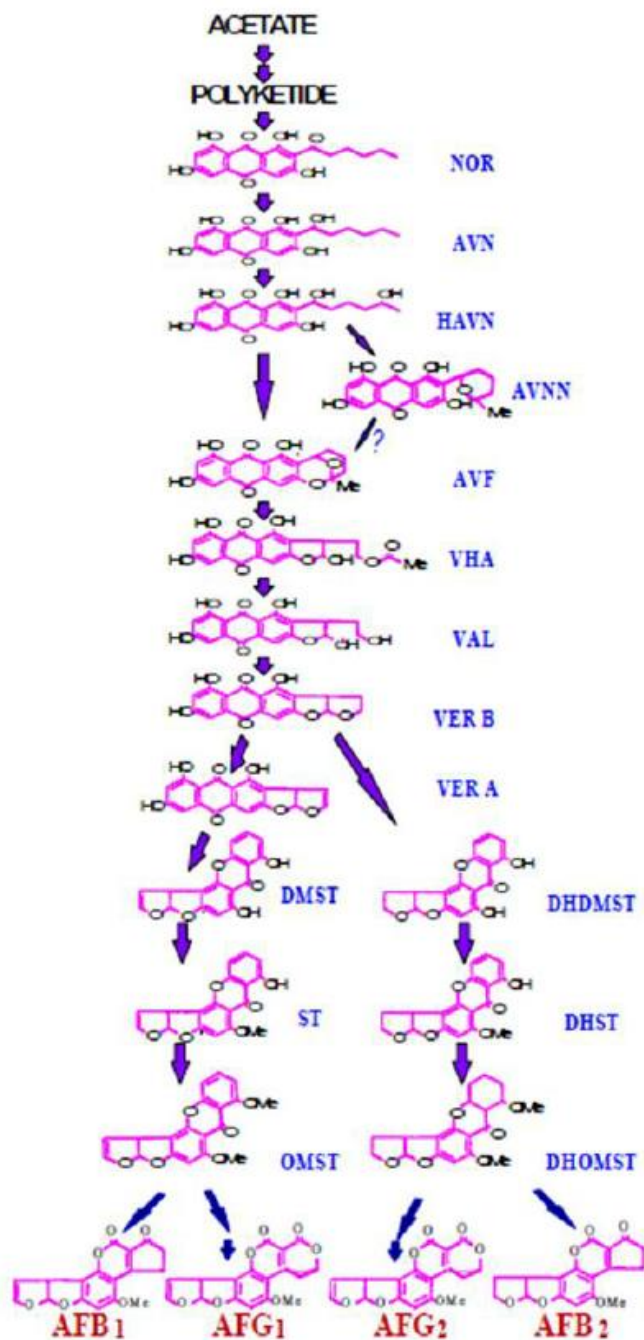
1.3 Aflatoxin biosynthesis pathway and gene cluster

A number of studies have attempted to relate the expression of specific mycotoxin biosynthesis genes with phenotypic mycotoxin production under different environmental conditions. They further integrate the correlation of eco-physiological conditions with gene expression and phenotypic toxin production (Schmidt-heydt *et al.*, 2008; Schmidt-Heydt *et al.*, 2009). At least 25 genes are clustered within a 70-kb DNA region involved in aflatoxin biosynthesis (Yu *et al.*, 2004a). One of these genes is *nor-1* (*afID*) which encodes an enzyme that catalyses the ketoreduction of norsolorinic acid (NA) (the first stable pathway intermediate) to averantin (Chang *et al.*, 1992). Disruption of this gene resulted in NA accumulation (Chang *et al.*, 1992), confirming the important function of *nor-1* gene product in aflatoxin synthesis and suggesting that NA is a substrate for this protein. Molecular tools have used and compared to traditional assessment methods and quantitative AFB₁ analyses in monitoring temporal changes in stored cereals. Experiments have also been carried out with *A. flavus*-inoculated grains stored at different environmental conditions to measure asexual reproduction of *A. flavus* colony-forming units, quantification of

structural and regulatory gene expression, aflatoxin production and transcription of selected aflatoxins biosynthetic genes (Abdel-Hadi *et al.*, 2011a).

Sweeney and Dobson (1999), described an aflatoxin biosynthesis pathway with at least 23 enzymatic conversions, through a series of pathway intermediates (Fig.1.7). Malonyl CoA and acetate are converted to a hexanoyl starter unit by a fatty acid synthase, and the starter extended by a polyketide synthase to norsolorinic acid, the first stable precursor in the pathway. The penultimate precursors of the aflatoxins are sterigmatocystin (ST) and dihydrosterigmatocystin (DHST) where ST is the precursor of AFB1 and AFG1 and the DHST the precursor of AFB2 and AFG2 respectively (Yu *et al.*, 2004a).

The complete genome of *A. flavus* NRRL 3357 was sequenced in 2005, and several other genome sequences from *Aspergillus* species and *A. flavus* strains have since been obtained (Georgianna *et al.*, 2010; Varga *et al.*, 2011). Ehrlich *et al.* (2015) demonstrated that genome sizes of *A. flavus*, distributed on 8 chromosomes, is estimated to be 37 Mb and encode for more than 12,000 functional genes. Compared to other *Aspergilli* [*A. niger* (34 Mb), *A. nidulans* (31 Mb), *A. fumigatus* (30 Mb), and *A. terreus* (30 Mb)] the genome of *A. flavus* is larger although all of the species have the same number of chromosomes (Birren *et al.*, 2004; Nierman *et al.*, 2005; Pel *et al.*, 2007).



KEY: **Nor**: Norsolorinic acid; **AVN**: Averantine; **HAVN**: 5'-hydroxy-averantine; **AVNN**: averafunin; **AVF**: averufin; **VHA**: versiconal hemiacetal acetate; **VAL**: versiconal; **VER B**: versicolorin B; **VER A**: versicolorin A; **DMST**: demethylsterigmatocystin; **DHDMST**: dihydrodemethylsterigmatocystin; **ST**: Sterigmatocystin; **DHST**: dihydrosterigmatocystin; **OMST**: O-methylsterigmatocystin; **DHOMST**: dihydro-O-methylsterigmatocystin; **AFB1**: Aflatoxin B1; **AFB2**: Aflatoxin B2; **AFG1**: Aflatoxin G1; **AFG2**: Aflatoxin G2.

Figure 1.7 Flow diagram showing aflatoxin biosynthetic pathway gene cluster. The arrows indicate the pathway steps involved from previous precursor to the next intermediate towards the formation of aflatoxins. The enzymes involved are fatty acid synthase, polyketide synthase, norsolorinic acid reductase, versiconal hemiacetal acetate reductase, esterase, versicolorin B synthase, versiconyl cyclase, desaturase, O-methyltransferase (adopted from Yu *et al.*, 2004a; Yu, 2012a).

Previously, it has been reported that the biosynthesis of mycotoxin genes is induced and not expressed constitutively (Peplow *et al.*, 2003; Price *et al.*, 2006). Studies have attempted to relate the expression of specific aflatoxin biosynthesis genes with aflatoxin production under different ecological conditions (Schmidt-heydt *et al.*, 2008; Schmidt-Heydt *et al.*, 2009). Their induction at the transcript level can be determined some time before the detection of mycotoxins using methods such as real-time qPCR which has become a diagnostic tool for possible aflatoxin contamination (Mayer *et al.*, 2003).

1.4 Metabolomics and fingerprinting approaches towards *Aspergillus flavus* and aflatoxin production

The availability of sequences from many different fungal genomes has allowed the prediction of many known and unknown biosynthetic metabolic pathways. The *A. flavus* metabolome is of interest due to its production of aflatoxins (Mehl *et al.*, 2012). Known secondary metabolites other than aflatoxins and their precursors produced by *A. flavus* include aflatrem, cyclopiazonic acid, pseurotin, kojic acid, pyrazines, aflavinines, paxillines, paspalinines, aspersitin; and metabolites related to pigment and melanin formation (Duran *et al.*, 2007; Rank *et al.*, 2012).

Studies of genes involved in secondary metabolite biosynthesis suggested that *A. flavus* is capable of producing many more metabolites than have so far been discovered (Khaldi *et al.*, 2010). *In silico* analysis of the *A. flavus* genome identified 55 gene clusters predicted to be associated with the production of secondary metabolites (Khaldi *et al.*, 2010). Approximately 20 metabolites have been identified in *A. flavus* cultures, but only six are known to be involved in the aflatoxin biosynthetic pathway gene cluster (Forseth *et al.*, 2014). Secondary metabolites are excellent phenotypic characters for species recognition and recent studies on full genome sequencing of important aspergilli concluded that major genomic differences between species are often related to the number and similarity of polyketide synthase and non-ribosomal peptide synthase genes (Pel *et al.*, 2007).

Chemoinformatic tools have been developed and applied to manage large amounts of spectroscopic data generated from analysis of numerous fungal strains (Smedsgaard & Nielsen, 2005; Piana *et al.*, 2012). Many types of polyketides and non-ribosomal peptides contain aromatic ring systems and other conjugated chromophore

systems allowing detection with a diode array detector (DAD), whereas non-ribosomal peptides and other alkaloids in general are readily protonated and easily detected using Quadrupole time-of-flight tandem mass spectrometry and nuclear magnetic resonance spectroscopy (Lindorff-Larsen *et al.*, 2005; Piana *et al.*, 2012; Silva-Stenico *et al.*, 2011).

1.5 Preventive measures to reduce aflatoxins contamination

Due to high economic losses as a consequence of aflatoxin contamination and the toxicity of these compounds, several approaches have been studied and applied to reduce the risks of contamination (Adda *et al.*, 2011; Udomkun *et al.*, 2017). Stopping the least infection process with host plant resistance, biocontrol strategies, pre-harvest and post-harvest management strategies including good agricultural practices, timely harvesting and proper drying of the product have all been practiced (Adda *et al.*, 2011; Udomkun *et al.*, 2017).

Aflatoxin biocontrol management is effective in consistently reducing aflatoxin contamination in a significant manner (Cotty, 2006; Mehl *et al.*, 2012; Atehnkeng *et al.*, 2014; Doster *et al.*, 2014; Atehnkeng *et al.*, 2016; Bandyopadhyay *et al.*, 2016; Udomkun *et al.*, 2017) Although several strategies have been applied to reduce pre-harvest aflatoxin contamination, biological control with atoxigenic strains of *A. flavus* is the most promising (Amaike & Keller, 2011). Atoxigenic strains can displace aflatoxin producers during crop development with a consequent reduction in aflatoxin contamination (Cotty, 2006; Atehnkeng *et al.*, 2008; Atehnkeng *et al.*, 2016).

Cotty, (2006) observed that when the spore number of atoxigenic strains in the soil is high, they may possibly compete with other strains, both aflatoxigenic and atoxigenic, for essential nutrients needed for growth. The International Institute of Tropical Agriculture (IITA) and the United States Department of Agriculture together with other partners have been investigating possible atoxigenic fungi that can be used as a biocontrol agents in mitigation of *A. flavus* for African farmers (Grace *et al.*, 2015). A country-specific indigenous aflatoxin biocontrol product was developed and generically named *Aflasafe*[™] (www.aflasafe.com), which can be used on maize and groundnut (Bandyopadhyay *et al.*, 2016). This product is an eco-friendly innovative biocontrol technology that utilizes native atoxigenic strains of *A. flavus* to naturally out-compete their aflatoxin-producing counterparts (Atehnkeng *et al.*, 2014). *Aflasafe*[™]

consistently reduces aflatoxin contamination in maize and groundnut by 80-99 % during crop development, postharvest storage, and throughout the value chain in several countries across Africa (Grace *et al.*, 2015).

1.6 Maize (*Zea mays L.*) as a staple food in sub-Saharan Africa

Maize is an important agricultural crop in sub-Saharan Africa and contamination with aflatoxins is one of the main factors compromising yield and its quality that may lead to possible health risks for consumers (Magan *et al.*, 2011). Maize grain is a good substrate for mould infection and production of potentially dangerous mycotoxins harmful to both humans and animals (Ngindu *et al.*, 1982). Some fungi are associated with grain mouldiness, but the most common are *A. flavus*, *A. parasiticus*, *Fusarium graminearum*, *F. verticillioides*, *penicillium* species and *Diplodia maydis* (Kpodo *et al.*, 2000; González-Salgado *et al.*, 2008a). The presence of mycotoxins in food is often overlooked in Africa due to public ignorance about their existence and lack of regulatory mechanisms. Furthermore, contaminated commodities are often introduced into the human food chain during food shortages that result from drought, political upheaval and economic instability. However, the majority of aflatoxin poisoning in Africa is associated with eating locally produced maize (Lewis *et al.*, 2005).

Aflatoxins have been particularly problematic in eastern and central parts of Kenya, as mentioned earlier, where there have been multiple outbreaks of aflatoxin poisoning among subsistence maize farmers (Ngindu *et al.*, 1982). Several cases of aflatoxicosis have been reported annually since 1981-2010 following consumption of maize contaminated with *A. flavus* and aflatoxins (Ngindu *et al.*, 1982; Gieseke 2004b; Cotty *et al.*, 2007; Probst *et al.*, 2012).

1.7 Research focus

1.7.1 Statement of the problem

Maize is an important agricultural crop in sub-Saharan Africa and particularly in Kenya. Contamination of maize kernels with *A. flavus* and aflatoxins is a major concern as it compromises the yield and quality of maize kernels and leads to health risks for consumers and livestock.

Makueni county has repeatedly been exposed to outbreaks of aflatoxicosis for decades due to consumption of contaminated maize kernels by *A. flavus*. Other

regions with similar climatic conditions that also produce maize (Nandi, Kisumu and Homa Bay) have no history of aflatoxicosis outbreaks. It is therefore not known if these regions are exposed to lower levels of *A. flavus* contamination or if the strains responsible for aflatoxin production are different from those found in Makueni.

1.7. 2 Specific Research questions

- i. Does the incidence and phenotypic characteristics of *A. flavus* isolates vary in different climatic regions of Kenya?
- ii. What are the Vegetative Compatibility Groups to which these *A. flavus* isolates belong too and is there a pattern in the VCG distribution?
- iii. Are selected aflatoxin biosynthetic genes present or absent in these *A. flavus* isolates?
- iv. What is the gene expression profile of selected aflatoxin biosynthetic genes under aflatoxin induced and non-induced conditions?
- v. Is there a difference in the aflatoxin and metabolite profile of selected *A. flavus* isolates?
- vi. Does the maize variety cultivated in different climatic regions of Kenya influence *A. flavus* colonisation?
- vii. Can characterised atoxigenic *A. flavus* isolates from this study suppress the growth of toxigenic strains and toxin production within maize tissue?

1.7.3 Research Aim

The aim of this study was to investigate the phenotypic and genetic variability within the *A. flavus* population, and the potential of atoxigenic strains for aflatoxin suppression.

The specific objectives were:

- i. To isolate, characterise and identify *A. flavus* from maize kernels collected from different climatic regions of Kenya
- ii. To determine Vegetative Compatibility Groups pattern within *A. flavus* strains from different climatic region of Kenya
- iii. To determine the presence of five aflatoxin biosynthetic genes in *A. flavus* isolates and how this affects aflatoxin production
- iv. To determine the metabolic profiles of *A. flavus* isolates from different climatic regions of Kenya

- v. To quantify *A. flavus* colonisation and aflatoxin accumulation in different maize varieties
- vi. To evaluate the ability of atoxigenic *A. flavus* strains to suppress growth of toxigenic strains within growing maize plants and kernels

CHAPTER TWO

Isolation and phenotypic characterisation of *Aspergillus* species

2.0 Introduction

Zea mays L. (maize) is an important agricultural crop in sub-Saharan Africa (Magan *et al.*, 2011). Maize grain is also a good substrate for mould infection and the production of potentially dangerous mycotoxins harmful to both humans and domesticated animals (Ngindu *et al.*, 1982; Okoth, 2016). The presence of mycotoxins in food is often overlooked in Africa due to public ignorance about their existence and the lack of adequate regulatory mechanisms to monitor their presence in food (Okoth, 2016). Furthermore, contaminated commodities are often introduced into the human food chain during food shortages that result from the drought, wars, or political and economic instability. However, most of aflatoxin poisoning in Africa has been associated with eating locally produced maize (Lewis *et al.*, 2005).

Although molecular methods continue to improve and have become more rapidly available for *Aspergillus* species identification, morphological methods using microscopy are still essential and have remained the most commonly used tool (McClenny, 2005). This preference was confirmed in a survey conducted by the American Society of Microbiology of laboratories performing mycological examination in which 89 % were morphological, 16 % were serological and less than 5 % used molecular tests for fungal identification (Warris, 2001). Moreover, morphological characterisation based on culturable and microscopic methods has been widely used to discriminate species in *Aspergillus* section *Flavi* (Klich, 2002; Varga *et al.*, 2011).

The defining characteristic of the genus *Aspergillus* is the aspergillum-like spore bearing structure (Klich, 2002; Klich, 2007). The structure has a conidial head and a basal “foot cell”. The foot cell bears long conidiophore (stipe) that terminate in a vesicle (spore head). The vesicle organs may have either single or double layers made of synchronised cells and asexually formed spores termed conidia. The conidia shapes may either be uniseriate or biseriate (Bennett, 2010). The size and arrangements of the conidial heads as well as the colour of the spores they bear are important identifying character (Klich, 2002; Varga *et al.*, 2011). For example, species in the *A. ochraceus* group bear yellowish to brown spores, in the *A. niger* group spores are

black, while *A. nidulans*, *A. fumigatus*, *A. parasiticus* and *A. flavus* all have green spores (Varga *et al.*, 2011; Okoth *et al.*, 2012; Nyongesa *et al.*, 2015).

In addition to conidiophores, other morphological characteristics used for identification include growth rate, thermo tolerance, colony colour, sclerotia, cleistothecia and Hulle cells (Pitt, J.I. 2007; Mitema *et al.*, 2018).

Under suitable laboratory conditions and culture media, *Aspergillus* spp. show prompt *in vitro* growth. In fact, *A. flavus*, *A. fumigatus*, *A. niger* and *A. terreus* can grow to 65-70 mm, 40-60 mm, 55-70 mm and 40-60 mm in diameter, respectively within seven days at 25 ° C on Czapek Yeast Extract Agar (Klich 2002). In the field, the fungus is present in the soil as conidia or sclerotia and in plant tissues as mycelia (Scheidegger & Payne, 2003). As an opportunistic pathogen, *A. flavus* can infect maize kernels, peanuts, tree nuts, cotton bolls and other cereals (Leger *et al.*, 2000). Additionally, *A. flavus* has a complex species morphotype that results in two groups (S and L) based on sclerotia size: L-morphotype strains have sclerotia greater than 400 µm in diameter and S-morphotype strains have sclerotia less than 400 µm in diameter (Horn, 2005).

In Kenya, maize is a staple food crop for both urban and rural areas with an estimated 1.6 million hectares under cultivation (Nyoro *et al.*, 2004). Aflatoxins have been problematic in eastern and central parts of Kenya since 1981 with cases reported in Makueni and neighbouring administrative counties following consumption of maize contaminated with *A. flavus* and aflatoxins (Ngindu *et al.*, 1982; Cotty, 1989; Giesecker, 2004a; Cotty *et al.*, 2007; Probst *et al.*, 2012). The worst outbreak occurred in 2004 with 317 incidences and 125 deaths, and the main causative morphotype of *A. flavus* was S-strain (Cotty, 1989; Cotty *et al.*, 2007; Probst *et al.*, 2012).

The S-morphotype strain is adapted to crops grown in warm environments and the strain is associated with lethal mycotoxin production (Cotty, 1989). Aflatoxicosis appears to be endemic in some Kenyan regions such as Makueni and neighbouring administrative counties, as every year cases are reported (Ngindu *et al.*, 1982; Cotty, 1989; Giesecker, 2004a; Cotty *et al.*, 2007; Probst *et al.*, 2012). Though cross-sectional and surveillance studies have been conducted in eastern Kenya (Makueni, Machakos and Kitui) and some parts of central Kenya (Thika in Kiambu county) (Ngindu *et al.*, 1982; Cotty, 1989; Giesecker, 2004a; Cotty *et al.*, 2007; Probst *et al.*,

2012), other regions with similar climatic conditions that also produce maize (Nandi, Kisumu and Homa Bay) have not been the focus of similar studies. It is not known if these regions suffer from the same level of aflatoxicosis due to maize consumption or if the strains responsible for aflatoxin production are different from those found in Makueni and neighbouring counties.

Determining the primary causal agents of aflatoxin contamination is critical for predicting the risk of contamination events and designing and implementing management system strategies. Given the relationship between the S-morphotype and aflatoxicosis outbreaks in Kenya; aflatoxin management strategies that reduce the frequency of the S-morphotype strain may be particularly effective at reducing contamination. This targeting requires information on the distribution of local *A. flavus* populations and strain morphotypes in different maize growing regions of Kenya so that findings can be compared to areas where aflatoxicosis is endemic.

The aim of this study was to determine phenotypic variation in *A. flavus* strains from maize kernels collected from different climatic regions of Kenya (Nandi, Kisumu, Homa Bay and Makueni). Specific objectives were to isolate, identify and characterise the gross morphology, anatomy (reproductive structures) of the *A. flavus* strains based on standard mycological techniques. This characterisation will enable identification of *A. flavus* strains and the corresponding morphotypes that are associated with maize grown in the region. Furthermore, identification of atoxigenic *A. flavus* strains in these regions could help develop of biocontrol strategies to mitigate the effect of toxigenic strains associated with aflatoxicosis in endemic regions.

2.1 Materials and methods

The schematic flow diagram of the protocol designed for the current research is illustrated in Fig.2.1.

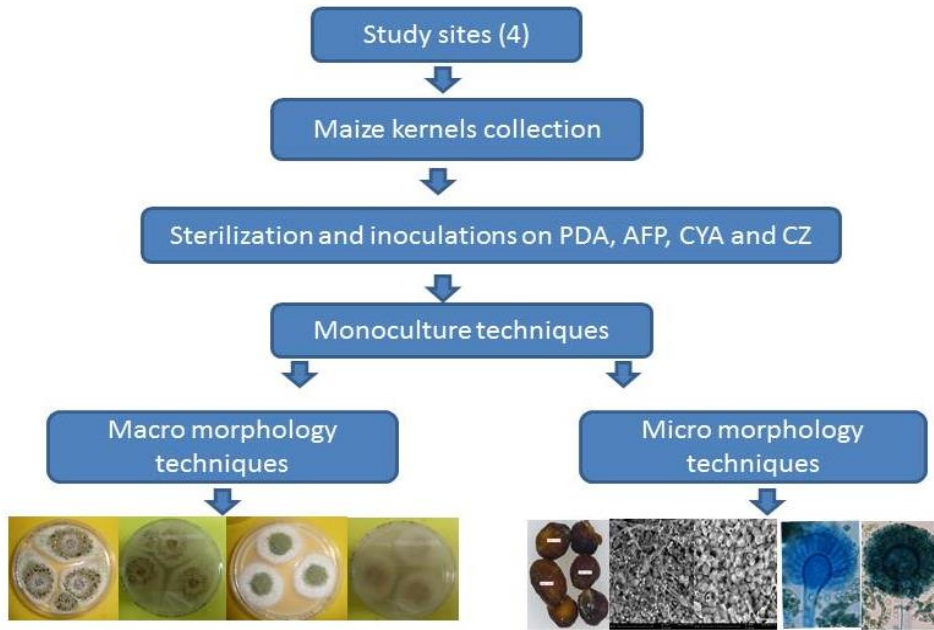


Figure 2.1 Schematic diagram showing methods employed from the field to the laboratory for isolating *Aspergillus flavus* strains from maize kernels collected from four different climatic Kenyan maize growing regions. Collected maize kernels were sterilized and placed on various *Aspergillus* growth media (PDA: potato dextrose agar; AFP: *Aspergillus flavus parasiticus* agar; CYA; Czapek yeast extract agar; CZ: Czapek dox agar). *Aspergillus flavus* strains were subsequently identified using macro- and micro-morphology techniques.

2.1.1 Study area

Collections were in November and December 2013, in four different climatic regions of Kenya that fall within four administrative counties; Makueni, Nandi, Homa Bay and Kisumu (Fig.2.2).

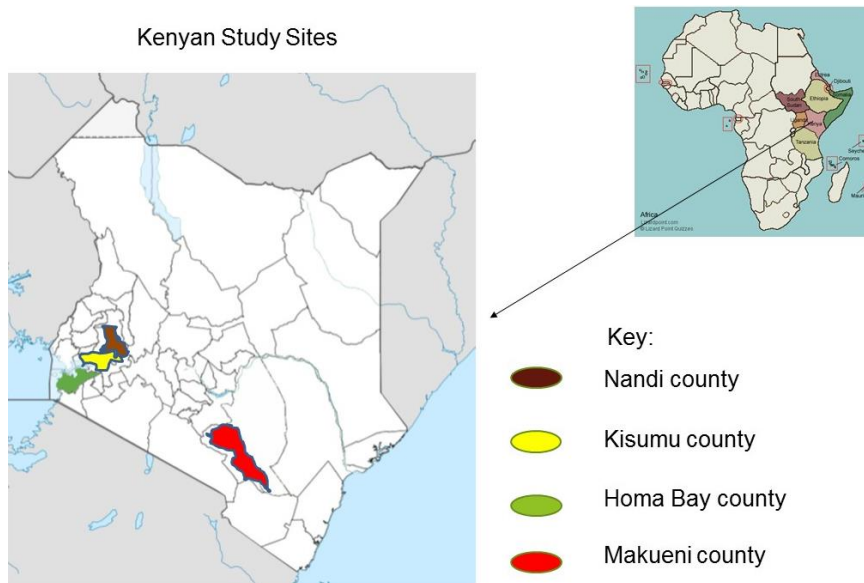


Figure 2.2 Map of Kenya showing four administrative counties in different climatic regions sampled out of 47; Makueni, Nandi, Homa Bay and Kisumu. The specific villages in the different counties from which maize kernels were collected for *Aspergillus flavus* isolation are mentioned in section 2.2: Table 2.1 for context and Fig.2.3.

Among the four administrative counties, Makueni is located in a drought-prone semi-arid zone of the former eastern province of Kenya at an elevation of between 800-1700 m above sea level (Fig.2.3 and Section 2.2: Table 2.1).

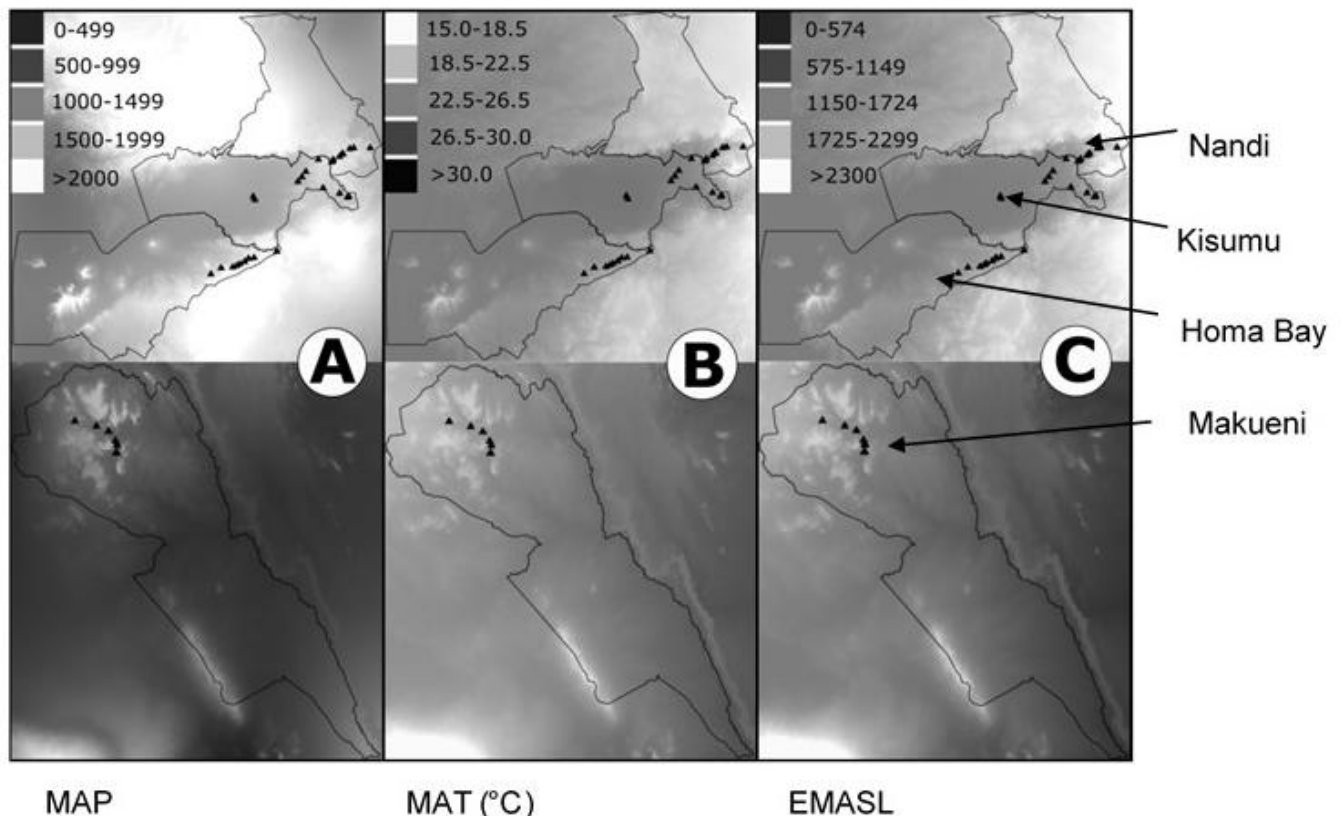


Figure 2.3 Map of Makueni, Nandi, Homa Bay and Kisumu, and GPS location of the sites in small black dots/triangle. [**A=MAP** (mean annual precipitation (mm)); **B=MAT** (mean annual temperature (° C)); **C=EMASL** (elevation mean above sea level (**m.a.s.l.**))].

Additionally, Makueni has an annual rainfall between 300-600 mm and mean temperature of 24 ° C (FAO, 2004; Lewis *et al.*, 2005). The county has two maize planting seasons, from March to May and from October to December where the weather pattern is characterized by extreme wet and hot conditions (FAO, 2004).

Nandi county is located in the former Rift Valley province of Kenya. Rainfall months extend from March to June, in which lengthy, heavy rains occur while the short rainfall months are from September to November (FAO, 2004). The average temperature is 20 ° C, with the highest temperature recorded in December and

January (23 ° C) and the lowest, 12 ° C, occurred in July and August (Fig.2.3; Section 2.2: Table 2.1).

Homa Bay and Kisumu counties are in the former Nyanza province of Kenya. Homa Bay county has an average temperature range of 21-35 ° C. Kisumu county has an annual relief rainfall of between 1200-1300 mm and a mean annual temperature 23-35 ° C. Nandi, Kisumu and Homa Bay counties have only one planting season from February to April (Fig.2.3; Section 2.2: Table 2.1).

2.1.2 Sampling

A randomised sample collection method was carried out between November and December 2013. The sites were identified based on maize cultivation and the diagonal transect method was applied to sample approximately eighty households according to the procedures described by Clark & Steel, (2007) and Corsi *et al.* (2012). The collected samples from each household within the proximity of each village were pooled to make a total of forty samples. Identified participants were compensated at the current market rates in Kenyan shillings. The household member(s) was informally interviewed by using a basic questionnaire as a template (Supplementary data, S1) to gauge their knowledge on fungal infection of maize and their maize yields. In the Nandi region, most households had a stock of stored maize kernels, whereas in Makueni, Homa Bay and Kisumu, the households lacked maize in their stores, granaries or fields due to poor harvest in these regions (source: interviewed local participants). The samples in these regions were not obtained by using the diagonal transect method. Instead, the search area was enlarged in these regions to obtain the same number of samples as in Nandi region.

Maize in storage facilities was collected by piercing the maize sacks at three different locations from which the maize kernels were drawn. Approximately 200-300 g of shelled maize kernels, or 2-3 maize cobs, were collected per household, stored in sterile brown paper bags and sealed in sterile zipped plastic polythene bags. Maize kernel samples were stored at 4 ° C in a coolant container before transport to the Mycology Laboratory at the University of Nairobi, Kenya for further analysis.

2.1.3 Chemicals and reagents

Potato dextrose agar (PDA), yeast extract, sodium chloride, ammonium acetate and tryptone were from Merck chemicals (USA). Lactophenol cotton blue stain, mycological peptone, malt extract agar (MEA), agar, water agar, ethanol, sucrose, Whatman No. 1 filter paper and sodium hypochlorite were from Sigma-Aldrich (USA). Sterile water was produced by a Milli Q water purification system (Millipore LTD, Bedford, MA, USA). All chemicals were of molecular biology grade and were used without further purification.

2.1.4 Cultivation and monoculture techniques

Four to five kernels were surface sterilized for 1 min in 2.5 % NaOCl, washed three times with water and dried on a disc of sterile Whatman No. 1 filter paper according to the methods described by Okoth *et al.* (2012). The kernels were plated on quarter strength PDA and incubated at 30 ° C for 3 days. Kernels observed with fungal growth, in different shades of green, yellowish brown, black or cottony white, were transferred onto full strength PDA plates to obtain pure colonies (Okoth *et al.*, 2012). The pure colonies were single-spored, cultured in water agar and incubated at 25 ° C for 24 hrs. Single growing mycelia were sub-cultured onto fresh PDA plates.

2.1.5 Morphological identification

Isolates were inoculated onto different media: Czapek yeast extract agar (CYA), Czapek dox agar (CZA), MEA, PDA and incubated for 7 days at 25 ° C based on methods of Klich (2002) with the slight modifications. Briefly, 2 µl suspensions of spores (1×10^6 conidia/ml) in water were dispensed at three points on the plates. Isolates were characterised based on their morphological characters, such as colony surface and reverse colours, presence or absence of sclerotia, colour of sclerotia, and growth diameter. Colours of the colony and sclerotia were assessed visually and described based on Ridgway's Colour Standards and Colour Nomenclature (Pitt, 2000). Microscopic examination were made with a Nikon stereomicroscope with bright field, phase contrast and Differential Interference Contrast (DIC) optics mounted with a Nikon camera for photomicrography (Nikon Corp., Japan). Microscope mounts were made in lactophenol cotton blue from colonies grown on CYA.

2.1.6 Production of sclerotia and identification

Plates containing CZA were inoculated by fungal mycelia from seven-day old cultures grown on MEA. The CZA plates were incubated in the dark at 30 ° C for 3 weeks. Sclerotia were harvested by pouring 10 ml of 0.01 % Tween 20 per plate and scraping the surface of the culture plates (3 replicate plates per isolate) over a disc of sterile Whatman No. 1 filter paper. Sclerotia were cleaned in a beaker with repeated rinses and then air-dried in a desiccator housed within a biosafety cabinet [Contained Air Solutions (CAS) BioMAT2, UK]. Individual strains that did not produce sclerotia on CZA agar were sub-cultured on CZA medium with 0.5 % sodium nitrate and 0.5 % sucrose at 30 ° C for 3 weeks and inspected for sclerotia formation visually. The isolates were further categorized as S or L-morphotypes based on the diameter of the sclerotia.

2.2 Results and Discussion

2.2.1 Samples, storage facilities and drying methods

Maize is an economically important subsistence and cash crop grown in the climatic regions selected for this study. Across these regions, farmers used different methods of harvesting, drying, storing and retailing their grain (Figs.2.4; 2.5). Pathogenic fungi such as *A. flavus*, can attack kernels in the field before harvest or post-harvest during storage (Shapiro *et al.*, 2004). Severe infections by *A. flavus* may cause huge economic losses, reduce crop yield and kernel quality, and hinder inter-trade activities and, critically degrade the health of humans and domestic animals.



Figure 2.4 Local maize on sale to consumers in Meteitei market, in Nandi county (a-b), maize in stacks on a farm near Ringa village, Homa bay county (c), maize cobs drying in the fields in Bongu Kachieng village, Homa Bay county with red arrow indicating green *Aspergillus* species growing on kernel surface (d).

Stacking maize (Fig.2.4c) could enhance fungal growth when the weather is favourable. The farmers interviewed indicated that stacks are left in the field for days or weeks before removing the cobs and sometimes the kernels are left on the ground. This practice often leads to the kernels being soiled and becoming susceptible to infection by fungal spores from the soil or the air.

Proper storage methods are essential to limit *A. flavus* contamination. Harvested kernels may contain mycelia or spores from the pathogenic fungi that are acquired in the field and then taken into the storage facilities. If proper precautions are not taken during harvesting to limit fungal contamination, this transfer may lead to the spread of fungal infection in a poorly constructed and ventilated storage facilities (Fig.2.5).



Figure 2.5 A farmer in Malooi village in Makueni county inspecting a homemade granary (a) in which maize is stored in sacks (b).

Shapiro *et al.* (2004), observed that certain fungi invade kernels during storage or in the field is based on environmental conditions, including growth conditions, intergranular air, temperature, dockage, water vapour and structure of the storage facility. Poor storage conditions can eventually lead to mouldy substrate (maize kernels).

Approximately 70 % of the households in the Makueni, Homa Bay and Kisumu regions lacked maize in their stores, granaries or fields due to poor harvest condition. Thus, the collection area was expanded to obtain the same number of samples as in Nandi county. The surveyed sites with associated temperature, rainfall, GPS coordinates and maize line grown (Table 2.1).

The mean temperature does not vary considerably across the four counties (Fig.2.3; Table 2.1), however there was a difference in rainfall between Makueni and the other three counties with Makueni receiving the least rainfall.

Table 2.1 Surveyed sites in each location of the administrative county showing specific Global Positioning System, annual temperature, rainfall and maize lines (cultivar). (NC-Nandi, KSM-Kisumu, HB-Homa Bay, and MC-Makueni) respectively.

County	Sampling site	Latitudes and longitudes	Isolate code	Temp. (° C)	Rainfall	Cultivar
Nandi	Labuywa (LBU)	0°00'31.9"N 35°19'18.1"E	NCLBU001	15-25 ° C	1200-2000 mm	H614, GAF 4 (Highland variety)
	Chebibi (CHB)	0°02'42.4"S 35°14'05.3"E	NCCHB002			
	Tinderet (TD)	0°01'06.9"S 35°16'34.4"E	NCTD003			
	Senetwo 1(STW)	0°02'54.2"S 35°13'52.3"E	NCSTW004			
	Timboroa (TBA)	0°01'02.5"S 35°16'30.1"E	NCTBA005			
	Kabargatuny (KBY)	0°02'18.7"S 35°14'26.0"E	NCKBY006			
	Maraba (MRB)	0°00'19.6"N 35°18'30.2"E	NCMRB007			
	Senetwo 2 (STW)	0°00'54.1"S 35°16'35.4"E	NCST008			
	Owiro (OWR)	0°01'33.1"S 35°15'56.0"E	NCOWR009			
	Meteitei (MTT)	0°00'25.4"N 35°23'28.9"E	NCMTT010			
Kisumu	Koru (KRU)	0°10'45.0"S 35°15'56.5"E	KSMKRU011	23-35 ° C	1200-1300 mm	KDV1, KDV2, GAF 4 and KH500 (Dryland, Medium and Low altitude varieties)
	Kowawa (KOW)	0°07'54.7"S 35°05'32.5"E	KSMKOW012			
	Sauset (SAT)	0°11'34.9"S 35°18'10.9"E	KSMSAT013			
	Kambi Awendo (KAB)	0°11'46.8"S 35°17'51.7"E	KSMKAB014			
	Office Ngeny (OFN)	0°06'40.9"S 35°06'39.0"E	KSMOFN015			
	Ahero (AHR)	0°11'29.0"S 34°54'35.8"E	KSMAHR016			
	Chemelil Round (CHRA)	0°05'19.5"S 35°07'34.4"E	KSMCHRA017			
	Ojere (OJR)	0°12'15.3"S 34°54'51.5"E	KSMOJR018			
	Muhoroni (MHN)	0°09'17.8"S 35°11'53.0"E	KSMMHN019			
	Kopere (KPR)	0°02'25.5"S 35°10'43.4"E	KSMKPR020			
Homa Bay	Bongu Kachieng (BOK)	0°27'32.8"S 34°52'29.3"E	HBOK021	26-34 ° C	250-1300 mm	KDV1, KDV2 and GAF 4 (Dryland, Medium and Low altitude varieties)
	Ringa 2 (NRG)	0°28'33.1"S 34°50'30.2"E	HBNRG022			
	Mikai Kakelo (MIK)	0°26'34.5"S 34°54'47.9"E	HBMIK023			
	Ringa 1(RNG)	0°28'13.2"S 34°51'12.7"E	HBRNG024			
	Chabera (CBR)	0°25'03.5"S 35°00'24.1"E	HBCBR025			
	Kadongo 2 (KDG)	0°26'50.4"S 34°53'27.9"	HBKDG026			
	Kadongo 1 (KDG)	0°26'50.4"S 34°53'27.9"	HBKDG027			
	Oyugis (OYG)	0°30'37.6"S 34°44'06.3"E	HBOYG028			
	Konditi (KDT)	0°29'08.2"S 34°46'38.3"E	HBKDT029			
	Kochieng Location (KOL)	0°28'59.7"S 34°49'35.0"E	HBKOL030			
Makueni	St. Lawrence (LAW)	1°48'08.4"S 37°31'53.3"E	MCSt.LAW031	23-27 ° C	300-834 mm	KDV1, KDV2 (Open pollinated Dryland varieties)
	Mukuyuni (MKY)	1°44'53.2"S 37°27'38.3"E	MCMKY032			
	Nziu 2 (2)	1°49'08.5"S 37°32'11.9"E	MCNZU'2'033			
	Nziu 1 (NZU)	1°49'04.1"S 37°32'13.7"E	MCNZU'1'034			
	Ukia (UKA)	1°45'53.0"S 37°30'15.8"E	MCUKA035			
	Kivani (KVN)	1°43'36.3"S 37°22'43.2"E	MCKVN036			
	Malooi 2009 (MAL)	1°50'46.1"S 37°32'03.1"E	MCMAL'09'037			
	Malooi 2011 (MAL)	1°50'46.1"S 37°32'02.9"E	MCMAL'11'038			
	Malooi 2012 (MAL)	1°50'46.1"S 37°32'03.1"E	MCMAL'12'039			
	Malooi 2013 (MAL)	1°50'46.1"S 37°32'03.1"E	MCMAL'13'040			

2.2.2 Characterisation of fungal isolates

Three sets of biological replicates were used to determine the statistical mean average. Five kernels were used from each site (15 kernels). The study revealed fungal growth incidence on maize kernels (Fig.2.6) with variation in *Aspergillus* strains recovered.

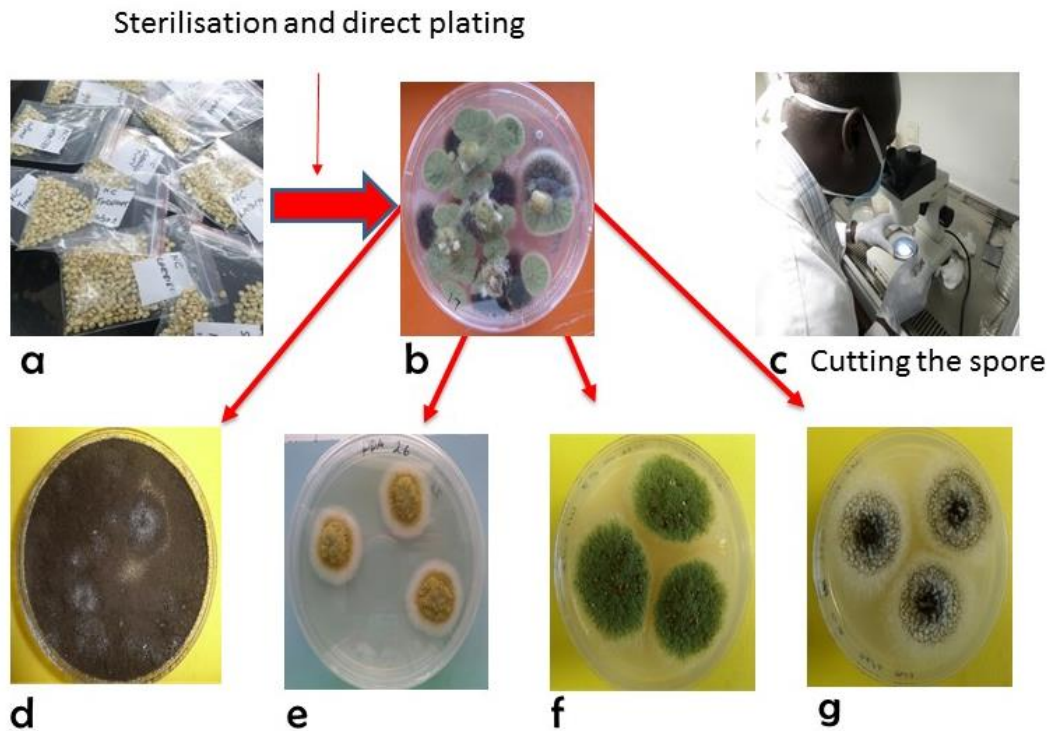


Figure 2.6 Schematic flow diagram illustrating how pure colonies from *Aspergillus* section *Flavi* isolates from maize kernels isolated from four different climatic regions of Kenya were obtained: (a) maize kernels in zipped plastic bags; (b) fungal growth on maize kernels on Petri plate; (c) monoculture technique and, (d, e, f and g) isolated pure colonies, respectively.

Thirty-seven mono-spore sub-cultures of *A. flavus* strains isolated from maize kernels were obtained and each isolate was assigned a code corresponding to the specific region of collection (Table 2.1). Macromorphological characteristics (colony diameter, colony colour, sclerotia, exudates) and micromorphology (conidia and vesicle shapes) characteristics of cultures observed on plates were used to identify isolates based on the keys of Klich (2002) and Nyongesa *et al.* (2015). The findings were consistent with those of Klich (2002); Geiser *et al.* (2007); Okoth *et al.* (2012) and Nyongesa *et al.* (2015) who all used pure cultures grown on known media at different temperatures and recognised several sub-groups within *A. flavus*. *A. flavus*

isolates displayed different phenotypic and morphological features depending on the growth media CYA and CZA respectively (Fig.2.7).

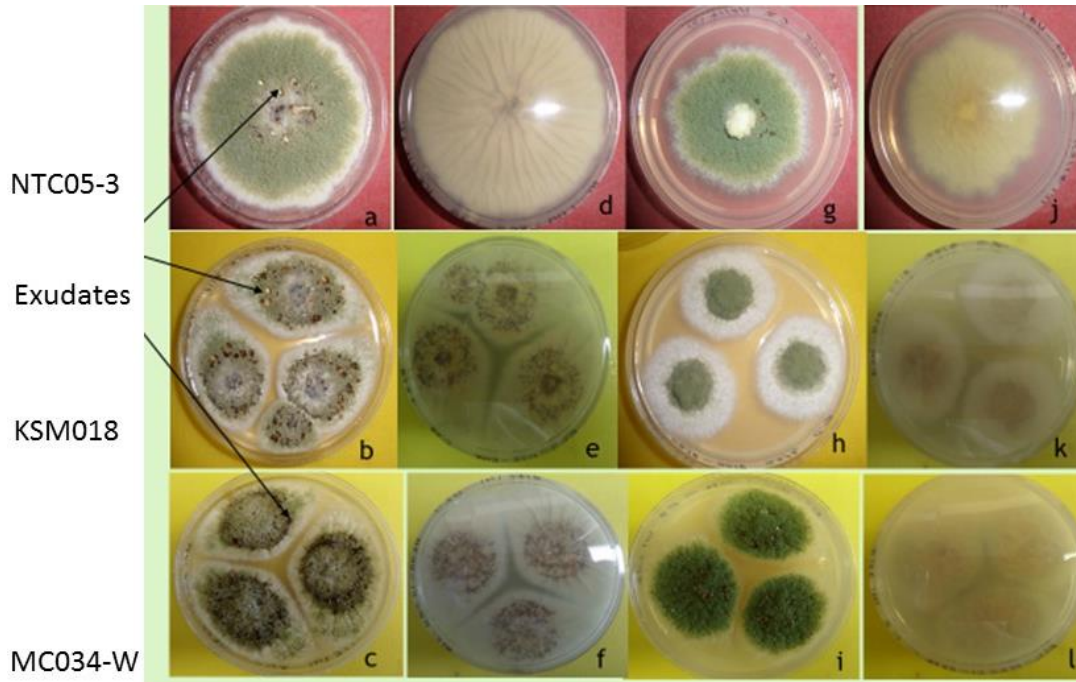


Figure 2.7 Macro-morphological characteristics for three of the 37 *Aspergillus flavus* strains isolated from maize kernels from four different climatic regions of Kenya. *Aspergillus flavus* isolates (NTC05-3, KSM018, MC034-W) were incubated at 25 ° C for 7 days. The characteristics observed on Czapek Yeast Extract Agar (colony **a-c**; reverse plate **d-f**) and Czapek Dox Agar (colony **g-i**; reverse plate **j-l**) media respectively.

Additionally, vesicle shapes ranging from radiate, globose/spherical, spathulate and pyriform were observed (Fig.2.8a). Moreover, some isolates produced unique sclerotia and different ultra-structural features (Fig.2.8b, c). Rough and smooth conidia also were produced by some isolates (Fig.2.8d). The micro-morphological characters observed were predominantly uniseriate to biseriate phialides with a smooth, fine to rough surface (Fig.2.8a).

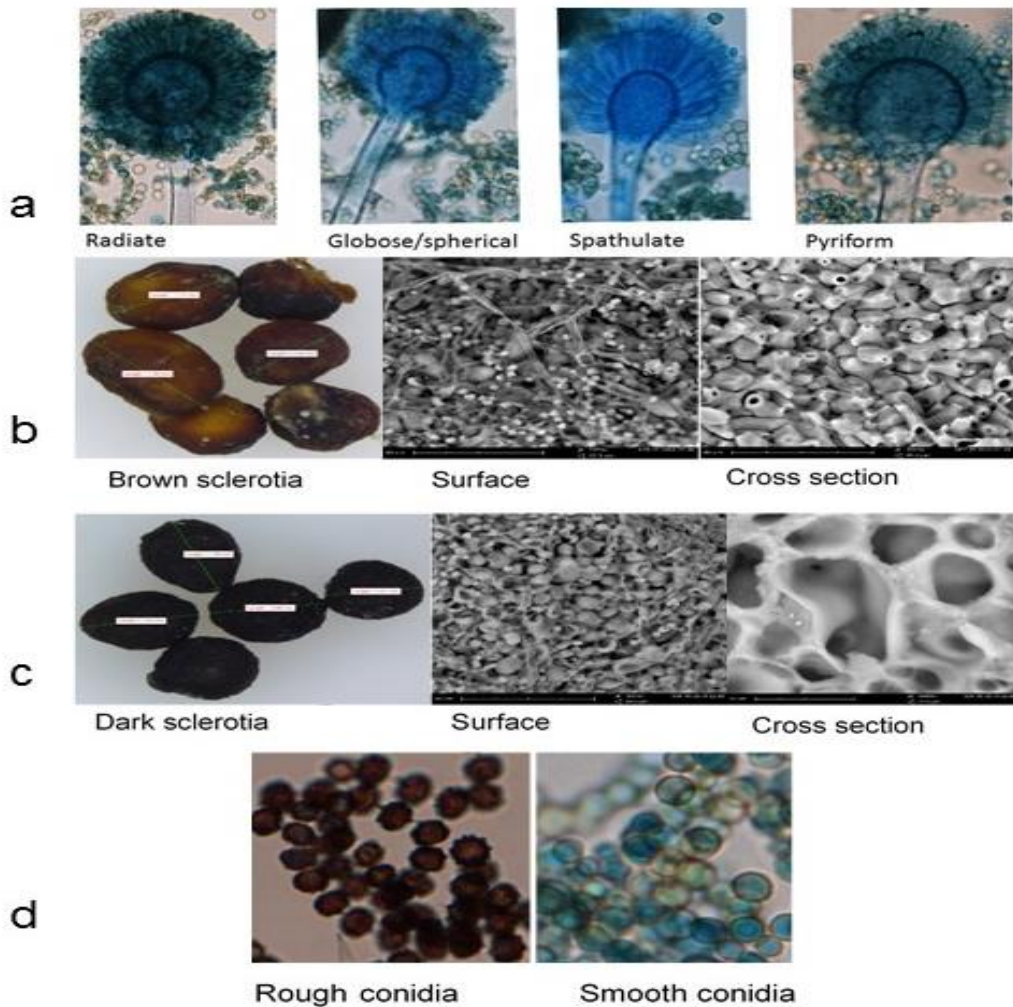


Figure 2.8 Micro-morphological characteristics for six of the 37 *Aspergillus flavus* isolates sampled from the four different climatic regions of Kenya. *Aspergillus flavus* isolates were incubated at 25 ° C for 7 days. The observations on Czepak Yeast Extract Agar media include: vesicle shapes (a); sclerotia (b-c) illustrating scanning electron micrographs of sclerotia surface and cross-section; conidia shapes (d) respectively (scale bar 10 µm and SEM X3000).

The growth rate of *A. flavus* mycelia and colony diameter was measured every second day on CYA and CZA. The experiment was performed in two biologicals per isolate and three technical replicates which exhibited colony diameters (Fig.2.9; Table 2.2). Rapid growth rate was observed on CYA and colony diameter ranged from 20-70 mm for CYA and from 15-60 mm for CZA media respectively (Fig.2.9; Table 2.2). The contemporary findings were consistent with those of Klich, (2002); Okoth *et al.* (2012) and Nyongesa *et al.* (2015) who observed different growth rates and colony diameters for various species of *Aspergillus*. Klich, (2002), observed *A. flavus*, *A. fumigatus*, *A. niger* and *A. terreus* colonies with growth diameters between 65-70 mm, 40-60 mm, 55-70 mm and 40-60 mm respectively within seven days at 25 ° C on CYA

media. Similarly, Araujo & Rodrigues (2004), found that germination rates at 37 ° C of some *Aspergillus* spp. varied significantly for the most common pathogenic species, while species with lower pathogenicity potential had an optimal temperature for growth around 30 ° C (Gock *et al.*, 2003; Torres *et al.*, 2003). Interestingly, isolate NC03 grew better on CZA than on CYA (Fig.2.9a) although the explanation for this result is not known, CYA media could be suitable for cultivation of the *A. flavus* isolates and may contain all of the necessary nutrients required for optimal growth.

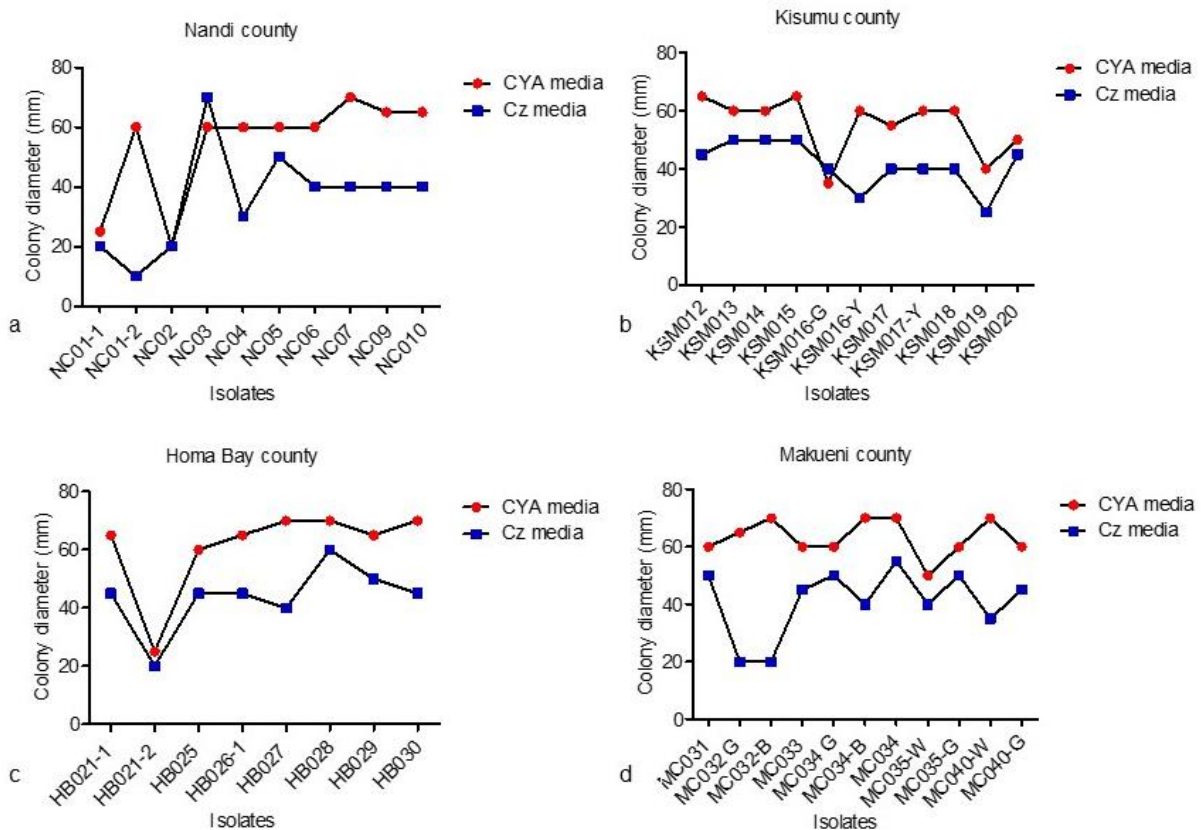


Figure 2.9 Growth rate (colony diameter, mm) for *Aspergillus flavus* strains isolated from four different climatic regions of Kenya cultured on Czapak Yeast Agar and Czapak Dox Agar media, for 7 days at 25 ° C. The isolates were obtained from: (a) Nandi; (b) Kisumu; (c) Homa Bay and (d) Makueni counties.

Samples from Makueni, Homa Bay and Kisumu regions had high levels of fungal contamination (Fig.2.10). Based on macro- and micro-morphological characters, 81 % of the isolates sampled across these four counties were from the genus *Aspergillus*. Of these isolates determined to be Aspergilli, 59 % exhibited characters associated with species such as *A. flavus*, 13 % exhibited characters of *A. niger*. Maize kernels from three regions (Kisumu, Homa Bay and Makueni) had higher

incidences of *Aspergillus* growth (64 %, 57 % and 71 %) respectively (Fig.2.10), while Nandi county had the lowest incidence (44 %).

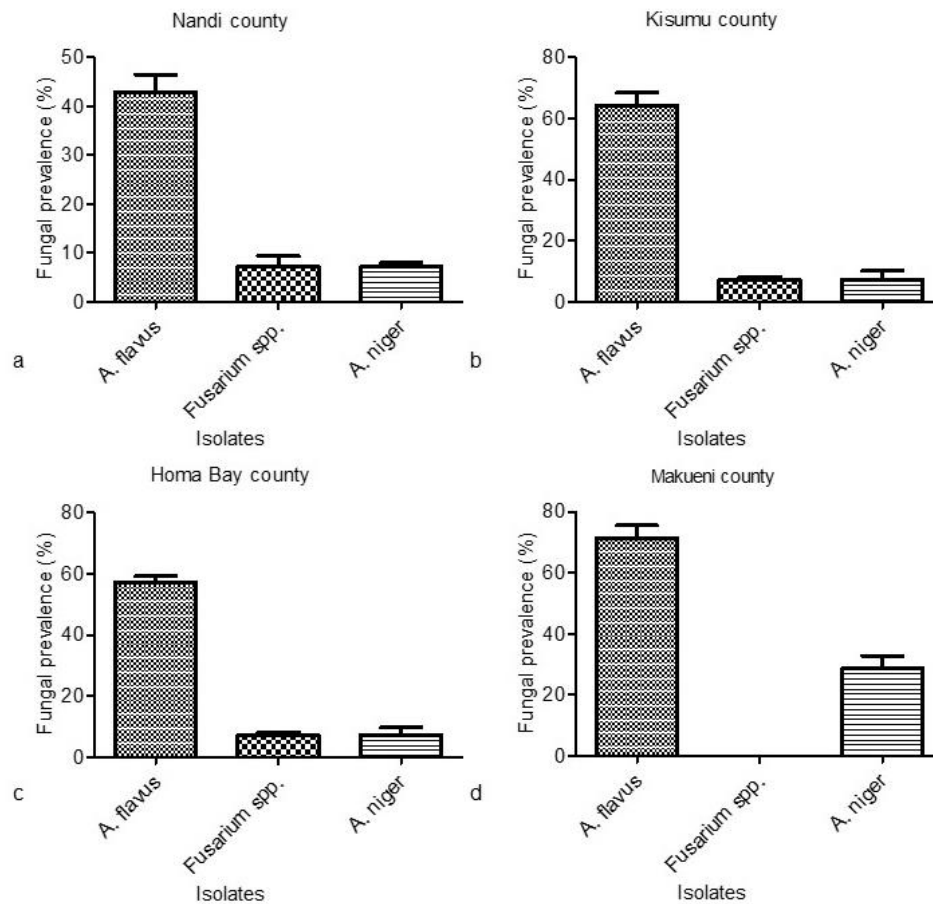


Figure 2.10 The incidence of fungal contamination of maize kernels collected from four different climatic regions of Kenya (a-d) showing the percentage fungal prevalence (*A. flavus*: *Aspergillus flavus*; *A. niger*: *Aspergillus niger*, *Fusarium spp.*: *Fusarium* species.) on maize kernels after 3-7 days upon incubation on potato dextrose agar at 30 ° C. Error bars show standard mean deviations ($n = 3$).

The frequency of *A. flavus* on maize kernels from the Nandi region was the lowest followed by Homa Bay (Fig.2.10a, c). The kernels from Kisumu and Makueni had higher incidence of *A. flavus* strains with the latter exhibiting the highest (Fig.2.10b, d). Probst *et al.* (2010) reported similar results when culture-based methods were used to monitor and describe the population structure of aflatoxin-producing fungi from maize cultivated in eastern Kenya, where Makueni county is located. Data on macro- and micro-morphology of the respective *Aspergillus* types recovered are given in the supplementary data (Figs.S2.1-S2.43).

The distribution of the sclerotia morphotypes across the four different climatic regions were 57 % for the L-type strain ($n = 21$), 7 % for the S-type ($n = 3$) and 36 %

for the S/L-type ($n = 13$) (Fig.2.11). Makueni was the only region whose *A. flavus* population included both S and L-morphotypes, whereas isolates from Nandi, Kisumu and Homa Bay regions lacked strains with the S-type morphotype (Fig.2.11d).

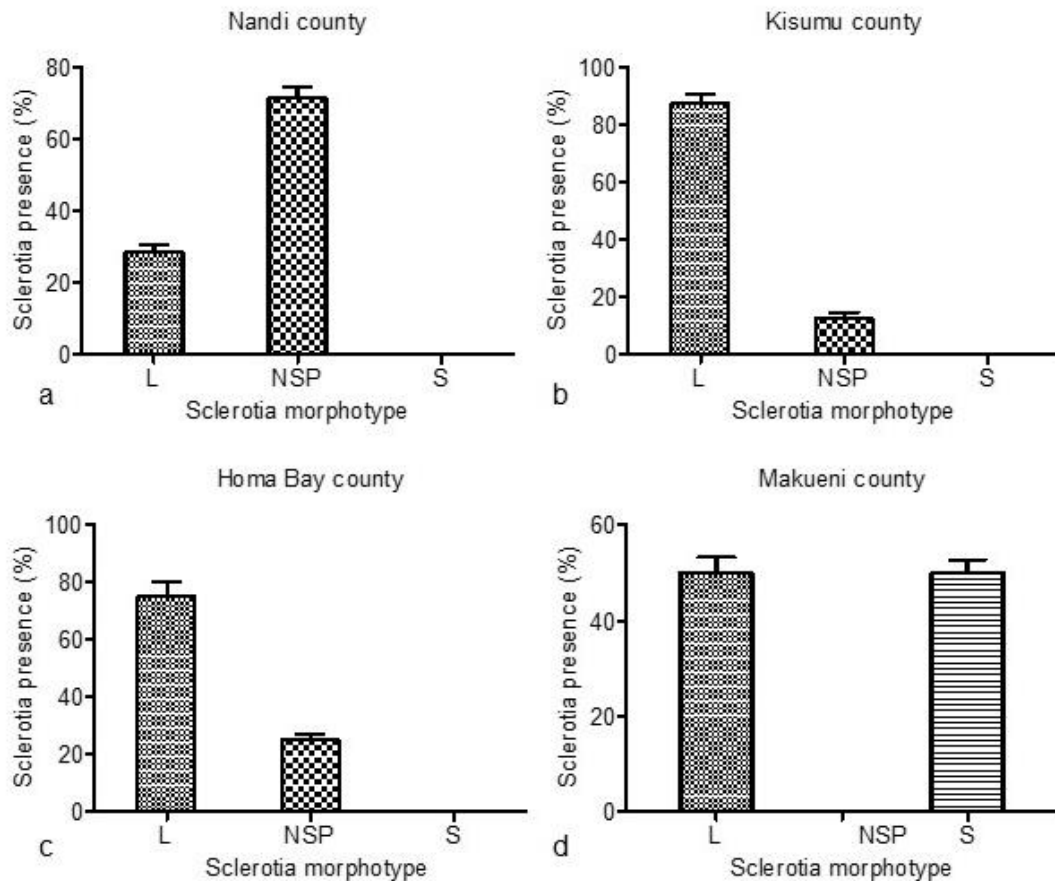


Figure 2.11 Incidence of sclerotia morphotypes in different four climatic regions of Kenya (a-d) showing the percentage distribution of the S and L-morphotypes of sclerotia for *Aspergillus flavus* per region, after 14 days of incubation on Czapek yeast extract agar media at 30 ° C. (L: large sclerotia; NSP: none sclerotia producer; S: small sclerotia).

S-morphotype (sclerotia $\varnothing < 400\mu\text{m}$) strain associated with high levels of mycotoxin production (Cotty, 1989). Related studies (Probst *et al.*, 2007; Probst *et al.*, 2010; Probst *et al.*, 2012) also identified S-morphotype strains as the most highly aflatoxigenic. S-morphotype strains have been postulated to be responsible for the human aflatoxicosis incidences previously reported in Kenya. L-morphotype (sclerotia $\varnothing > 400\mu\text{m}$) strains dominated Kisumu, Homa Bay and Nandi regions, and are known to produce little or no aflatoxins (Cotty, 1989; Probst *et al.*, 2007; Probst *et al.*, 2010; Probst *et al.*, 2012). Strains producing no sclerotia were most common in Nandi, followed by Homa Bay and Kisumu respectively. The dominance of L-morphotype

strains and lack of S-morphotype strains could be one reason why there have been no reports of human aflatoxicosis in Nandi, Kisumu and Homa Bay.

The Makueni region experiences periodic outbreaks of aflatoxin poisoning during bumper harvests. The presence of S-morphotype strains in Makueni increases the risk of aflatoxin poisoning in this region. S-morphotype isolates produce numerous small sclerotia and relatively large quantities of conidia (Probst *et al.*, 2012). Makueni suffers intermittent prolonged droughts and has two maize planting seasons, from March to May and from October to December. The weather pattern in Makueni is characterised by extreme wet and dry conditions (Probst *et al.*, 2007; Probst *et al.*, 2010; Probst *et al.*, 2012) which create ideal conditions for the growth and proliferation of *Aspergillus* propagules. Thus, climate change has the potential to alter both the incidence and the severity of aflatoxin contamination events (Probst *et al.*, 2007; Jaime-Garcia & Cotty, 2010). Contamination by *A. flavus* is favoured by hot and dry climates where hot climates lead to thriving of higher densities of *A. flavus* and incidences of the S-morphotype strain (Jaime-Garcia & Cotty, 2006).

Previously, Cotty *et al.* (2007) found that S and L-morphotype strains have different **environmental adaptations** with the S-type strain better adapted to crops grown in warm environments. Repeated and severe aflatoxin contamination in the eastern parts of Kenya is due in part to high incidences of isolates with S-type strain morphology (Probst *et al.*, 2007; Probst *et al.*, 2010; Probst *et al.*, 2012). Thus, Jaime-Garcia & Cotty, (2010) suggested that as temperature influences the frequency of these aflatoxin producing fungi, food safety and human health could be threatened by warmer temperatures resulting from climate change. Determining the primary causal agents of aflatoxin contamination is critical for predicting the risk of contamination events, and for designing and implementing management strategies. Thus, aflatoxin management strategies that reduce frequencies of the S-morphotype strain may be particularly effective at reducing contamination.

Table 2.2 Summary of phenotypic characteristics of *Aspergillus flavus* strains isolated from maize kernels collected from four different climatic regions of Kenya. The identification was based on macro- and micro-morphological characteristics.

County	Isolate	Colony colour	Phialides	Reverse colour	Ø (mm) CYA	Ø (mm) CZA	Exudates	Conidia shape	Vesicle shape
Nandi	NC001-1	Torque green	none	Brown orange	25-25	15-20	Colourless	none	none
Nandi	NC001-2	Dull green	none	Dull yellow	55-60	20-25	Colourless	none	none
Nandi	NC002	Torque green	none	Brown yellow	20-25	20-25	Colourless	none	none
Nandi	NC003	White purple	none	Dull yellow	65-70	55-60	Colourless	none	none
Nandi	NC004	Light orange	uniseriate	Dull yellow	55-60	25-30	Colourless	rough surface	globose/spherical
Nandi	NC005-2	Dull green	none	Dull yellow	55-60	45-50	Brown yellow	none	none
Nandi	NC005-3	Dull yellow	uniseriate	Dull yellow	70-80	45-55	Green yellow	smooth surface	globose/spherical
Nandi	NC006	Light brown	none	Dull yellow	55-60	35-40	none	none	none
Nandi	NC007	Dull green	biseriate	Dull yellow	60-70	45-50	Colourless	smooth surface	globose/spherical
Nandi	NC009-1	Dull brown	biseriate	Dark tan	45-65	35-40	Brown	s rough surface	globose/spherical
Nandi	NC009-2	Torque green	none	Brown green	25-30	40-45	none	none	none
Nandi	NC010	Dull brown	uniseriate	Dark tan	55-65	35-40	Brown	none	pyriform/globose
Kisumu	KSM012	Dull green	uniseriate	Dull yellow	55-65	40-45	Pink brown	smooth surface	globose/spherical
Kisumu	KSM013	Dull green	uniseriate	Dull yellow	55-60	45-50	Colourless	smooth surface	pyriform/globose
Kisumu	KSM014	Brown green	uniseriate	Brown yellow	55-60	45-50	Brown	smooth surface	globose/spherical
Kisumu	KSM015	Dull yellow	uniseriate	Dull yellow	60-65	45-50	Green brown	smooth surface	spathulate/pyriform
Kisumu	KSM016-1	Dull green	none	Brown yellow	30-35	35-40	colourless	none	none
Kisumu	KSM016-2	Dull green	uniseriate	Brown yellow	55-60	25-30	Colourless	smooth surface	spathulate/pyriform
Kisumu	KSM017	Brown green	uniseriate	Brown yellow	50-55	35-40	Pink brown	smooth surface	globose/spherical
Kisumu	KSM017-1	Brown green	uniseriate	Brown yellow	55-60	35-40	Pink brown	smooth surface	globose/spherical
Kisumu	KSM017-2	Blue green	none	Brown yellow	20-25	20-25	none	none	none
Kisumu	KSM018	Brown green	uniseriate	Brown yellow	55-60	35-40	Pink brown	fine rough	pyriform/globose
Kisumu	KSM019	Torque green	none	Brown yellow	35-40	20-25	Light green	smooth surface	none
Kisumu	KSM020	Dull green	uniseriate	Brown yellow	45-50	35-45	Brown yellow	fine rough	pyriform/globose
Homa Bay	HB021-1	Dull green	uniseriate	Dull yellow	55-65	40-45	Colourless	fine rough	globose/spherical
Homa Bay	HB021-2	Torque green	none	Green yellow	20-25	15-20	Colourless	none	none
Homa Bay	HB025	Dull green	uniseriate	Brown yellow	55-60	40-45	Brown yellow	fine rough	globose/spherical
Homa Bay	HB026-1	Brown green	uniseriate	Dark tan	60-65	20-45	Brown yellow	fine rough	pyriform/globose
Homa Bay	HB027	Brown green	uniseriate	Brown yellow	60-70	35-40	pink brown	rough surface	globose/spherical
Homa Bay	HB028-1	Brown green	biseriate	Dull yellow	65-70	55-60	Brown yellow	fine rough	pyriform/globose
Homa Bay	HB028-2	Brown green	none	Brown yellow	65-70	55-60	Brown yellow	none	none
Homa Bay	HB029-2	Brown white	uniseriate	Dark tan	60-65	45-50	Brown yellow	smooth	spathulate/pyriform
Homa Bay	HB030	Dull green	biseriate	Dull yellow	55-70	40-45	Colourless	smooth	pyriform/globose
Makueni	MC031	Dull green	biseriate	Dull yellow	55-60	45-50	Colourless	smooth	globose/spherical
Makueni	MC032-1	Brown green	uniseriate	Brown yellow	60-65	15-20	Pink brown	smooth	pyriform/globose
Makueni	MC032	Brown green	uniseriate	Dull yellow	65-70	15-20	Pink brown	smooth	globose/spherical
Makueni	MC033	Cotton white	biseriate	Dull yellow	55-60	35-45	Colourless	smooth	pyriform/spathulate
Makueni	MC034-1	Dull green	none	Dull yellow	55-60	40-50	Colourless	smooth	none
Makueni	MC034-2	Cotton white	biseriate	Dull yellow	65-70	35-40	Colourless	rough	globose/spherical
Makueni	MC034-3	Dull green	uniseriate	Brown white	65-70	50-55	Pink brown	smooth	globose/spherical
Makueni	MC035-1	Torque green	none	Pink red	45-50	35-40	Light green	smooth	none
Makueni	MC035-2	Cotton white	uniseriate	Dull yellow	55-60	45-50	Colourless	smooth	pyriform
Makueni	MC040-1	Grey purple	none	Yellow	60-70	30-35	Yellow green	none	none
Makueni	MC040-2	Dull green	uniseriate	Dull yellow	55-60	40-45	Colourless	smooth	pyriform

N/B: CYA-Czapek Yeast Extract Agar; CZA-Czapek-Dox medium; UV-light at 360nm; S-small and L-large sclerotia

2.3 Conclusion

Maize samples from Makueni had the highest frequency of *A. flavus* sub-groups and was the only region where both S- and L-morphotypes were found. Identification of *A. flavus* strains in areas with no history of aflatoxicosis, Nandi, Kisumu and Homa Bay, suggests that consumers in these areas are exposed to lower levels of mycotoxin producing *Aspergillus* spp. than those that live in Makeuni.

The classification and identification of *Aspergillus* spp. based on phenotypic characteristics in the last decades, is influenced by molecular and chemotaxonomic characterisation (Probst *et al.*, 2010; Probst *et al.*, 2012). The next chapter describes molecular markers for *A. flavus* and vegetative compatibility groups of the *A. flavus* strains from across the four different Kenyan counties. Moreover, genomic DNA was extracted from *A. flavus* isolates, amplified, sequenced and a phylogenetic analysis conducted with selected barcode genes as a target for discrepancy to understand the taxonomic relationships between *A. flavus* isolates in the surveyed regions.

CHAPTER THREE

Determining genetic diversity in *Aspergillus flavus* by using vegetative compatibility groups and molecular markers

3.0. Introduction

In the previous chapter, culturable methods together with microscopy were used to evaluate and identify *A. flavus* phenotypically following standard identification keys (Klich, 2002; Nyongesa *et al.*, 2015). In the current chapter, genetic variation within the *A. flavus* population and its distribution across different climatic regions of Kenya were investigated by using genetic markers and the ability to produce aflatoxins.

A. flavus populations designated as the L and S-morphotypes based on colony and sclerotia morphology characteristics can be further subdivided into VCGs by a heterokaryon (vegetative) incompatibility system (Cotty, 1989). Heterokaryon formation between different *A. flavus* isolates is a vital component of the life cycle of numerous filamentous fungi (Cotty, 1989). Heterokaryon incompatibility is a genetic phenomenon (*het* or *vic* loci) that limits heterokaryosis amongst entities which differ at one or more *het* or *vic* loci (Xiang & Glass, 2004). Heterokaryon incompatibility is circumvented only during sexual recombination (Horn *et al.*, 2009). Successful mating events may occur when the parent strains have different chemotype profiles, are of complementary mating type (MAT1-2 + MAT1-1), and are from different VCGs. Leslie, (1993) described VCG diversity through genomic differences at the *het* loci. The determination of the VCG of a specific *A. flavus* strain is done by complementation tests with nitrate non-utilising auxotroph's (Leslie, 1993; Grubisha & Cotty, 2010; Atehnkeng *et al.*, 2016). Members of the same VCG are presumed to have the same clonal lineage (Leslie, 1993; Grubisha & Cotty, 2010; Atehnkeng *et al.*, 2016), and the similarity of aflatoxin production is greater within a given VCG than between VCGs.

VCG analysis also has been used to evaluate genetic diversity within *A. flavus* populations and multiple VCGs can be recovered from the same geographic region including fields or crops from which isolates were obtained (Bayman & Cotty, 1993; Pildain *et al.*, 2004; Mehl *et al.*, 2011; Atehnkeng *et al.*, 2008; Atehnkeng *et al.*, 2016). Sweany *et al.* (2011) recognised 16 VCGs of *A. flavus* amongst isolates from soil and

maize ears obtained from 11 corn fields in Louisiana. Similarly, Habibi & Banihashemi, (2008) identified 16 VCGs of *A. flavus* amongst isolates from sesame seeds collected in Iran. Ideally, atoxigenic *A. flavus* strains for biological control of aflatoxin production should be from a VCG that has no aflatoxigenic strains (Cotty, 2006; Mehl *et al.*, 2012; Ehrlich, 2014). This isolation ensures that there is no possibility for genetic exchange between aflatoxigenic and atoxigenic strains which might confer any benefit to the aflatoxin producers (Ehrlich *et al.*, 2007; Ehrlich *et al.*, 2014). Yard *et al.* (2013), observed that the use of genetically similar atoxigenic VCGs from local soil communities of *Aspergillus* can increase growth efficacy, minimize the potential for sexual recombination, and results in biocontrol of aflatoxigenic *A. flavus* strains (Donner *et al.*, 2010; Mehl *et al.*, 2012; Ehrlich, 2014).

Morphological characters; sclerotia size and aflatoxin-production ability usually are preserved within a VCG (Pildain *et al.*, 2004). Different levels of aflatoxin production in *A. flavus* populations is linked to their VCGs with only isolates having compatible *het* loci forming stable hyphal fusions (Horn *et al.*, 2009).

The genus *Aspergillus* contains diverse species and the identification of *A. flavus* is complicated by many factors; especially the presence of overlapping morphological features among closely related species (Klich, 2002; Varga *et al.*, 2011). *Aspergillus* spp. have been described on morphological and phenotypic parameters such as colony colour, diameter and size, the texture of conidia, and the structure of conidiophores (Klich, 2002; Varga *et al.*, 2011). Asao, (1996), observed that species classification in *Aspergillus* section *Flavi* is challenging due to extensive divergence and genetic variability. In contrast, molecular approaches for differentiating species in *Aspergillus* that rely on DNA sequence based techniques result in more robust species identifications (Balajee *et al.*, 2007).

The internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) have been proposed by several groups (Pryce *et al.*, 2003) to be the best regions for sequence-based discrimination of fungal species. Additionally, the more conserved 18S and 28S regions which flank the ITS region, have been used in the design of *Aspergillus* species probes (Wu, 2006). Turner *et al.* (2003), noted that commercial molecular detection kits that used conserved rDNA regions for *Aspergillus* identification may identify strains from multiple species as the same species. Analysis

of sequences from the most conserved rDNA regions may enable identification to a relatively large taxonomic group, whereas, the ITS1 and ITS2 regions may allow for more precise identification of species (Pryce *et al.*, 2003).

The ITS regions have been used in both ecological and molecular systematic investigations of fungi which has led to a repository of more than 200,000 Sanger-derived fungal ITS sequences in nucleotide databases such as GenBank (Clark *et al.*, 2016). The ITS regions of fungi vary, roughly, between 450-750 bp in length and consist of 3 sub-regions: an intercalary 5.8S gene (highly conserved) and more variable ITS1 and ITS2 spacer regions. The spacer regions provide resolution within the genus, often to the species level (Nilsson *et al.*, 2008). These genomic regions are being used as the official DNA barcode for classification in fungal studies (Schoch *et al.*, 2012).

Aspergillus flavus isolates also differ in the ability to produce aflatoxins, the formation of sclerotia and sporulation (Bayman & Cotty, 1991). Some isolates lack the ability to produce aflatoxins (atoxigenic), while aflatoxigenic isolates can produce low (<100 ng/g) to very high (>1000 ng/g) levels of the toxin (Bayman & Cotty, 1991). The ability of *A. flavus* to produce aflatoxins can be preliminary identified by culturing on coconut agar medium and exposing the growth to ultra violet light (365 nm) (Davis *et al.*, 1987; Pitt, 1994; Mitema *et al.*, 2018). The resultant emission of blue and green fluorescence is associated with toxin production (Mitema *et al.*, 2018).

Frisvad *et al.* (2005) and Pildain *et al.* (2008) both demonstrated that multiple species in *Aspergillus* section *Flavi* cannot be completely resolved based solely on morphological characters. Therefore, the objective of the current study was to evaluate VCG genetic diversity and variation among the isolates, identify toxigenic and atoxigenic *A. flavus* strains, and to conduct phylogenetic analysis of the *A. flavus* strains. Thirty-seven *A. flavus* isolates from Nandi, Kisumu, Homa Bay and Makueni were examined to clarify their taxonomic status based on their morphological characters. A phylogenetic analysis of ITS1 and ITS2 sequences and, the 5.8 S rRNA gene cluster of the isolates made. This analysis provides a deeper understanding of the taxonomic relationship between the *A. flavus* isolates and could help contextualise the data obtained for each isolate with respect to morphotype, VCG genetic diversity and aflatoxin production.

3.1 Materials and methods

The schematic flow diagram (Fig.3.1) show the steps followed for VCG complementation tests.

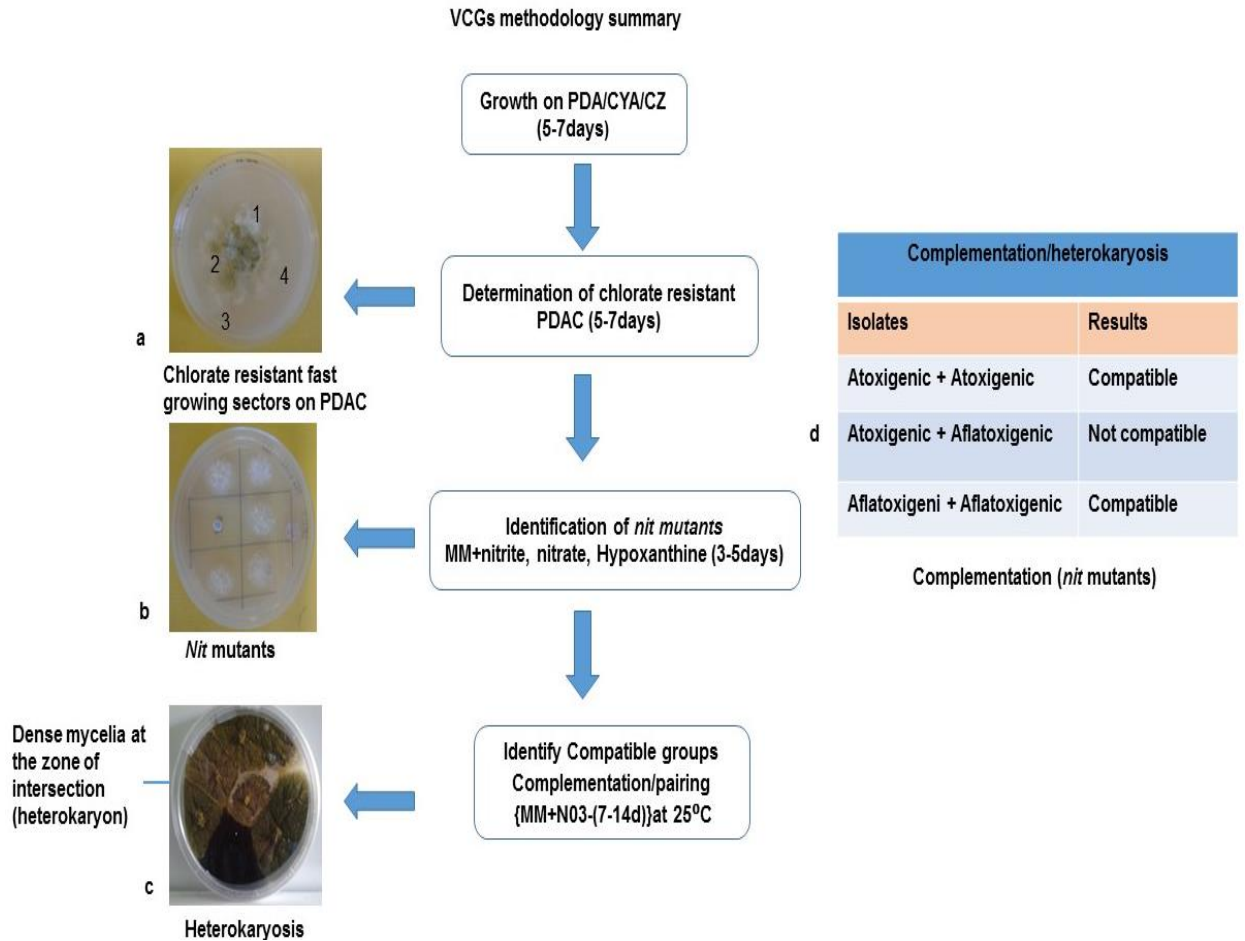


Figure 3.1 Complementation test steps for vegetative compatibility group in *Aspergillus flavus* strains isolated from maize kernels from four different climatic regions of Kenya used for identification of heterokaryons; **a.** chlorate resistant fast-growing sectors; **b.** *nit*-mutant identification; **c.** formation of dense mycelia at the zone of intersection (heterokaryosis) and **d.** compatibility tests table for generation of nit mutants. The isolates were cultured on selective and minimal media (PDA: potato dextrose agar; PDAC: potato dextrose agar chlorate; CYA: Czapek yeast agar; Czapek dox agar; MM: minimal media).

The schematic flow diagram (Fig.3.2) shows cultivation of *A. flavus* isolates, DNA isolation and phylogenetic analysis to species level.

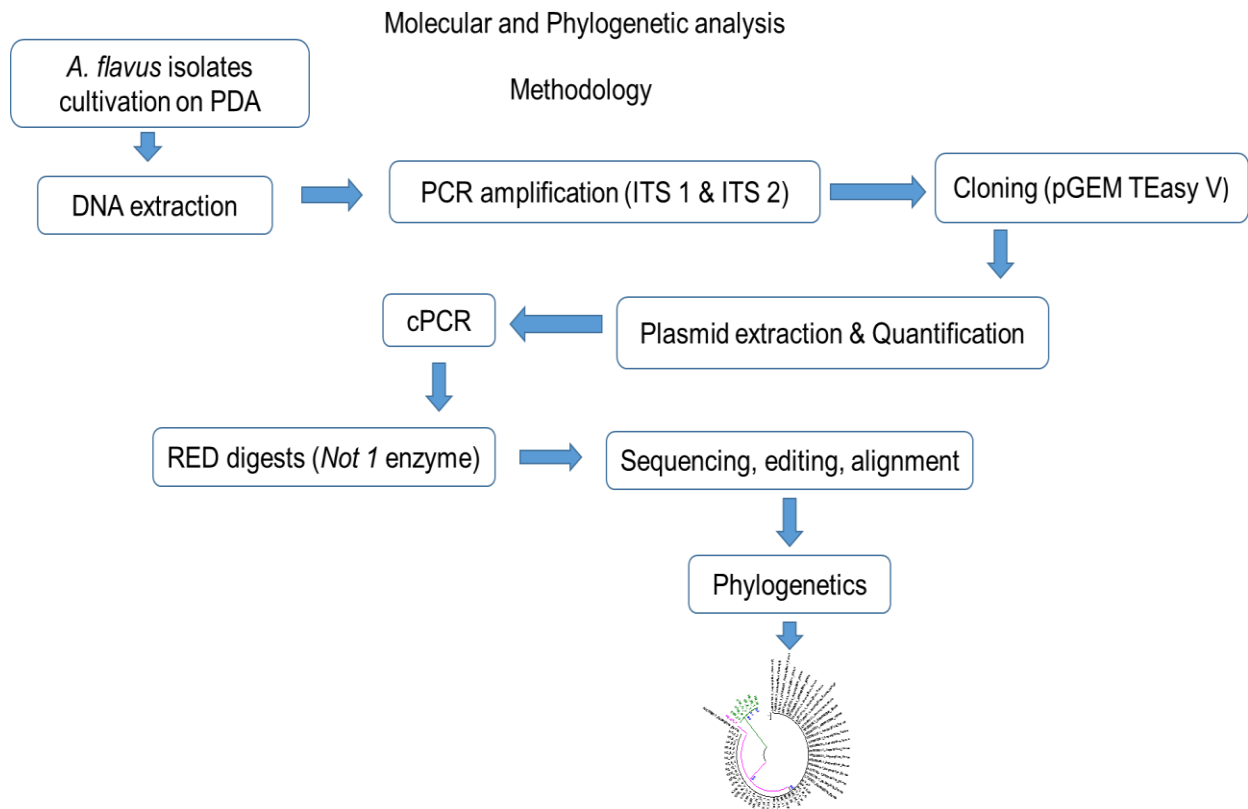


Figure 3.2 Procedure employed in the cultivation, isolation and identification of *Aspergillus flavus* isolates to species level. Genomic deoxyribonucleic acid was extracted from the cultures cultivated on potato dextrose agar and amplified on polymerase chain reaction, using ITS1 and ITS2 primers. PCR products were then cloned into pGEM TEasy vector and subsequently, plasmid DNA was extracted and quantified using Nano Drop spectrophotometer before conducting chromosomal deoxyribonucleotide tests to ascertain the presence or absence of the insert. Restriction digestion was then investigated using *Not1* enzyme and thereafter sent for sanger sequencing before editing, alignment and phylogenetic tree reconstruction to determine the relationships of the isolates from the four different climatic regions surveyed.

3.1.1 Reagents and chemicals used

Chemicals used are listed in chapter 2 section 2.1.3. Additionally, *Not1* enzyme, *Taq* DNA Polymerase and detection kits were from Thermo™ Scientific (USA). Ampicillin, Isopropylthio-β-D-galactoside (IPTG), chloroform, agarose gel, 5-bromo-chloro-3-indolyl-β-D-galactopyranoside (X-gal), isoamyl alcohol, glass beads and ethylene-diamine-tetra acetic acid (EDTA) were from Sigma-Aldrich (USA). Pure and ultra-pure water was from Molecular and Cell Biology laboratory (Millipore LTD, Bedford, MA, USA). Ligations were performed with the pGEM® T Easy vector and T4 DNA Ligase kit (Promega Corporation, USA).

3.1.2 Fungal cultivation techniques

The 37 *A. flavus* isolates were routinely cultivated and maintained on PDA as described previously (Chapter 2, section 2.1.4).

3.1.3 Vegetative Compatibility Tests

To determine the genetic diversity and distribution patterns of VCGs across different climatic zones, 37 isolates of *A. flavus* were used for tester-pair development (Chapter 2, Table 2.2). Nitrate non-utilising (*nit*⁻) mutants were generated using a modified method (Bayman & Cotty, 1991). Briefly, fungal isolates were grown on a selective medium [Czapek–Dox broth (Difco)] containing 25 g l⁻¹ potassium chlorate, 50 mg l⁻¹ Rose Bengal and 20 g l⁻¹ agar with pH adjusted to 7. The selective medium was inoculated with a conidial suspension of *A. flavus* at the centre of the Petri plate. Cultures were then incubated at 30 ° C, and margins of colonies with restricted growth were periodically examined for fast growing sectors containing sparse mycelium. Hyphal tips from sectors arising from different colonies were transferred to Petri plates containing Czapek–Dox broth with 15 g l⁻¹ potassium chlorate (Sigma Aldrich, USA) and 20 g l⁻¹ agar with pH adjusted to 7 to stabilize the mutants and confirm their inability to utilize nitrate.

The *nit*⁻ mutant phenotypes, *niaD*⁻ (defective in the structural gene for nitrate reductase), *nirA*⁻ (defective in the nitrate reductase) and *cnx*⁻ (defective in the molybdenum cofactor) were determined by growing the mutants on a medium with nitrite, hypoxanthine or ammonium as sources of nitrogen as previously described by Cove, (1976) and Bayman & Cotty, (1991). A complementary pair of nitrate non-utilising auxotroph's composed of either a *niaD*⁻ and a *cnx*⁻ or a *niaD*⁻ and a *nirA*⁻ mutant was obtained for each isolate, and complementary pairing was first conducted to establish self-compatibility (Bayman & Cotty, 1991). Complementary pairs of mutants from an isolate were used as tester pairs and complementation with one or both of the tester mutants of a VCG-defined membership in that VCG (Atehnkeng *et al.*, 2016).

The *nit*⁻ mutants were used to determine the distribution of VCGs in the areas studied. Subsequently, the *nit*⁻ mutants were used to perform complementation tests with each VCG tester-pair. Complementation tests were conducted by placing 10 µl of

a spore suspension of each member of a VCG-defining tester-pair and a *nit*⁻ mutant (unknown phenotype) of one of the isolates in 3 mm wells cut into complementation medium. The wells were arranged approximately 15 mm apart in a triangular pattern so that each tester may react with both the *nit*⁻ mutant and the other tester. The Petri plates with the VCG tester-pair were incubated at 30 ° C from 7 to 14 days. Compatibility was identified by a line of prototrophic growth, where the mycelia interacted. This interaction was frequently associated with formation of sclerotia. Inter-location complementation test was determined as described previously (Atehnkeng *et al.*, 2016).

3.1.4 Coconut agar medium test for aflatoxins

Spores from seven-day old cultures on PDA plates of *Aspergillus* spp. were suspended in sterile water. One microliter of suspension from each strain was centrally inoculated in triplicate on 50 % Coconut Agar Medium (CAM) as described previously (Davis *et al.*, 1987) with slight alterations. Briefly, CAM was supplemented with 0.05 % chloramphenicol and incubated at 30 ° C in the dark for 3-7 days and visualised on the reverse side of the plate under ultra-violet long wavelength light at 365 nm. Greenish blue or bright blue fluorescence region surrounding colonies were used as guidelines to indicate the presence or absence of aflatoxins based on the methods of Davis *et al.* (1987) and Pitt, (1994).

3.1.5 Primer design

Oligonucleotide primer pairs (F-5'-TCA TTA CCG AGT GTT GGG TTC CTA G-3' and R-5'-GGT CAA CCT GGA AAA GAC TGA TTT G-3') were designed in Primer3 ver. 4.0 programme (Untergasser *et al.*, 2012) based on sequence alignment of the ITS1 and ITS2 regions of *A. flavus* strains retrieved from NCBI (<http://www.ncbi.nlm.nih.gov/>). The secondary structure formation for the primers were evaluated in DNAMAN software ver. 6.0 (Lynnon LLC., USA) and further verified in OligoAnalyzer Tool version 3.1 (Integrated DNA Technologies, Inc., USA). The standard methods were used to synthesize the primers as provided by the Synthetic DNA Laboratory (MCB, University of Cape Town in South Africa). The PCR analysis was performed (GeneAmp PCR system 9700, Applied Biosystems) to detect both non-specific and specific amplification.

3.1.6 Fungal genomic DNA isolation

Briefly, *A. flavus* mycelia from seven-day-old cultures were scrapped off the plates, flash frozen and ground in liquid nitrogen in a sterile mortar with a pestle prior to DNA extraction. Fungal DNA was recovered from ground cultures (100 mg) by using a ZR Fungal/Bacterial DNA Kit (Zymo Research, USA) according to manufacturer's instructions. The DNA yield and integrity were evaluated, using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) and further assessed on 1 % (w/v) agarose/EtBr gel run at 80 volts for 45 min. The DNA was diluted to 50 ng/μl, tested for suitability for PCR amplification and, stored for further analysis at -80 ° C.

3.1.7 Polymerase Chain Reaction amplifications

PCR amplification was performed in a 20 μl reaction mixture containing 10 x 25 (1.5) mM MgCl₂ buffer, 10 mM dNTPs, 5U/μl Kapa Taq DNA polymerase (Kapa Biosystems Ltd., UK), 1 μl of template DNA and 10 μM of ITS1 and ITS2 forward and reverse primers (see section 3.1.5). The amplification procedure comprised of a pre-denaturation step at 96 ° C for 3 min, followed by 30 cycles of denaturation at 96 ° C for 30 secs, annealing at 55 ° C for 1 min and extension at 72 ° C for 1 min, plus a final at 72 ° C elongation for 5 min before being stored at 4 ° C for 1 min. The integrity of PCR products was assessed on 1 % (w/v) agarose/EtBr gel run at 80 volts for 45 min. One kilo base pair (1kb) DNA ladder (Promega BioSciences, CA, USA) was used to detect the product sizes visualised under low radiation ultra violet (UV) trans-illuminator (ChemiDoc™ XRS+ Bio-Rad ver.5.1, USA).

3.1.8 Gene cloning, plasmid DNA extraction and quantification

Purified PCR products were cloned using a 1:3 vector/insert ratio into the pGEM®-T-Easy vector (Promega Corporation, USA) according to the manufacturer's instructions. Ligated plasmid DNA was transformed into chemically competent *E. coli DH5α* cells (*E. coli*™, Lucigen, WI). Transformed cells were selected on LB agar plates (based on blue/white screening) with 100 μg/mL ampicillin, 80 μg/mL X-gal and 0.5 mM IPTG. Positive colonies were screened by PCR with M13 universal primers using the described PCR profile (section 3.1.7). Plasmid DNA from positive colonies was subsequently isolated by using a Plasmid DNA extraction kit (Biospin Bioer Technology Co. Ltd, China) and quantified on NanoDrop ND-1000 spectrophotometer

(NanoDrop Technologies, USA). Plasmid DNA were confirmed by restriction enzyme digest mapping and the final products were shipped for Sanger sequencing (Macrogen Europe, UK).

3.1.9 Phylogenetic analysis

The successfully sequenced amplicons were analysed, errors detected and corrected using DNAMAN software ver. 6 (Lynnon LLC., USA). Species were identified following with the Basic Local Alignment Search Tool (BLAST), which is implemented within the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). When assigning an isolate, a species name, only BLAST search results showing > 98 % identity with a species' ITS sequence were considered.

Isolates were identified based on percentage identity to the *RefSeq* strain *A. flavus* GenBank: EU982012.1. Sequence comparisons of *RefSeq* strains and the isolates were aligned by using MUSCLE in MEGA ver. 6 (Tamura *et al.*, 2013). Each locus was aligned separately and thereafter concatenated into a super gene alignment for construction of a phylogenetic tree. The phylogenetic tree construction was inferred by using the Maximum Likelihood method under Tamura 3-parameter model (Tamura, 1992). The reconstructed phylogeny was tested for statistical support by bootstrapping using 1000 replicates (Felsenstein 1985). This phylogeny enabled the evolutionary history of the taxa under analysis to be studied in detail (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown above the branches (Felsenstein 1985). Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among site [4 categories (+G, parameter = 1.6511)]. The analysis involved 60 nucleotide sequences where all positions containing gaps and missing data were removed prior to analysis. In the final dataset, there was a total of 174 positions. Evolutionary analyses were finally performed in MEGA ver.6 (Tamura *et al.*, 2013).

3.1.10 Statistical diversity index

VCG diversity was projected and analysed as described by Horn & Greene, (1995) and Martins *et al.* (2008). The number of VCGs divided by the number of isolates was used to quantify VCG genetic diversity, and the diversity indices were calculated from each of the four regions. The graphs and analyses were achieved using GraphPad Prism version 5.02 (GraphPad Software, Inc., USA), Tukey's Multiple Comparison Test, One-way analysis of variance (1-way ANOVA), Post-test for linear trend and R statistical software (www.r-project.org), version 3.2.5.

3.2 Results and Discussion

3.2.1 Characterisation of the fungal isolates based on *nit*- mutants

Three sets of biological replicates were performed and VCG tests on selective media revealed 4-8 chlorate resistant sectors (Fig.3.3). The sectors sporulated at different rates displaying characteristics of whitish mycelia growth at hyphal tips (see red arrows) of the colonies at the edges of the plates (Fig.3.3).

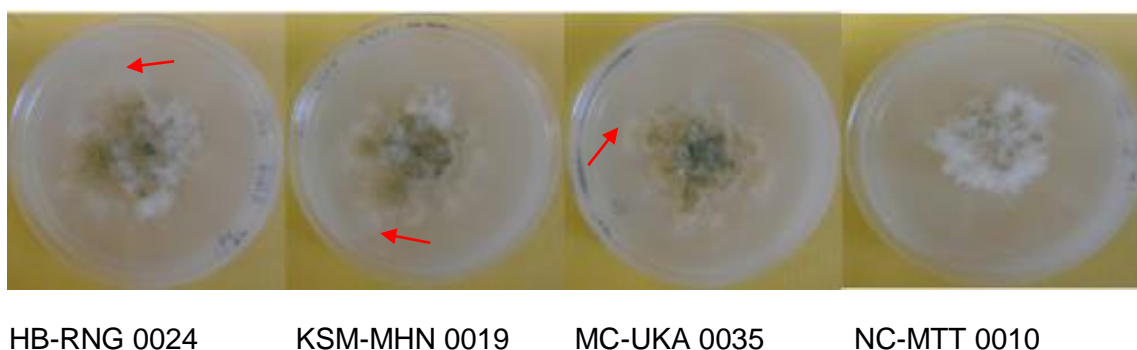


Figure 3.3 Four of the 37 *Aspergillus flavus* strains isolated from four regions of Kenya growing on minimal media showing different growth patterns after 10 days of incubation at 30 ° C. The extended radial hyphal tips growth (red arrows) is an indication of fast growing chlorate resistant sectors.

Some chlorate-resistant sectors were observed to grow very rapidly away from the colony while others grew more slowly. These different growth patterns could be attributed to chlorate, which is an analogue of nitrate and preferred by fungi for growth (Papa, 1986; Bayman & Cotty, 1991; Pildain *et al.*, 2004; Barros *et al.*, 2006). Upon reduction by the nitrate reductase, the chlorate would become chlorite which is poisonous substance to fungi (Papa, 1986; Bayman & Cotty, 1991; Pildain *et al.*, 2004; Barros *et al.*, 2006) hence the different growth patterns.

Different sources of nitrogen were tested on the *Nit 1*, *Nit 2*, *Nit 3*, *Crn* (chlorate resistant nitrate-utilizing) and wild type isolates (Table 3.1). The generated *nit* mutants were then classified based on three classes: *niaD* (nitrate non-utilising), *nirA* (nitrate and nitrite non-utilising) and *cnx* (nitrate hypoxanthine non-utilising). The results showed that the presence or absence of a nitrogen source plays a major role in complementation tests in determining the sensitivity of the *A. flavus* isolates. Total sporulation of *nit* mutant's isolates was observed mainly in NH₄ source (data not shown) whereas the wild type exhibited growth in all except in ClO₃ source. *Nit 1*, *Nit 2*, and *Nit 3* displayed no growth in NO₃ whereas wild type, *Nit 1*, *Nit 2* and *Crn* exhibited growth on hypoxanthine.

Table 3.1 Complementation tests of *Aspergillus flavus* isolates and *nit*-mutants on different sources of nitrogen (+ indicates presence and – absence of growth).

Isolate	Media				
	NH ₄	NO ₃	NO ₂	Hypoxanthine	ClO ₃
Wild type	+	+	+	+	-
<i>Nit1</i>	+	-	+	+	+
<i>Nit2</i>	+	-	-	+	+
<i>Nit3</i>	+	-	+	-	+
<i>Crn</i>	+	+	+	+	+

Amongst the thirty-seven isolates, there were twenty VCGs (Fig.3.3). These VCGs were assigned the prefix “KVCG”, where “K” represented Kenya and consequently assigned numbers 1 to 20 based on contemporary findings. KVCG14 and KVCG15 ($n = 13$; 10.8 %) were the most frequent and KVCG10 and KVCG20 ($n = 1$; 0.8 %) were the least frequent, respectively (Fig.3.3).

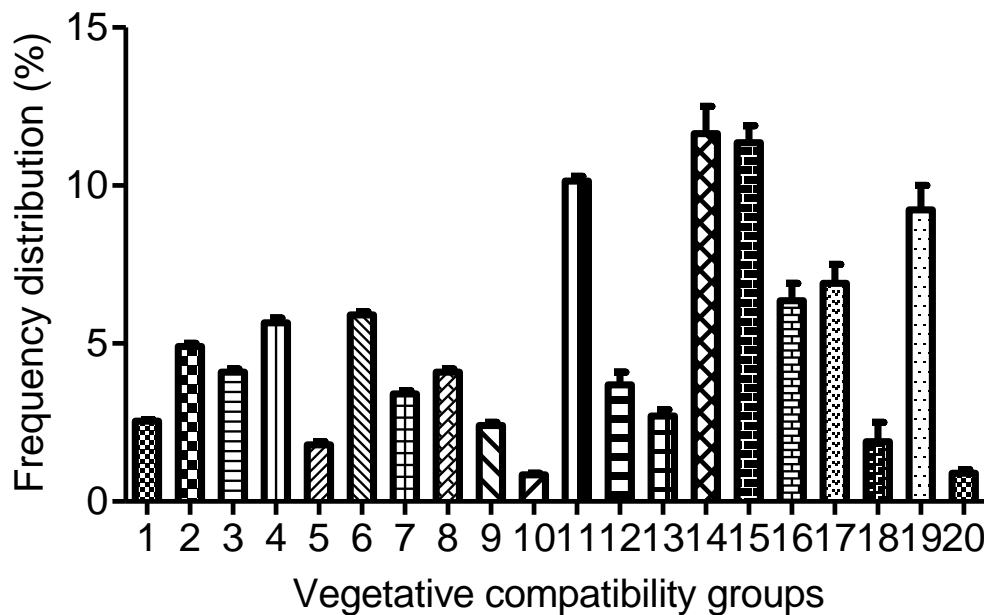


Figure 3.4 Vegetative compatibility groups distribution of *Aspergillus flavus* strains isolated from maize kernels collected from four different climatic regions of Kenya. KVCG14 and KVCG15 were highly distributed compared to KVCG20 which had the least distribution. Each bar represents the standard error ($n = 3$) of the mean frequency of VCGs ($P < 0.05$). (KVCG: Kenya vegetative compatibility groups)

Isolates from each region were self-compatible within the locality but incompatible between the regions with a few exceptions. Heterokaryon incompatibility, an indicator of distance association amongst the strains was detected between counties. Strong heterokaryon incompatibility was observed between Nandi region isolates ($n = 6$; 67 %) and Makueni ($n = 3$; 33 %). Isolated strains from Nandi, Kisumu, and Homa Bay were vegetatively compatible and formed dense hyaline mycelia at the point of intersection. Kisumu and Makueni region isolates were observed to be widely distributed (Fig.3.4). The findings of this study were similar to studies conducted by Horn & Greene, (1995) and Pildain *et al.* (2004) who noted that *A. flavus* strains are composed of many VCGs. Additionally, VCG diversity could be interrelated to variations in specialised regions within the *A. flavus* populations displaying competitive advantages in specific agroecological zones. Pildain *et al.* (2004) and Atehnkeng *et al.* (2008); Atehnkeng *et al.* (2016) further demonstrated that, frequencies of strains and VCGs vary by farm land and crop, and that some VCGs are frequently isolated whereas others are uncommon.

3.2.2 Characterisation based on VCG diversity index of the isolates

Diversity index is the number of VCGs divided by total number of isolates. The overall diversity index of VCG across the surveyed regions was ($H = 0.54$). The populations from the four climatic regions of Kenya had different diversity indices from low to moderate with Kisumu ($H = 0.324$) the highest followed by Makueni ($H = 0.27$), Homa Bay ($H = 0.22$) and Nandi ($H = 0.108$) as the lowest respectively (Fig.3.5). Moreover, Kisumu region isolates were observed to be highly diversified compared to other regions with Nandi region being the least diverse (Fig.3.5).

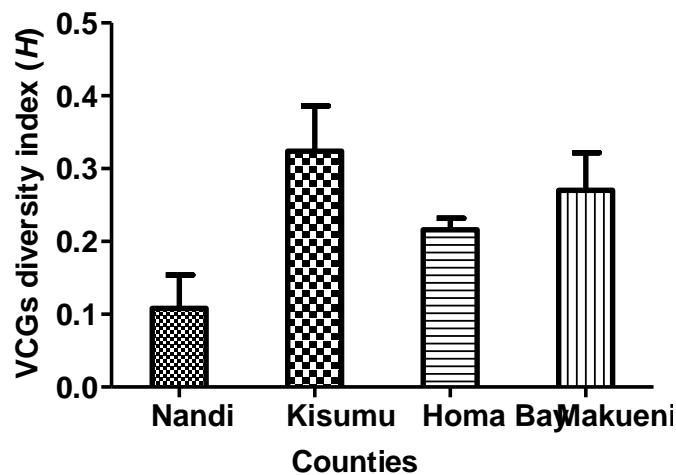


Figure 3.5 Vegetative compatibility group diversity index of *Aspergillus flavus* strains isolated from maize kernels from four different climatic regions of Kenya. Each bar represents the standard error of the mean diversity index ($n = 3$) ($P < 0.05$).

The current findings for VCG diversity index ($H = 0.54$) are similar to those reported by Bayman & Cotty, (1991) who found a diversity index of $H = 0.54$ for *A. flavus* isolates from Arizona. Higher H -indices for *A. flavus* was obtained in Argentina; $H = 0.64$ (Pildain *et al.*, 2004) in Italy; in Georgia; $H = 0.69$ (Papa, 1986); $H = 2.40-3.20$ (Mauro *et al.*, 2013) and Nigeria; $H = 2.60$ (Atehnkeng *et al.*, 2016). In the present, the H -index was relatively low, which could be attributed to the sample size evaluated. It was difficult to obtain substantial amount of samples in Kisumu, Homa Bay and Makueni due to poor harvests. In contrast, the Nandi region had an above average maize harvest and it was easier to obtain a large number of samples.

VCG distribution frequency and diversity indices of the isolates were closely linked to specific regions (Fig.3.4). Bock *et al.*, (2004) and Cotty, (2006) suggested that different *A. flavus* lineages might be distributed according to niche speciality

adaptations and have competitive advantages in specific regions, soils, hosts or seasons.

The genetic diversity and distribution of the Kenyan *A. flavus* isolates within the fungal communities in local populations have been under reported probably due to lack of adequate research infrastructure and funding. This study is believed to be the first to provide data on the VCG genetic diversity of *A. flavus* from Nandi, Kisumu, and Homa Bay regions. Makueni and its neighbouring regions for example Makindu, Kibwezi, Kitui and Machakos have been studied previously due to their history of aflatoxicosis outbreaks (Ngindu *et al.*, 1982; Gieseke 2004a; Cotty & Jaime-Garcia, 2007; Probst *et al.*, 2012).

3.2.3 Comparison of *Aspergillus flavus* isolates phenotypes with VCGs

The generated heat map identified interesting clusters of the *A. flavus* isolates based on VCG diversity that were correlated with the phenotypes of the isolates (Fig.3.6). The dark blue colours generated on the heat map (Fig.3.6) indicates higher similarity and the presence of a specific VCG for a strain whereas, the light blue colours indicate the absence of a VCG for a strain or low similarity. The higher the intensity of the blue colours on the heat map, the closer the similarity of the isolates with regard to the VCGs macro- and micro-morphological characteristics and genetic diversity. The histograms further show inter-location VCGs genetic diversity across the surveyed regions. Some isolates from Homa Bay and Nandi were observed to cluster together, whereas, others from Kisumu and Makueni appeared mixed. This indicates the possibility of likelihood or compatibility of the isolates between these climatic regions. KVCG 10 (Homa Bay) and KVCG 20 (Kisumu) were restricted to specific regions (Fig.3.6).

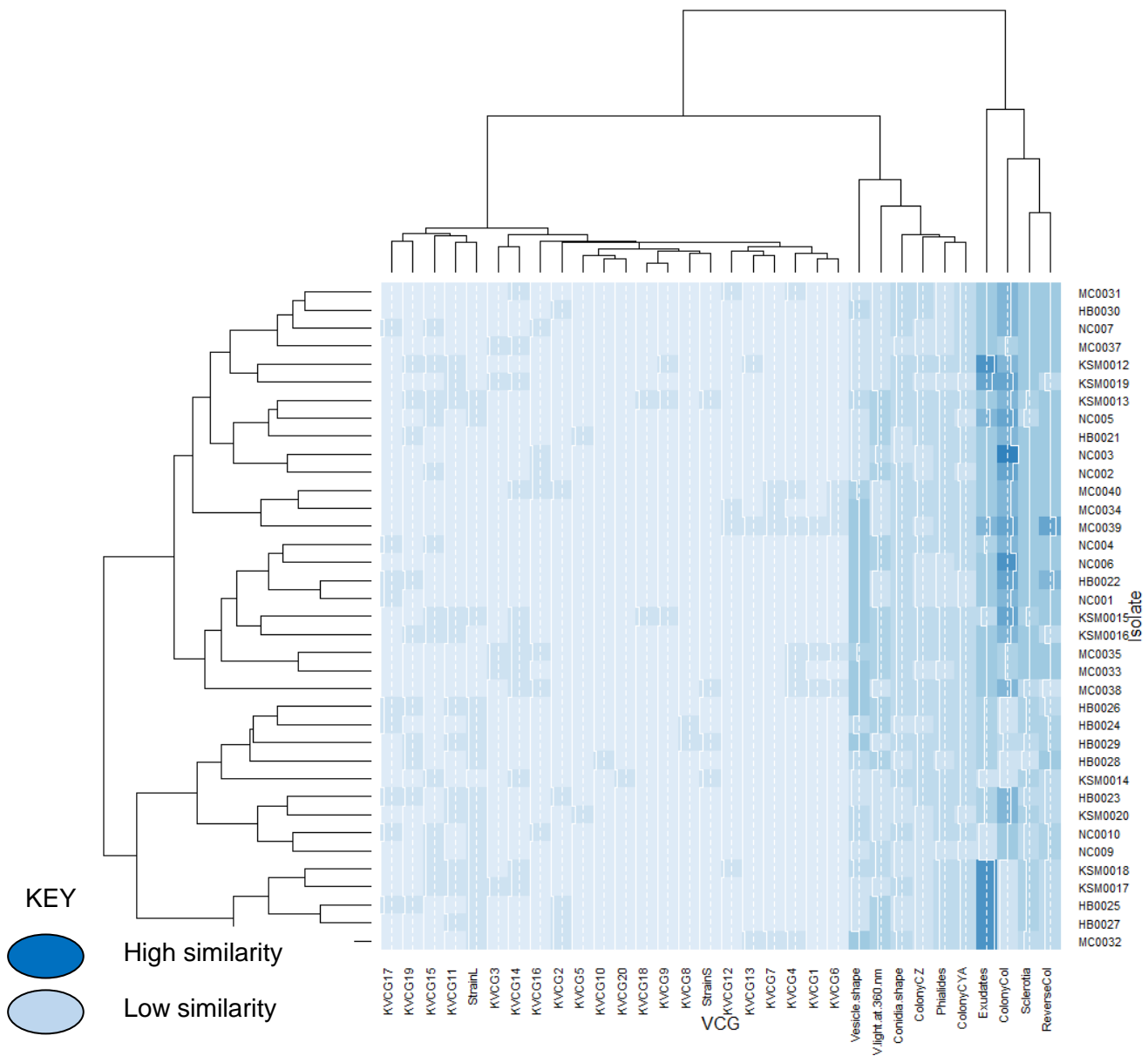


Figure 3.6 Heat map showing the relationship between vegetative compatibility groups genetic diversity, macro- and micro-morphological characters of thirty-seven *Aspergillus flavus* strains isolated from maize kernels from four different climatic regions of Kenya. Regions of high similarity are indicated by dark blue while regions of low similarity are indicated by light blue.

3.2.4 Characterisation based on Coconut agar medium test

Blue and green fluorescence produced by *Aspergillus* spp. subjected to long wavelength ultraviolet light (365 nm) on CAM is an indication of the presence of aflatoxins AFBs and AFGs respectively (Cotty, 1989; Mitema *et al.*, 2018). Screening the *A. flavus* isolates on CAM showed that some isolates could produce blue or green fluorescence (Figs.3.6; 3.7). Across the four climatic regions examined, 43 % of the strains fluoresced blue ($n = 16$); 24 % fluoresced green ($n = 9$) and 32 % fluoresced

blue/green ($n = 12$) respectively. Blue fluorescence was exhibited by 33 % of the isolates from Makueni, 29 % of the isolates from Kisumu, 24 % of the isolates from Homa Bay and 14 % of the isolates from Nandi, whereas green fluorescence frequencies were 12 %, 19 %, 31 % and 38 % respectively (Figs.3.7; 3.8).

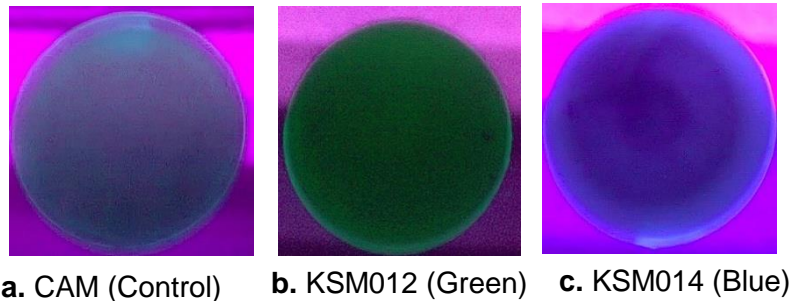


Figure 3.7 Images of fluorescence of plates of *Aspergillus flavus* cultures on coconut agar medium at long wavelength ultraviolet light (365 nm) for two of the 37 *Aspergillus flavus* strains isolated from maize kernels collected from four different climatic regions of Kenya showing the presence or absence of aflatoxins. (a). control plate, coconut agar medium with no *Aspergillus flavus* isolate inoculation; (b). positive plate with atoxigenic isolate KSM012 inoculation (green fluorescence) and (c). positive plate with aflatoxigenic isolate KSM014 inoculation (blue fluorescence). (CAM: coconut agar medium; KSM: Kisumu).

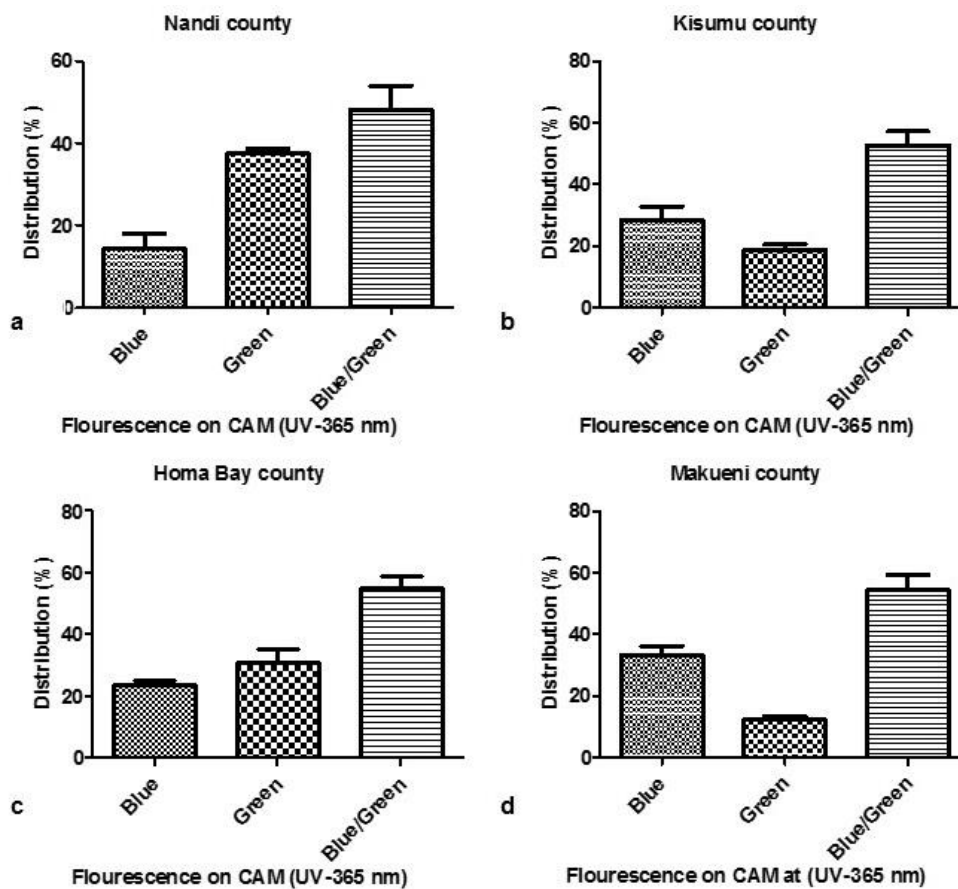


Figure 3.8 Toxigenic potential of *Aspergillus flavus* strains isolated from maize kernels from four different climatic regions of Kenya; fluorescence on coconut agar medium (**a-d**) showing possible aflatoxins expressed as percentage distribution. The isolates were cultured on coconut agar medium and visualised in a UV-cabinet chamber at 365 nm. The bar graphs illustrate the standard error deviations of the mean ($n = 3$) fluorescence distribution ($P < 0.05$).

Makueni and Kisumu region isolates exhibited high incidences of blue fluorescence in comparison to Nandi and Homa Bay (Fig.3.8a, d) suggesting higher potential for AFB1 and AFB2 contamination of maize kernels in these regions. Similar trends were observed for isolates producing blue/green fluorescence in the same regions, whereas the production of green fluorescence was higher among isolates from Nandi and Homa Bay.

The high frequency of *A. flavus* isolates producing blue fluorescence in Makueni may be linked to the frequently reported incidences of aflatoxicosis in that region. The current findings are in agreement with those of Probst *et al.* (2007); Probst *et al.* (2010) and Probst *et al.* (2012) who also observed a similar trend in the eastern region of Kenya where aflatoxin producing *A. flavus* isolates were found in the soil and in maize kernels. They also suggested that the identified aflatoxigenic isolates could be the causal agent of the human aflatoxicosis epidemic in Kenya in 2004.

The ability to produce aflatoxin is not a useful character in discriminating species within *Aspergillus* section *Flavi*. Some authors (Moore & Horn, 2009) observed wide variation in *A. flavus* populations and Varga *et al.* (2011) found that many strains can lose their ability to produce aflatoxins overtime.

3.2.5 Genomic DNA integrity

Raeder & Broda, (1985) observed that fungi (especially filamentous) possess a tough cell wall which is resilient to standard DNA extraction procedures. However good quality gDNA was successfully extracted from all of the *A. flavus* isolates (Fig.3.9)

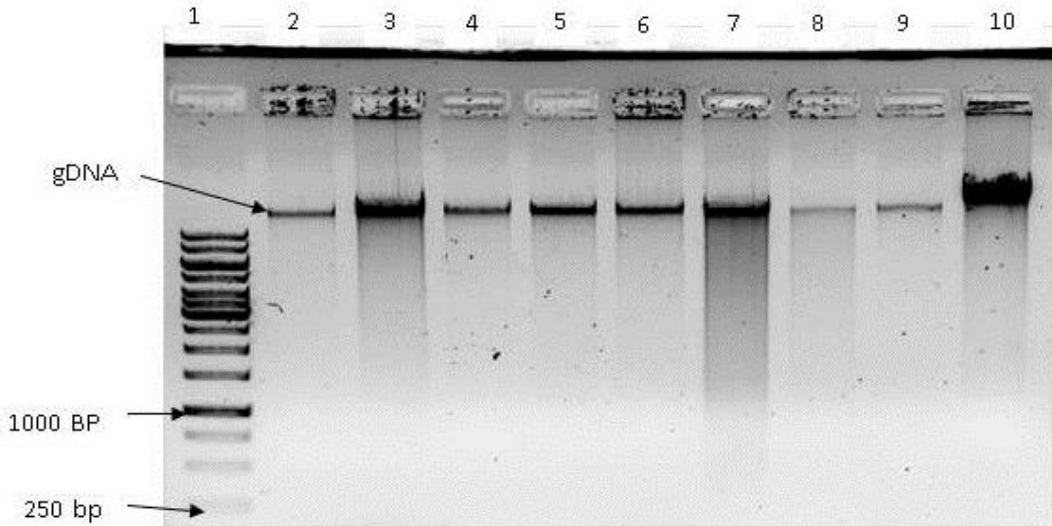


Figure 3.9 Gel electrophoresis of gDNA extracted from *Aspergillus flavus* strains isolated from maize kernels collected from four different climatic regions of Kenya assessed on 1 % agarose/EtBr gel run at 80 volts for 45 min. Lanes: 1. 1Kb Ladder; 2. NC01; 3. NC04; 4. KSM012; 5. KSM016 Y; 6. HB025; 7. HB027; 8. MC031; 9. MC034W; 10. MC040 G. (NC: Nandi county; KSM: Kisumu; HB: Homa Bay; MC: Makeni).

3.2.6 PCR amplification and primer specificity

Restriction digests with *Not1* followed by PCR with primer pairs (M13F and ITS1/ITS2R) identified the genes of interest at 518 bp (blue arrow) (Fig.3.10). Lanes 1. 1kb marker; 2 and 3 shows undigested isolates whereas, lanes 4-13, positive digests. The ITS 1 and ITS 2 primers designed also amplified a 518 bp. Thus, the primers were specific and suitable for use either for *A. flavus* identification.

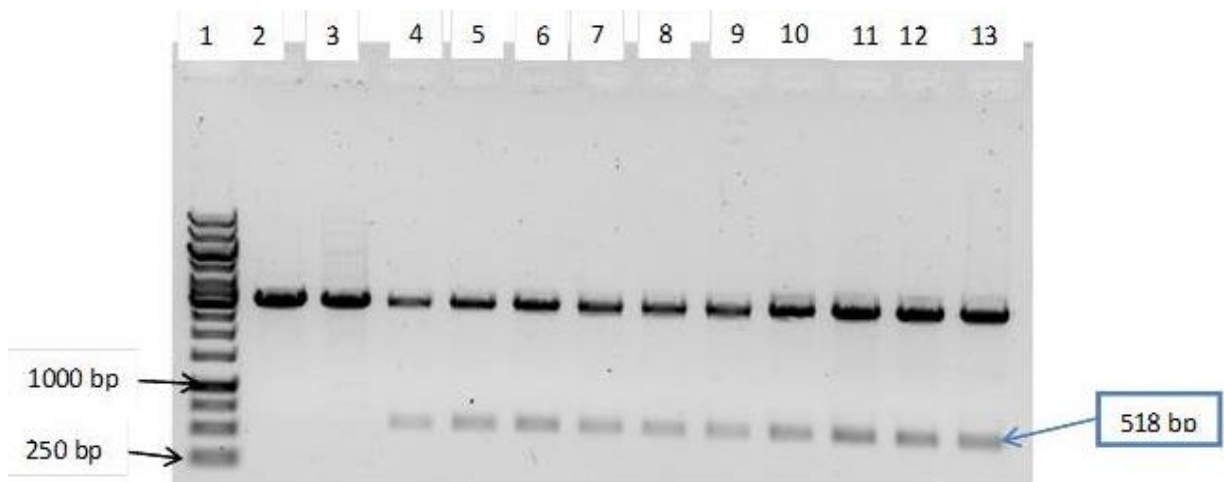


Figure 3.10 Images of restriction digests with *Not1* enzyme amplified on PCR machine using primers (M13F and ITS1/ITS2R) identified the genes of interest at 518 bp run on 1 % agarose/EtBr gel at 80 volts for 45 minutes. 1. 1Kb Ladder; 2. NC01 (-); 3. KSM012 (-); 4. NC01; 5. NC04; 6. KSM012; 7. KSM016; 8. HB025; 9. HB027; 10. MC031; 11. MC034; 12. MC035; 13. MC040. (NC: Nandi county; KSM: Kisumu; HB: Homa Bay; MC: Makeni).

3.2.7 *Aspergillus flavus* NCBI generated sequences

Sequences were isolated from 37 *A. flavus* isolates [Supplementary data, S3; Table S3.1, numbers in front of the letters refers to laboratory codes assigned (NC: Nandi county; KSM: Kisumu; HB: Homa Bay; MC: Makueni county respectively)].

3.2.8 Phylogeny and statistical analysis of *A. flavus* isolates from selected Kenyan counties

Phylogenetic analysis of the ITS1 and ITS2 domain sequences exhibited variable clustering with some isolates clustered together whereas others clustered with the *RefSeq* from the GenBank. Clusters had bootstrap support ranging from 64 % - 100 % (Fig.3.11).

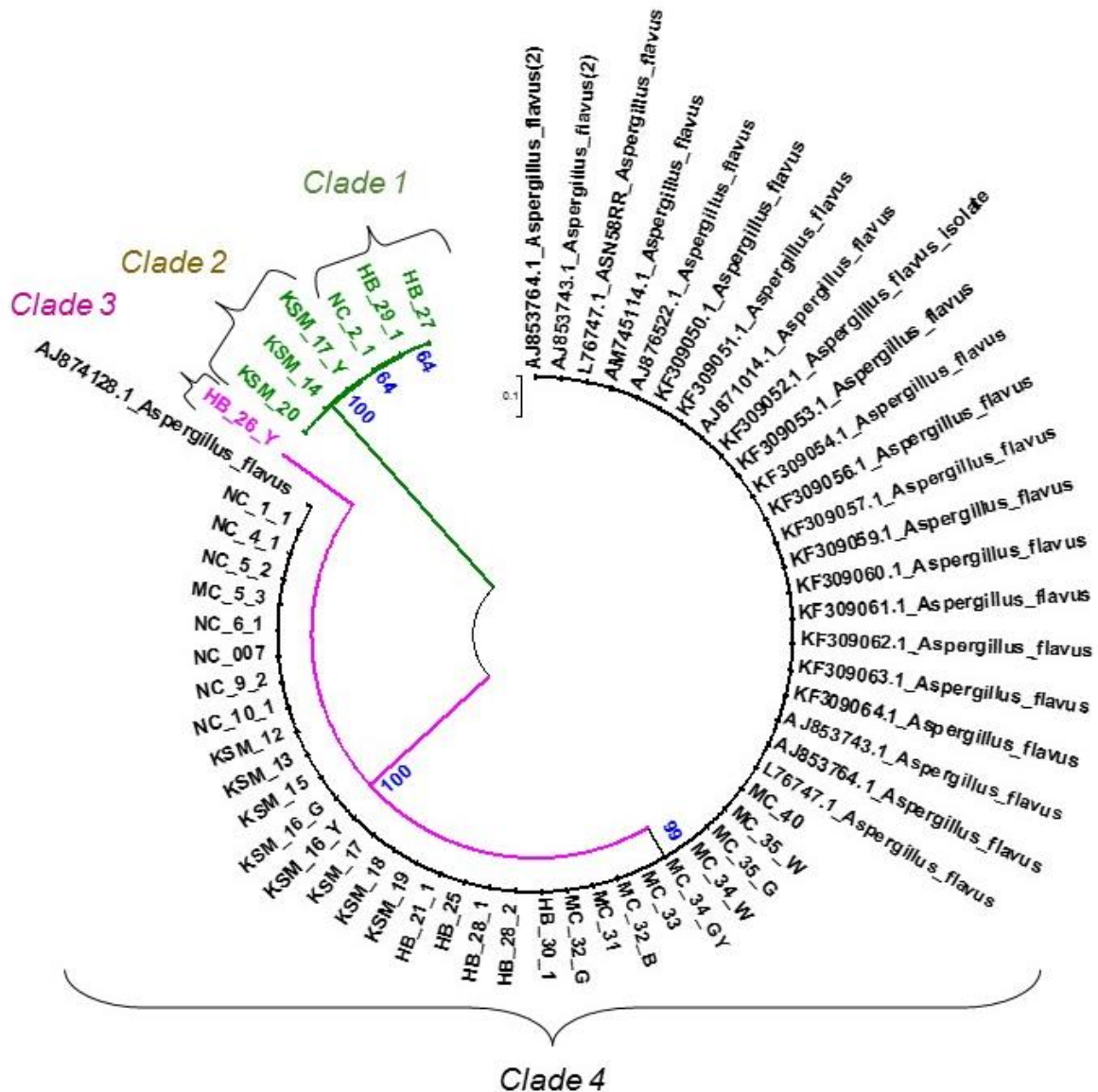


Figure 3.11 Maximum Likelihood Phylogenetic tree constructed from aligned DNA sequences of the ITS domain using MUSCLE, MEGA version 6. Nucleotide sequences generated are presented in bold, whereas the retrieved *RefSeq* from GenBank are labelled with their respective accession numbers. Clade 4 shows distribution patterns of the isolates with *RefSeq* from GenBank and surveyed counties (NC-Nandi county; KSM-Kisumu; HB-Homa Bay and MC-Makueni county and the numerical in front of the letters represent assigned codes).

Out of thirty-seven isolates tested, ITS primers discriminated *A. flavus* isolates; 10.8 % ($n = 4$) as 100 % bootstrap support, 81.1 % ($n = 30$) as 99 % and 8.1 % ($n = 3$) as 64 % bootstrap support respectively. Certain isolates (HB026Y) (clade 3) was discriminated as 100 % bootstrap support from other isolates (Homa Bay, Nandi, Kisumu and Makueni regions) and formed its own clade from the members of the group as revealed by Maximum Likelihood (ML) and Tamura 3-parameter model. The current findings were similar to the analysis of the ITS1 and ITS2 sequences

conducted by Gonçalves *et al.* (2012) which revealed high molecular heterogeneity of *A. flavus* strains and their close relatives.

From the previous chapter, isolate HB026Y was found to have characteristics of a S/L morphotype strain and this could have been the reason for discrimination to isolated clade. The phylogeny reconstruction tree also revealed that some isolates (KSM014, KSM020, KSM017Y and HB026Y) formed highly supported clusters with 100 % bootstrap support (Clade 1 and clade 3) (Fig.3.11). In the Maximum Parsimony model method, the four isolates also exhibited similar trend with 100 % bootstrap support as in ML model.

Further phylogenetic assessment of the tree showed that, the *RefSeq* from the GenBank clustered at 99 % bootstrap support with isolates across the regions; Makueni (100 %, $n = 10$), Homa Bay (65 %, $n = 5$), Kisumu (72 %, $n = 8$), and Nandi (91 %, $n = 7$) (Fig.3.11; clade 4). Clade one isolates were from Homa Bay ($n = 2$) and Nandi ($n=1$) region exhibiting 64 % bootstrap support. Clade 2 isolates were observed to have originated from Kisumu exhibiting bootstrap support 100 % (Fig.3.11). Interestingly, none of the isolates from Makueni region clustered in clade 1, 2 and 3. Additionally, strains from Makueni were more diverse compared to other regions. The findings were similar to Samson *et al.* (2014), who found that, *Aspergillus* is a diverse genus and the species occurs worldwide in various habitats. *A. flavus* isolates from Kisumu region were observed to be more closely related to strains from Nandi than to those from Homa Bay region. Clade 1 and 2 isolates originated from regions with little or no risk of aflatoxicosis over the years.

A. flavus is a genetically complex species (Geiser *et al.*, 2000; Pildain *et al.*, 2004; Chang *et al.*, 2006) and numerous cryptic species have been identified (Gonçalves *et al.*, 2012). A phenotypic and molecular investigations conducted by Gonçalves *et al.* (2012) on a set of isolates of *A. flavus* and related species to infer the different phylogenetic groups and, to determine possible cryptic speciation in *A. flavus*, as well as to assess whether or not the isolates form discrete groups revealed that primer sets used gave respective varying amplicon lengths. ITS primers gave an amplicon that was 520-535 bp in length, *amd* was 540-550 bp, *omtA* was 465-480 bp and BT2 was 508-522 bp. Their findings were similar to the current observation though, only ITS1 and ITS2 primer sets were used due to financial constraints. A

similar range of amplicon sizes for *A. flavus* strains from the four different climatic regions of Kenya were obtained using ITS1 and ITS2 marker genes.

The current results were consistent with those of Geiser *et al.* (2000) and Moore & Horn, (2009), who both demonstrated that the aflatoxin cluster genes could be used for phylogenetic studies in *Aspergillus*. section *Flavi*. Kanbe *et al.* (2002), identified the values of the β -tubulin genes as important in discriminating genetically and distant *Aspergillus* spp. of section *Flavi*.

Further phylogenetic and phenotypic studies are needed to evaluate speciation of *A. flavus* strains and should involve a greater number of loci. Hendolin *et al.* (2000) and Hinrikson *et al.* (2005) proposed that a specific DNA capture probe might be essential for recognition of *A. flavus* and some close relatives recognition but cannot be distinguished alone by ITS amplicon. In contrast the presence of intraspecies ITS variability did not hamper *Aspergillus* species and the ITS sequences determined in the current study which yielded high-ranking BLAST scores with corresponding reference GenBank data and the current findings were in agreement with those of Haugland *et al.* (2004) (Fig.3.10, clades 2-4).

3.3 Conclusion

The study revealed significant variation in many characters displayed by individual *A. flavus* isolates. VCG, aflatoxin-producing ability, fluorescence, and macro- and micro-morphological characters were the most common differences between the isolates of *A. flavus* examined. Strong heterokaryon incompatibility was observed between Nandi and Makueni isolates. Strains from Nandi, Kisumu, and Homa Bay were more vegetatively compatible while those from Kisumu and Makueni were widely distributed. *A. flavus* isolates from Makueni, Homa Bay and Kisumu region exhibited high fungal contamination with isolates from Makueni county showing high levels of suspected aflatoxin contamination compared to other regions. The study further showed a correlation between VCGs, S/L-morphotype strains and aflatoxin production which was evident in Makueni region isolates.

Aspergillus flavus isolates from Makueni region were more diverse than those from Nandi, Homa Bay and Kisumu isolates. There were no isolates from Kisumu region that clustered with strains from Makueni. Kisumu isolates were closely related

to strains from Nandi and Homa Bay. Within *A. flavus*, the ITS1 and ITS2 markers did not reveal significant information on intraspecies differentiation. The high genetic similarity of species of *Aspergillus* together with the high degree of intraspecific inconsistency might have led to the inability to detect the isolates, and in the future other molecular markers for distinguishing the strains at species level should be considered.

In the next chapter, the focus is on the expression of aflatoxin biosynthesis genes (structural and regulatory) in *A. flavus*. The study gives a better understanding of the genes that are responsible for aflatoxin production and confirms the phenotypes of the toxigenic and atoxigenic isolates identified in this and previous chapters.

CHAPTER FOUR

Detection of aflatoxin biosynthetic genes cluster in atoxigenic and aflatoxigenic *Aspergillus flavus* isolates

4.0 Introduction

In the previous chapters, sclerotia morphotype and the type of fluorescence exhibited when *A. flavus* isolates were cultured on CAM were used to characterise whether an isolate was potentially aflatoxigenic. This data was contextualised with the phylogenetic analyses of the *A. flavus* isolates, where in addition to observing the formation of different clades among the isolates and its distribution across the four different climatic regions surveyed, it was also noted that some regions had more toxigenic *A. flavus* strains than others (Chap 3, Fig.3.10).

Though phenotypic characterisation of *A. flavus* isolates may identify an isolate as possibly aflatoxigenic, these methods are not definitive or precise (Frisvad *et al.*, 2005; Pildain *et al.*, 2008). Molecular techniques, such as Reverse Transcriptase-PCR have therefore also been applied to differentiate aflatoxigenic and atoxigenic *A. flavus* strains, using the expression of regulatory and structural aflatoxin pathway genes as markers for aflatoxin production (Mayer *et al.*, 2003; Scherm *et al.*, 2005). Despite the complexity of aflatoxin pathway involving at least 25 structural and 2 regulatory genes (Yu *et al.*, 2004a), some studies found good correlation between gene expression and aflatoxin production (Scherm *et al.*, 2005). Scherm *et al.* (2005) further reported that the expression profiles of the genes *afID*, *afIO* and *afIP* were correlated with the strains' ability to produce aflatoxins.

In *A. flavus*, expression of *afID* (*nor-1*), a gene encoding an enzyme that catalyses the conversion of the first stable aflatoxin biosynthesis (Fig.4.1) intermediate, norsolorinic acid, to averantin (Trail *et al.*, 1994; Zhou & Linz, 1999) is a key structural gene in the aflatoxin biosynthetic pathway. Studies conducted by Abdel-Hadi *et al.* (2010); Abdel-Hadi *et al.* (2011) found that *afID* transcription was a good marker to discriminate between aflatoxigenic and atoxigenic strains but that *afIR* transcription was not a good marker. Cary & Ehrlich, (2000) showed that *afIR* is a pathway regulatory gene coding for a protein involved in transcriptional activation of most of the structural genes. Additionally, studies (Schmidt-Heydt *et al.*, 2009) have

also shown that there may be a relationship between the ratio of *aflR* and *aflS* genes which is influenced by environmental factors.

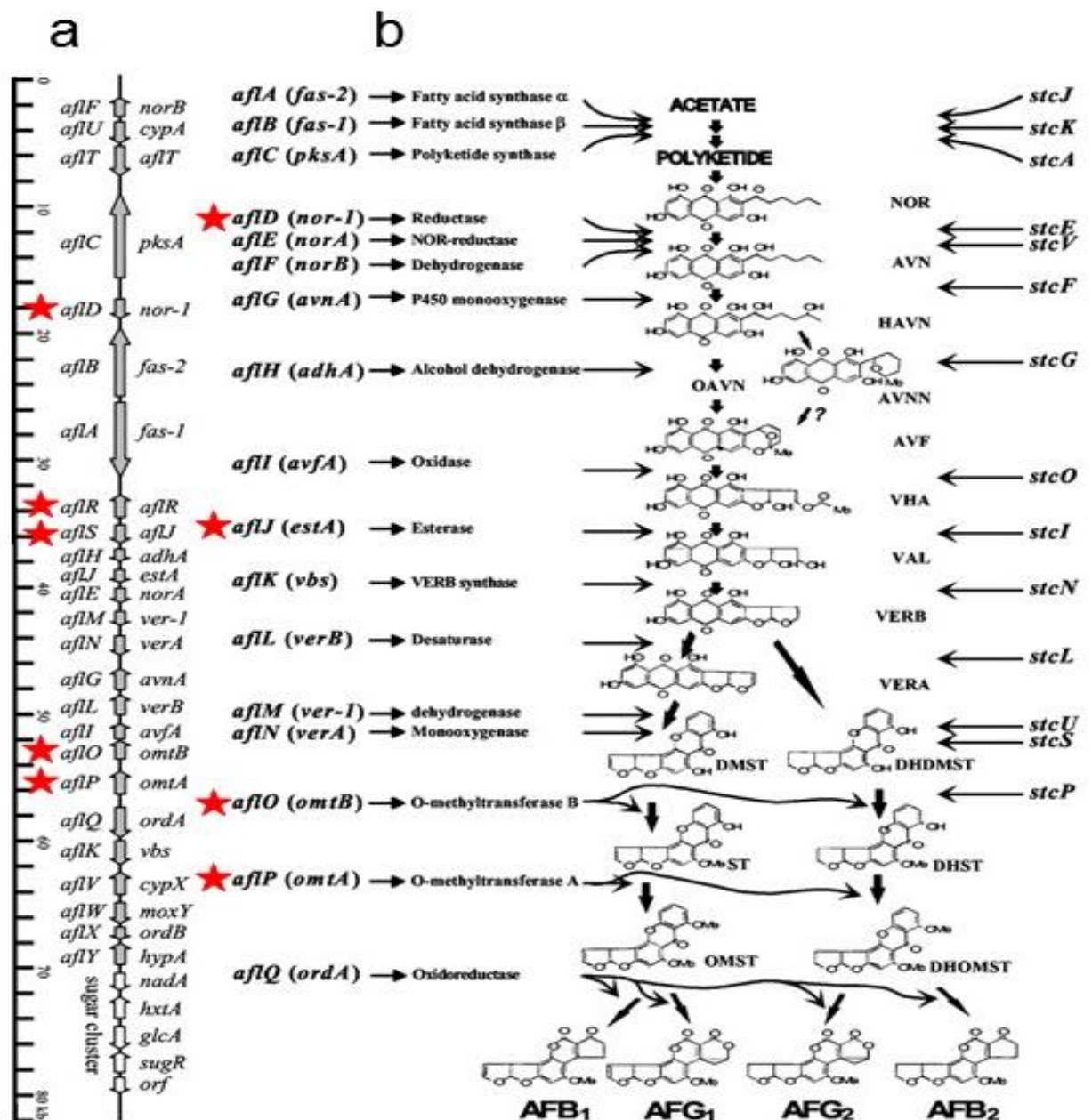


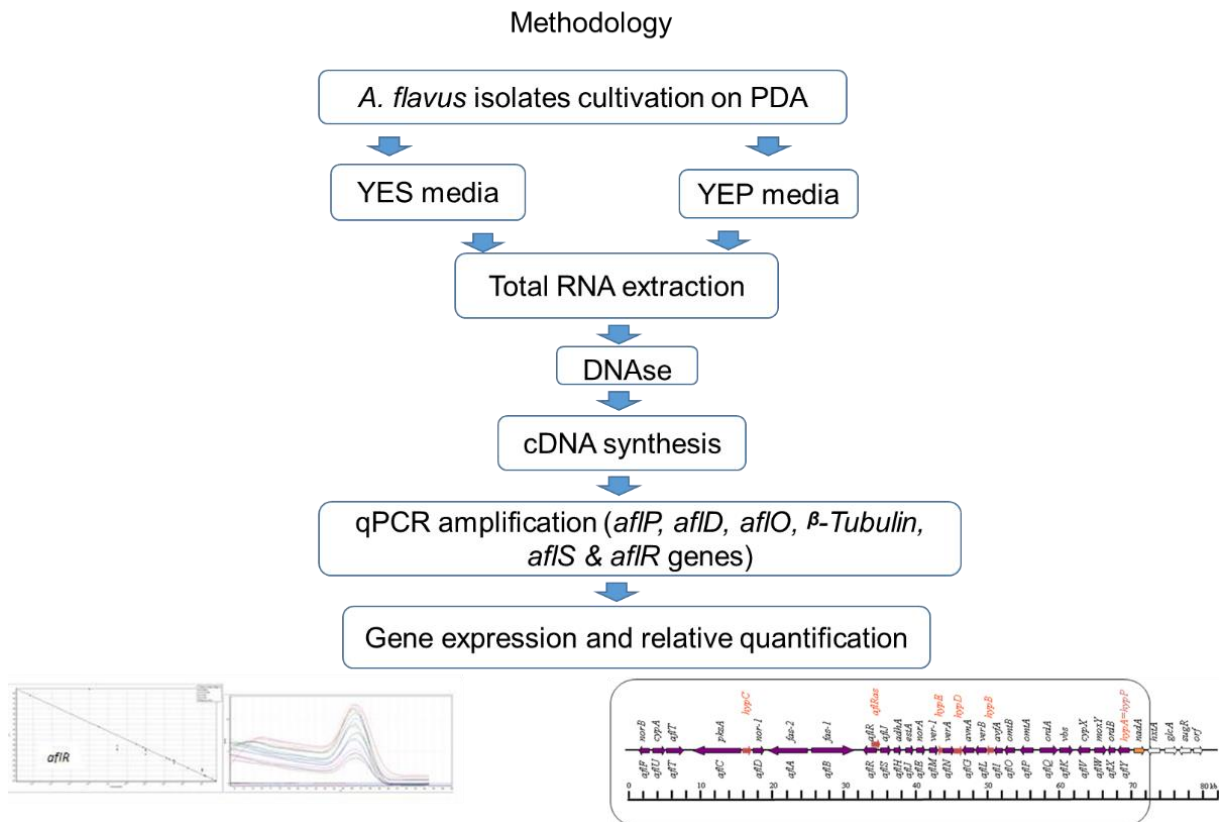
Figure 4.1 A flow diagram showing clustered genes (a) and the aflatoxin biosynthetic pathway (b). The corresponding genes and their enzymes involved in each bioconversion step are shown in panel (a). The vertical line represents the 82-kb aflatoxin biosynthetic pathway gene cluster and sugar utilization gene cluster in *Aspergillus flavus*. The new gene names are given on the left of the vertical line and the old gene names are given on the right. The enzymes involved: fatty acid synthase, polyketide synthase, norsolorinic acid reductase, versiconal hemiacetal acetate reductase, esterase, versicolorin B synthase, versiconyl cyclase, desaturase, O-methyltransferase (MT-II), O-methyltransferase, O-methyltransferase (MT-I); AFB₁: aflatoxin B1; AFB₂: aflatoxin B2; AFG₁: aflatoxin G1; AFG₂: aflatoxin G2 (Adopted from Sweeney & Dobson, 1999; Yu *et al.*, 2004b; Yu, 2012). Asterisks (red star) represents the specific genes studied.

Ehrlich *et al.* (2007), noted that molecular mechanisms responsible for the loss of aflatoxin production are diverse, however, for most atoxigenic *A. flavus* strains, the specific genetic mechanisms resulting in atoxigenicity have not been elucidated.

In the current chapter, the expression profiles of specific genes responsible for aflatoxin production in aflatoxigenic and atoxigenic *A. flavus* strains were examined by using real-time qPCR. These five specific *A. flavus* isolates (KSM012, KSM014, HB021, HB026 and HB027) were chosen from the four climatic regions based on certain characteristics (S and L-morphotype and ability to produce sclerotia).

4.1 Materials and methods

The schematic flow diagram (Fig.4.2) shows the methodology and techniques applied in the study of aflatoxin biosynthetic path way genes cluster responsible for aflatoxin production in aflatoxigenic and atoxigenic isolates. In this chapter, terms “induced” and “un-induced” was used interchangeably with aflatoxigenic and atoxigenic respectively. The isolates were maintained on PDA before culturing onto inducing (YES) and non-inducing (YEP) media, prior to RNA extraction, DNase treatment, cDNA synthesis and thereafter monitored the gene expression profiles of individual aflatoxin biosynthetic pathway cluster genes using RT-qPCR machine.



PDA: potato dextrose agar; YES: yeast extract sucrose; YEP: yeast extract peptone; cDNA: complimentary deoxyribonucleic acid; RNA: ribonucleic acid.

Figure 4.2 Steps involved in gene expression profile study of aflatoxin biosynthetic pathway cluster genes (*aflD*, *aflO*, *aflP*, *aflS* and *aflR*) by real time quantitative polymerase chain reaction machine (RT-qPCR).

4.1.1 *Aspergillus flavus* cultures

Fungal cultures from previous studies (Chapter 2) were routinely maintained on PDA supplemented with chloramphenicol to inhibit bacterial growth. Mono-conidial cultures were obtained by transferring a small amount of mycelium into 1000 μ l of sterile water which served as an inoculation mixture for agar plates. One hundred microliters of the inoculum were then transferred onto the water agar (WA) plate, swirled for a few seconds with conidial suspension and liquid culture discarded off the plate, thereafter, the plate was incubated overnight at room temperature. Mono-conidial colonies were assessed under stereomicroscope, excised, transferred onto new PDA plates and incubated at 25 ° C in the dark. For aflatoxin induction, each *Aspergillus* isolate was grown in aflatoxin-inducing medium (YES) and non-inducing medium (YEP) plates and incubated for 5 days at 25 ° C prior to RNA extraction.

4.1.2 Total RNA extraction

RNA was extracted from each *A. flavus* isolates grown on YES and YEP medium respectively. Mycelia were scrapped off the plates, flash frozen and ground in liquid nitrogen in a sterile mortar and pestle. Approximately 200-300 mg of ground mycelium was overlaid with 750 μ l of Trizol[®] (Sigma Aldrich, USA) in 2 ml tubes containing 0.3 mm diameter glass beads. The mixture was vortexed and incubated for 10 min at room temperature (RT). Chloroform (200 μ l) was added to the homogenized sample and mixed gently for 1 min. The tubes were incubated for 5 min at RT, followed by centrifugation for 15 min (14000 x g) at 4 ° C. The aqueous phase was transferred to a fresh 1.5 ml tube and the organic phase was kept for DNA and protein extraction at -80 ° C. Isopropanol (500 μ l) was added to the aqueous phase and incubated for 10 min at RT to allow RNA precipitation. The mixture was then centrifuged for 10 min (14000 x g) at 4 ° C and the supernatant discarded. The pellets were washed in 1 ml of cold 75 % ethanol for 1 min, then centrifuged for 5 min (14000 x g) at 4 ° C. The supernatant was discarded, and the RNA pellet air-dried for 10 min. The pellets were re-suspended in DEPC water (100 μ l), incubated at 55 ° C for 10-15 min and thereafter stored at -80 ° C for further downstream analysis.

4.1.3 DNase treatment

The RNA extracts were treated with deoxyribonuclease I (DNase I; New England Biolabs, USA) to digest and remove any genomic DNA contaminants. The DNase I reaction mixture consisted of 89 μ l of isolated RNA, 10 μ l of 10 X DNase buffer and 2 units of DNase I in a total reaction volume of 100 μ l. The reaction was mixed gently and incubated for 10 min at 37 ° C. The sample was purified using the Zymo Research Fungal/Bacterial RNA Miniprep Kit (Inqaba Biotec, South Africa) according to the manufacturer's instructions. The RNA was quantified using the Nano Drop ND-1000 spectrophotometer (Nano Drop Technologies, USA) and the integrity was assessed on a 1.2 % agarose/EtBr gel.

4.1.4 First strand cDNA synthesis

Circa 500 ng of RNA was used for cDNA synthesis. The reaction was performed in triplicate using M-MLV Reverse Transcriptase Kit (Promega, Corporations, USA) according to the manufacturer's instructions with modifications. Briefly, a ratio of 1:1

instead of 1:10 random hexamers to Oligo (dT) was selected for cDNA synthesis from the fungal RNA in order to increase sensitivity. The primer mix (reaction volumes of 10 μ l consisted of 5 μ l nuclease free water, 1 μ l 50 ng/ml Random hexamer, 0.1 μ l of 500 ng/ml Oligo dT and 500 ng RNA) was incubated at 70 ° C for 5 min and immediately cooled on ice for 5 min, then briefly centrifuged. The annealed primer mix was added to the master mix (reaction volumes of 14.5 μ l nuclease free water, 10 μ l 5 X M-MLV reaction buffer, 2 μ l M-MLV-RT point mutant, 2.5 μ l dNTP Mix and 1 μ l inhibitor) in the ratio of 1:3, gently mixed, and then aliquoted onto PCR tubes. The reaction conditions consisted of four cycles of 25 ° C for 20 min, 37 ° C for 40 min, 42 ° C for 90 min, followed by an incubation at 70 ° C for 15 min and a final step at 4 ° C for 1 min. To assess for successful cDNA synthesis, samples were initially run on a 1.2 % (w/v) agarose/EtBr gel at 120V for 5 min, thereafter, at 80V for 45 min and visualised as previously described (Chapter 4, section 4.1.5). The synthesised samples were combined and stored at -20 ° C for subsequent use and at -80 ° C for later analyses.

4.1.5 qPCR and primer design

To detect the presence or absence of aflatoxin genes in the induced or un-induced isolates, six sets of primers (Table 4.1) for one reference gene (β -tubulin) and five genes of interests (structural and regulatory) were designed and assessed as previously described (section 3.1.3). The PCR and melt curve analysis were used to identify both specific and non-specific amplification.

4.1.6 qPCR efficiency

The expression profiles and analysis of the genes were investigated using Rotor Gene 6000 2 plex HRM (Corbett Life Science Research, Australia). Serial dilutions of pooled cDNA (10-fold) from induced and un-induced isolates were used to generate standard curves. The Kapa SYBR Fast Kit (Kapa BioSystems, South Africa) master mix containing reaction buffer, heat activated DNA polymerase, dNTPs and a working concentration of 3 mM MgCl₂ were used for each qPCR reaction. The reaction consisted of a final concentration of 1 X Kapa SYBR green, 10 μ M gene specific primers (0.4 μ l), 1 μ l of cDNA and nuclease free water to a total volume of 20 μ l. The primer sets (Table 4.1) were used in separate reactions. Each dilution-point reaction was performed in triplicate along with no reverse transcription control, and a no

template control (NTC) in each real-time run. Amplification was carried out under the following conditions: 95 ° C for 10 min; and 35 cycles of 95 ° C for 3 s, 60 ° C for 20 s, and 72 ° C for 1 s.

4.1.7 Expression stability analysis of aflatoxin biosynthetic genes

The qPCR reaction mixes and conditions were set up as described (Section 4.1.6). To minimise variations between qPCR runs, all the reactions containing one primer pair were performed in a single run. The average expression levels were calculated from three technical repeats and by importing the relative standard curve into each run. Relative gene expression was determined by the amplification threshold in the exponential phase of the PCR, identifying the threshold cycle (Ct) value and comparing the Ct value to the standard curve (Fischer *et al.*, 2000). The stability and potential of the reference genes were evaluated using both GeNorm and NormFinder (GenEx, MultiD, Sweden) based on the Pfaffl equation (Pfaffl, 2001).

Table 4.1 Primer sequences used in the study, amplicon sizes and annealing temperatures (Ta, ° C).

Primer sequences					
Primer code	Target gene	Forward Primer	Reverse Primer	Product size (bp)	Ta (° C)
<i>aflS</i>	<i>aflJ</i>	CTg CAg CTA	TAA ACC CAg gCA	117	60
		TAT TgC CCA CA	gAg TTg gT		
<i>aflO</i>	<i>omtB</i>	gCC Agg ggT ATT	TTC TCC CgA gAg	120	60
		TgT Tgg TA	CAT CTA gC		
<i>aflD</i>	<i>nor-1</i>	CAC TTg gAC gTg	Agg ACg ggA gCA	116	60
		gTg gTT g	TAC ATg TT		
<i>alfR</i>	<i>alfR</i>	CCg ggA TAg CTg	gAT ggT CgC CgA	168	60
		TAC gAg TT	gTT gAA TC		
<i>alfP</i>	<i>omtA</i>	ggC CgC CgC TTT	ACC ACg AC CgCC	123	60
		gAT CTA gg	gCC		
β -Tub	reference	TCC CTT Cgg	TgT TAC Cag CAC	118	60
		CAA gCT TTT C	Cgg ACT gA		

4.1.8 Biostatistics and relative quantification analysis

The threshold cycle (Ct) values of the gene of interest were normalised by that of the reference gene. The average values calculated were used for relative quantification of the gene of interest. The values obtained for transcript levels were used as a calibrator to determine whether a significant change in expression has occurred. Relative quantification levels were determined with the GenEx software (MultiD, Sweden). The equation describes one sample as the ratio of the gene of interest (target) versus a calibrator sample (control) and the reference gene (reference) versus a calibrator sample (control). The amplification efficiencies (E) are calculated as: $E = 10\left(\frac{-1}{\text{slope}}\right)$. The difference in Ct values of the target gene in the control and sample ($\Delta\text{Ct target}$) and in the reference gene in the control and sample ($\Delta\text{Ct reference}$) were considered (Pfaffl 2001). The equation used to calculate the ratio was:

$$\text{Ratio} = \frac{(E_{\text{target}})\Delta\text{Ct target (control-sample)}}{(E_{\text{reference}})\Delta\text{Ct reference (control-sample)}}$$

The experiments were carried out in four biological and three technical replicates. The relative expression level profiles of genes correlated with production of aflatoxins by the isolates were log transformed and assessed. Graphs and analyses were made with GraphPad Prism version 5.02 (GraphPad Software, Inc., USA), One-way analysis of variance (1-way ANOVA), Tukey's Multiple Comparison Test (TMCT), Post-test for linear trend and R statistical software (www.r-project.org), version 3.2.5.

4.2 Results and Discussion

4.2.1 RNA extraction

The quality and integrity of RNA extracted from *A. flavus* strains cultivated on YES and YEP medium was good (Supplementary data, Table S4.1) with clear ribosomal RNA bands (Fig.4.3a). Additionally, there was successful cDNA synthesis, indicated by a continuous smooth cDNA smear (Fig.4.3b).

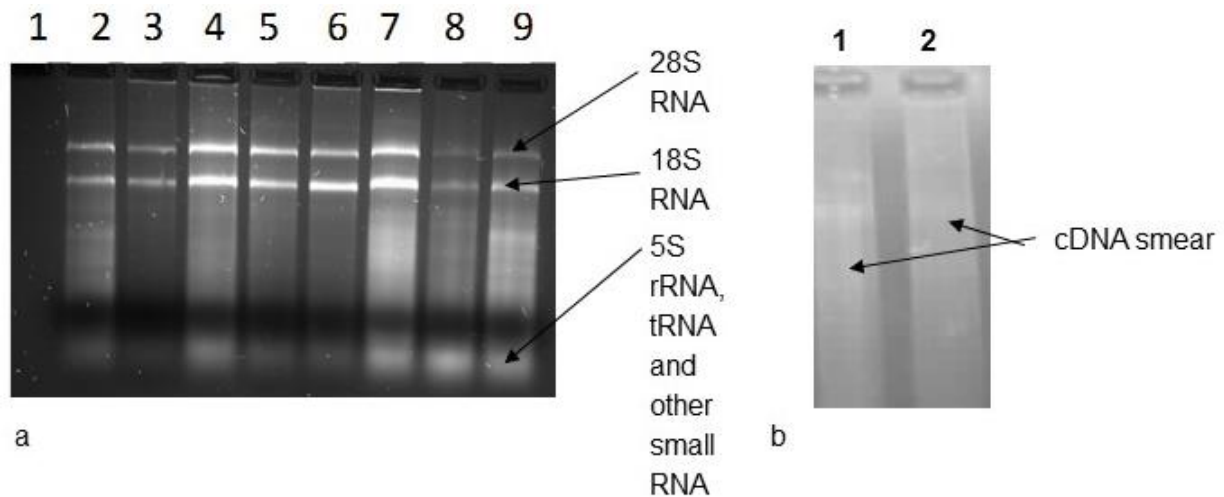


Figure 4.3 Gel electrophoresis of RNA extracted from selected *Aspergillus flavus* strains assessed on a 1.2 % agarose/EtBr gel at 80V for 45 min showing; (a). Total RNA; Lane: 1. No template control; Lanes 2. NC05; 3. KSM012; 4. KSM014; 5. HB021; 6. HB025; 7. HB26; 8. HB027; 9. HB028; (b). cDNA synthesis (Lane: 1. KSM012 and 2. KSM014). (NC: Nandi county; KSM: Kisumu; HB: Homa Bay; EtBr: ethidium bromide; cDNA: complimentary deoxyribonucleic acid; RNA: ribonucleic acid).

4.2.2 qPCR and primer efficiency analysis

The amplicons generated by qPCR showed, the primers used were specific, appropriately designed and suitable for studying *A. flavus* and aflatoxin genes (Tables 4.1; 4.2; 4.3). The standard melt curves exhibited statistical linear regression values and efficiency range (Table 4.2). *afIR* and *afID* displayed unique expression profiles and deletion patterns of transcripts compared to other genes (Table 4.3). Studies conducted by Abdel-Hadi *et al.* (2010) and Abdel-Hadi *et al.* (2012) demonstrated that, the decrease in mRNA expression of *afID* level caused a subsequent decrease in aflatoxin production. Additionally, they found that, changes in aflatoxin production in relation to mRNA level of *afID* showed a good correlation ($r^2 = 0.88$, $p = 0.00001$). In regards to their findings, it could be suggested that *afID* might be absolutely essential for aflatoxin biosynthesis and, inhibition of *afID* gene expression may lead to accumulation of intermediate compounds that blocks aflatoxin biosynthesis. Bennett, (1981) observed that disruption or deletion of *afID* gene leads to the accumulation of norsolorinic acid and thereafter blocks the synthesis of aflatoxins and their intermediates beyond norsolorinic acid. Similarly, Abdel-Hadi *et al.* (2010) and Abdel-Hadi *et al.* (2012) observed that, aflatoxin production could also be disrupted if any step in the aflatoxin biosynthesis pathway is completely blocked by a specific inhibitor.

Table 4.2 Clustered aflatoxin biosynthesis pathway genes showing enzymes involved, functions, statistical linear regression and efficiency.

Genes	Target gene	Enzyme/product	Function in the pathway	Linear regression	Efficiency
<i>β -tub</i>			Reference housekeeping gene	0.99	0.32
<i>afIO</i>	<i>omtB</i>	O-methyltransferase B	<i>DHDMST</i> (dihydrodemethylsterigmatocystin) → <i>DHST</i> (dihydrosterigmatocystin)	0.86	0.44
<i>afIR</i>	<i>afIR</i>	Transcription activator	Pathway regulator	0.82	0.32
<i>afIS</i>	<i>afIJ</i>	Transcription enhancer	Pathway regulator	0.82	0.80
<i>afIP</i>	<i>omtA</i>	O-methyltransferase A	sterigmatocystin (<i>ST</i>) → O-methylsterigmatocystin (<i>OMST</i>)	0.59	1.51
<i>afID</i>	<i>nor1</i>	<i>NOR</i> reductase	norsolorinic acid (<i>NOR</i>) → averantin (<i>AVN</i>)	0.64	0.12

Table 4.3 Different gene expression profiles or deletion patterns exhibited by *Aspergillus flavus* strains, threshold cycle value ratios, mean \pm standard deviation error based on three biological replicates.

Isolates	County	Strain	UV 365nm	Sclerotia			Mean normalised Ct value ratios (log ₁₀)				
				S	L	Status	aflP	aflD	aflO	aflR	aflS
12	KSM	atoxigenic	green	-	-	induced	0.150 \pm 0.017	0.117 \pm 0.008	0.059 \pm 0.012	-0.007 \pm 0.010	0.034 \pm 0.010
						Un-induced	0.093 \pm 0.032	0.099 \pm 0.003	0.067 \pm 0.004	-0.027 \pm 0.006	0.033 \pm 0.004
14	KSM	aflatoxigenic	blue	++	-	induced	0.254 \pm 0.029	0.278 \pm 0.007	0.139 \pm 0.011	0.144 \pm 0.024	0.233 \pm 0.008
						Un-induced	0.218 \pm 0.065	DL	0.181 \pm 0.010	DL	0.259 \pm 0.007
21	HB	afl/atoxigenic	blue/green	-	-	induced	0.177 \pm 0.006	DL	0.113 \pm 0.003	DL	0.153 \pm 0.011
						Un-induced	0.112 \pm 0.026	DL	0.066 \pm 0.003	DL	0.024 \pm 0.004
26	HB	afl/atoxigenic	blue/green	-	++	induced	0.089 \pm 0.007	DL	0.029 \pm 0.012	DL	0.069 \pm 0.004
						Un-induced	0.137 \pm 0.013	DL	0.051 \pm 0.004	DL	0.012 \pm 0.006
27	HB	afl/atoxigenic	blue/green	-	++	induced	0.130 \pm 0.022	DL	0.078 \pm 0.056	DL	0.175 \pm 0.004
						Un-induced	0.109 \pm 0.008	DL	0.051 \pm 0.004	DL	0.036 \pm 0.010

DL: deletion

- or ++: absence or presence of sclerotia

S or **L**-morphotype of sclerotia

KSM: Kisumu

HB: Homa Bay

Additionally, the results showed similarity to the findings obtained (Scherer *et al.*, 2005), where they demonstrated the reduction in aflatoxin was accompanied by a decrease in *aflD* (*nor-1*) transcripts. They concluded that, the expression profiles of *aflD* (*nor-1*), *aflO* (*omtB*), and *aflP* (*omtA*) were consistently correlated with the production of aflatoxins, whereas *aflS* (*aflJ*) and *aflR* were not.

In uninduced state, *aflD* and *aflR* exhibited deletion patterns for the *A. flavus* strains HB021, HB026, HB027, KSM014 except for strains KSM012 and KSM014 (induced) (Table 4.3). In contrast, all *A. flavus* strains (induced/uninduced) were expressed by *aflP*, *aflS* and *aflO* genes. From the observation, it can be suggested that *aflP*, *aflS* and *aflO* might not be appropriate for consideration as a suitable aflatoxin biosynthetic gene for discrimination of *A. flavus* and aflatoxins. The current observation was similar to other research group findings (Trail *et al.*, 1994; Abdel-Hadi *et al.*, 2011). They demonstrated that, in *A. flavus* and *A. pararsiticus*, the expression of *aflD* (*nor-1*), a gene encoding an enzyme that catalyses the conversion of the first stable aflatoxin biosynthesis intermediate, norsolorinic acid to averantin is a key structural gene in the biosynthetic pathway.

Moreover, Cary & Ehrlich, (2000), established that, *aflR* is a pathway regulatory gene coding for proteins shown to be involved in transcriptional activation of most structural genes. Similarly, studies performed by Schmidt-Heydt *et al.* (2009) on peanuts showed that, there might be a relationship between the ratio of associated regulatory genes (*aflR* and *aflS*) which is influenced by environmental factors. Abdel-Hadi *et al.* (2010) and Abdel-Hadi *et al.* (2011) demonstrated the potential use of *aflD* transcription as a good marker to discriminate between aflatoxin producing and non-producing strains isolated from peanuts while *aflR* failed to differentiate between these isolates.

4.2.3 Aflatoxin biosynthesis pathway genes expression profiles

The expression profiles of three structural genes (*aflD*, *aflO*, *aflP*) and two regulator coding genes (*aflR*, *aflS*) were analysed. One-way analysis of variance (1-way ANOVA) and Post-test for linear trend revealed aflatoxin biosynthetic cluster genes exhibiting significant difference between atoxigenic and aflatoxigenic isolates ($P < 0.05$) (Fig.4.4A-E).

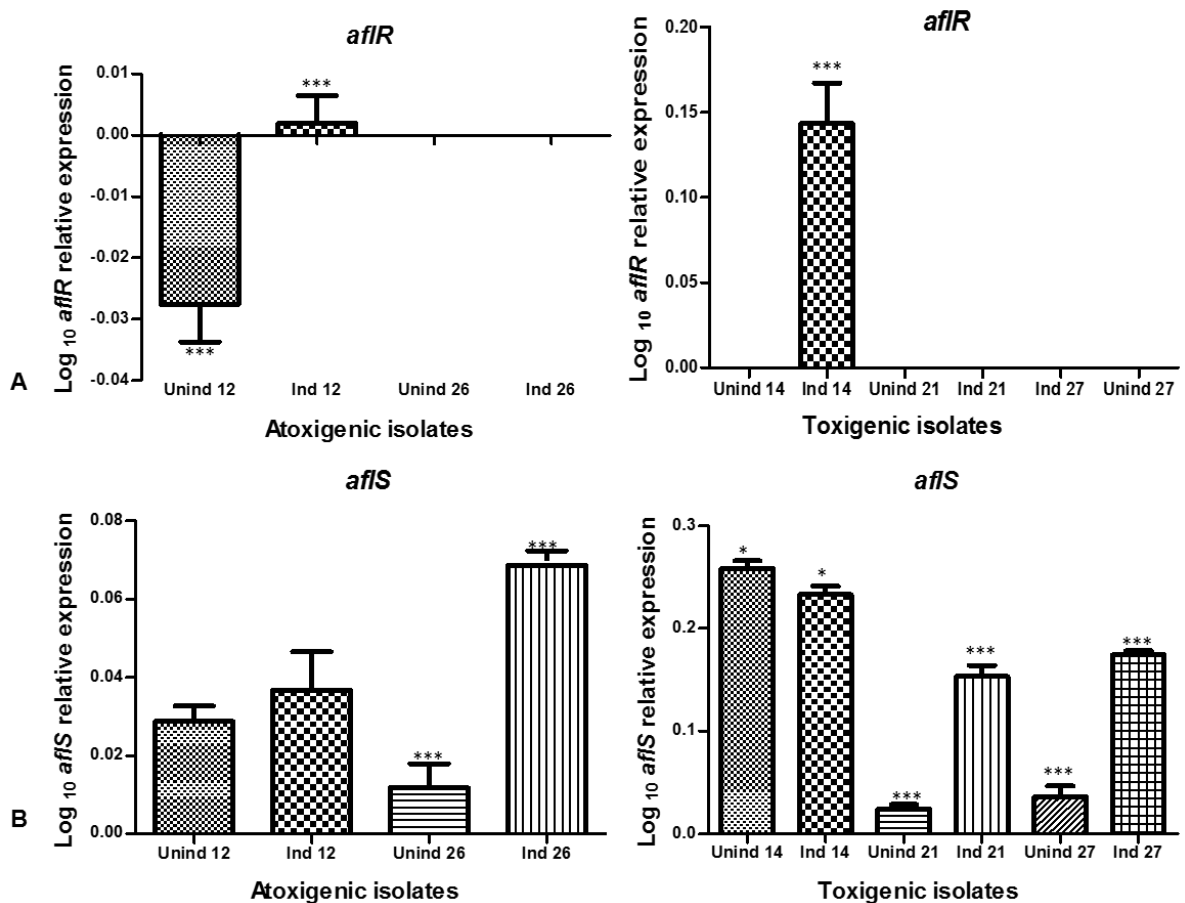


Figure 4.4 Aflatoxin biosynthetic gene cluster expression profiles for *aflR* and *aflS*. **A.** Significant difference in expression were noted in *aflR* for isolates 12 and 14 (both induced), with isolate 14 upregulated significantly. Isolates HB021, HB026 and HB027 displayed no significant expressions except, uninduced isolate KSM012 which was significantly down regulated. **B.** *aflS* exhibited expression profiles for both isolates displaying significant expressions. The expression values were normalised, and log transformed (\log_{10}). Asterisks and the error bars shows significance variance and standard mean deviations ($n = 3$), 1-way ANOVA and Tukey's Multiple Comparison Test ($P < 0.05$). (KSM: Kisumu; HB: Homa Bay; Unind: uninduced; Ind: induced; 12-27: isolates).

Tukey's Multiple Comparison Test (TMCT) revealed significant variances for different biosynthetic genes (Fig.4.4A-E). Significant decrease in transcript abundance in *aflR* gene was exhibited by *A. flavus* strains KSM012, HB021, HB026 and HB027 (Fig.4.4A). In contrast, *A. flavus* KSM014, an aflatoxin producing strain showed significant increase in transcript abundance in induced and decrease in uninduced state (Fig.4.4A). This observation suggests that *aflR* could possibly be considered as a marker for differentiation of toxin and non-toxin producers in the current study.

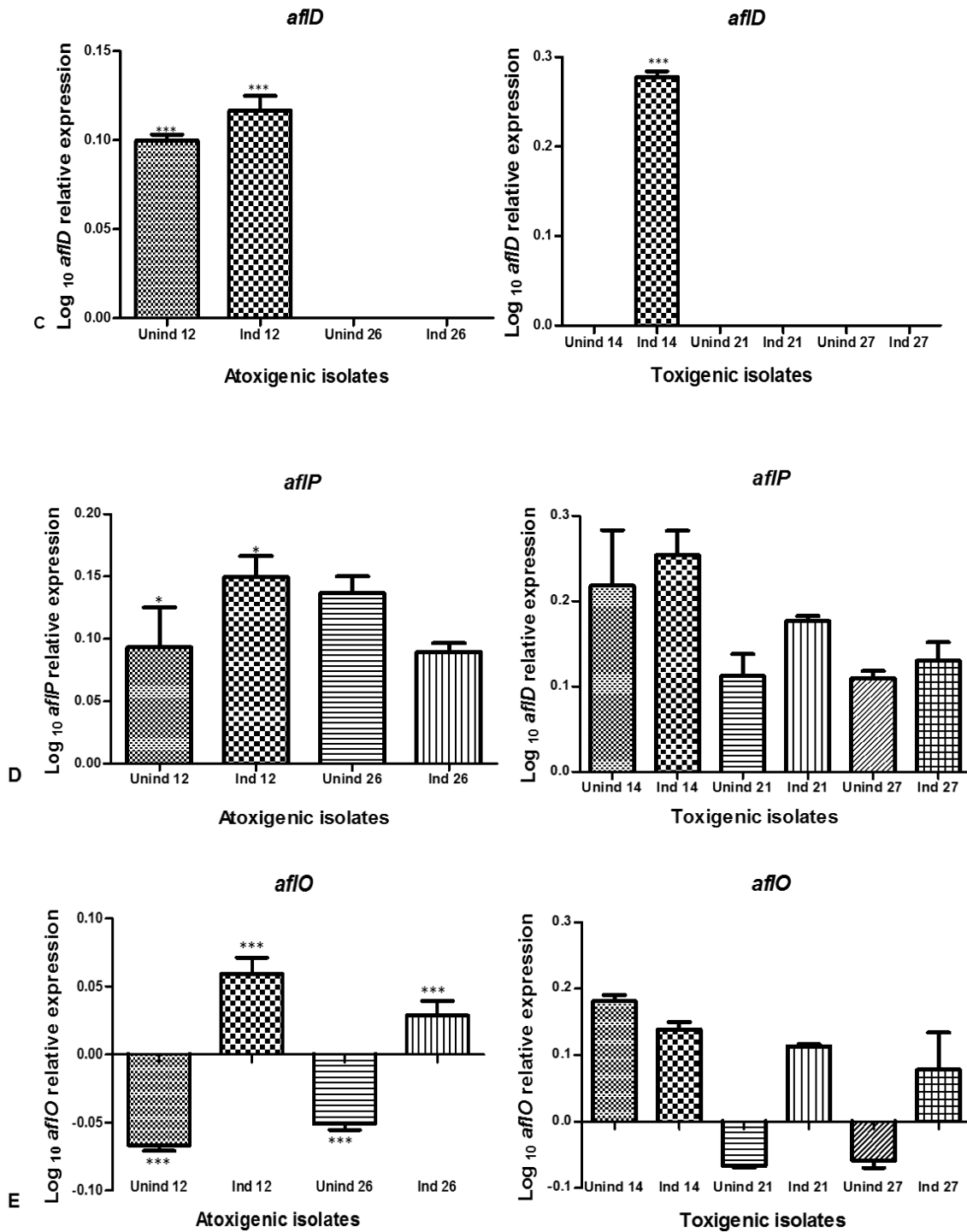


Figure 4.5 Aflatoxin biosynthetic gene cluster expression profiles. **C.** *aflD* exhibited significant expression profiles for *Aspergillus flavus* KSM012 and KSM014, with isolate KSM014 highly expressed. In contrast, no expression profiles were observed for isolates HB021, HB026 and HB027. **D.** *aflP* exhibited expression profiles for both isolates which was not statistically significant except for isolate KSM012. **E.** *aflO* displayed unique expression profiles for both isolates, with significant difference in atoxicogenic isolates and no significant difference for toxigenic isolates. The expression values were normalised, and log transformed (\log_{10}). [Asterisks and the error bars shows significance variance and standard deviations of the mean, 1-way ANOVA and Tukey's Multiple Comparison Test ($P < 0.05$)]. (KSM: Kisumu; HB: Homa Bay; Unind: uninduced; Ind: induced; 12-27: isolates).

In the current study, an aflatoxin producing strain, KSM014, always had higher transcript levels than the other isolates across all of the studied genes (Fig.4.4A-E). In *afIR* and *afID*, there was no significant increase in transcript abundance or fluorescence signals in RT-qPCR observed in isolate KSM014 (uninduced state). This phenomenon probably suggests that the substrate (non-inducing media) could have some suppression effect in aflatoxin production as revealed by both *afIR* and *afID*. In contrast, *afIO* showed that toxigenic isolate KSM014 was over expressed in uninduced state compared to induced. Similar pattern was observed in *afIS* for isolate KSM014. This observation suggests that there could be a possible relationship between *afIO* (structural gene) and *afIS* (regulatory gene) brought about either or due to environmental influence in substrate utilisation. Furthermore, *afIO* showed significant difference in transcript abundance between induced and uninduced isolates (Fig.4.5E). Moreover, *afIO* exhibited decreased transcript abundance in uninduced isolates KSM012, HB021, HB026 and HB027 respectively with an increased transcript abundance in isolate KSM014 (Fig.4.5E).

Expression of the *afIP* gene did not vary significantly between induced and uninduced isolates KSM014, HB021, HB026 and isolate HB027 according to 1-Way ANOVA and TMCT test (Fig.4.5D). *afIP* gene expression was higher for isolate KSM012 (both induced and un-induced states). Expression by, uninduced isolate HB026 was higher than in the induced isolate but the difference was not significant based on a TMC test. This observation suggests that *afIP* is not a good marker for differentiating aflatoxigenic and atoxigenic isolates (Fig.4.5D). *afID*, *afIR* and *afIS* all differed significantly in expression in KSM014 in between induced and uninduced states (Fig.4.4 and 4.5).

All induced isolates except HB026, had higher levels of expression of *afIS* and *afIO*. KSM012, and KSM014 had higher *afID* and *afIR* transcript levels in induced than in uninduced culture (Fig.4.4; Fig. 4.5; Table. 4.3). *afIR* expression decreased significantly in uninduced isolate KSM012 (Fig.4.4A). The *afID* gene transcript was detected in both induced and uninduced isolate KSM012 which is atoxigenic, (Table 2.1) (Fig.4.5C; Table 4.3). False positive and negative transcription signals have been previously observed (Bhatnagar *et al.*, 2003). The regulation of aflatoxin biosynthesis in *Aspergillus* spp. involves a complex pattern of positive and negative transcriptional

regulatory factors, which are affected by physiological response to both external and internal stimuli (Bhatnagar *et al.*, 2003). Chiou *et al.* (2002), postulated that chromosomal location of key genes may also play a role in aflatoxin gene expression. Subsequently, they further observed that, while rapid and accurate, the screening methods based on PCR detection of genes in the biosynthetic pathway of aflatoxins may fail to distinguish true aflatoxigenic isolates from the complex of *Aspergillus* species contaminating food.

Further investigations by Chang *et al.* (2005), showed that all aflatoxin and non-aflatoxin producers harbour the three genes (*afID*, *afIP* and *afIR*). This discounts the possibility that a lack of aflatoxin production in certain strains is due to loss of the genes from the genome. Additionally, (Chang *et al.*, 2005), reported that the loss of production of AFB1 and AFB2 in many non-aflatoxigenic *A. flavus* isolates is not caused by large deletions or a complete loss of the aflatoxin gene cluster, but probably results from point mutations.

Moreover, RT-PCR results have shown that *afID* transcription may be used as a marker to discriminate between aflatoxin and non-aflatoxin producers, while *afIP* and *afIR* failed to differentiate between aflatoxigenic and non-aflatoxigenic strains (Scherer *et al.*, 2005). Similarly, they further reported that *afID* (*nor-1*), gave the best correlation of aflatoxin production and gene expression on inducing and non-inducing media. Their results contrasts Rodrigues *et al.* (2009) findings, where they tested two genes, *afID* and *afIQ*, in 31 isolates of *Aspergillus* section *Flavi* originating from Portuguese almonds and concluded that *afID* expression was not a good marker for differentiating between aflatoxigenic and non-aflatoxigenic isolates based on testing 35 isolates (31 almonds isolate and 4 control strains). Only one almond isolate gave a false positive transcription. The current results also relates to Rodrigues *et al.* (2009), where it was observed that, *afID* and *afIR* transcripts may or may not give a clear distinction between aflatoxigenic and atoxigenic strains. This could be due to either point mutations or large deletions in the genome of aflatoxin genes cluster involved in aflatoxin production (Fig.4.4A, C).

The current study further demonstrates that, *afIO* probably might be considered as marker for differentiation of atoxigenic and aflatoxigenic strains based on the findings (Fig.4.4E; Table 4.3). The current research used more sensitive qPCR

compared to Scherm *et al.* (2005) and Rodrigues *et al.* (2009) who used RT-PCR and got contradictory findings. In contrast, due to the inconsistency of the information from different groups, it is strongly suggested that, further trials and investigations to be conducted to come up with a clear cut molecular marker suitable for differentiation of atoxigenic and aflatoxigenic strains apart from contemporary findings. The qPCR used showed some promising results and therefore, recommended for future research especially with *A. flavus* strains.

Criseo *et al.* (2001), applied molecular techniques widely attempting to distinguish between aflatoxigenic and non-aflatoxigenic strains of *A. flavus* through the correlation of presence or absence of one or several genes involved in the aflatoxin biosynthetic pathway with the ability or inability to produce aflatoxins. Some groups managed to distinguish these species from other food-borne fungi (Criseo *et al.*, 2001), but none was capable of distinguishing aflatoxin producing strains from non-aflatoxin producers.

Studies on aflatoxin production and aflatoxigenic strains differentiation have been assessed by monitoring the expression of aflatoxin genes using the reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR methodologies. Such systems have been applied to monitor aflatoxin production and aflatoxin gene expression based on various regulatory and structural aflatoxin pathway genes in *A. flavus* (Mayer *et al.*, 2003; Degola *et al.*, 2007). It is thus, not surprising that the protocols that can fully differentiate between aflatoxin producers and non-producers have not yet been successfully established. Furthermore, one should be aware that some genes are not exclusive to the aflatoxin biosynthetic pathway, which could create false-positives from sterigmatocystin producing fungi (Paterson, 2006).

4.3 Conclusion

Many enzymatic steps are involved in the aflatoxin biosynthetic pathway. Measuring the expression level of the genes coding for these enzymes or the absence or presence of these genes should provide information on whether a strain is aflatoxigenic. However, despite previous work (Scherm *et al.*, 2005; Rodrigues *et al.*, 2009), there is no agreement on a suitable gene marker for aflatoxin production, other than the measurement of aflatoxin production itself (Mayer *et al.*, 2003; Degola *et al.*, 2007). High genetic similarity between species of *Aspergillus*, as well as a high degree

of intraspecific variability has prevented identification of a suitable molecular markers capable of consistently differentiating the various species in their aflatoxigenic potential.

The present study showed that the expression of certain genes in the aflatoxin biosynthetic pathway to be significantly upregulated compared to others in the same pathway more than the general cluster gene expression level. The *afIP*, *afIS*, *afIR* and *afIO* transcripts were the most upregulated genes across the tested isolates. We observed that *afIS* and *afIO* were always found to be expressed in both induced and uninduced isolates while *afIP* and *afIR* failed to give clear distinction between toxin and non-toxin producing strains.

The modifications and optimisation of the RT-qPCR method used in this chapter gave relatively better discrimination of the isolates with respect to their possible toxigenicity or non-toxigenicity. Therefore, the use of this sensitive tool and modifications developed can be recommended in future research work especially when working with *A. flavus* strains.

CHAPTER FIVE

Infection, quantification and detection of *Aspergillus flavus* (KSM014) in KDV1 and GAF4 *Zea mays* L. lines

5.0 Introduction

In the previous chapters, thirty-seven *A. flavus* strains were isolated and identified across four different Kenyan climatic regions and characterised based on their morphology, genetic diversity and phylogeny. Specific isolates were selected from among these strains to measure expression of regulatory and structural genes involved in aflatoxin biosynthesis associated with aflatoxin production. It was noted that *afIP*, *afIS* and *afIO* were up-regulated in *A. flavus* when grown on medium that induced aflatoxin production (Chapter 4, section 4.2.2-4.2.3).

In the present chapter, two maize lines grown in different climatic regions of Kenya were infected with an aflatoxigenic *A. flavus* isolate (KSM014) and fungal colonisation of the maize plant tissues was monitored by measuring fungal biomass load after 14 days in a controlled environment. The objective of the study was to determine whether the maize line colonised was a factor in increasing or limiting the growth of an aflatoxigenic strain.

Fungal biomass quantification is critical in understanding the interactions between the pathogen and susceptibility or resistance of the host plant as well as identifying competition between individual fungal spp. in disease progression (Coninck *et al.*, 2012).

qPCR has been used to detect and quantify fungal biomass in various plant host tissues (Sanzani *et al.*, 2014, Mackay *et al.* 2007). Sanzani *et al.* (2014) demonstrated that, the high level of sensitivity of qPCR enables the measurement of very low infection titres, which could correspond to the amount of a pathogen present at the time of infection or during latent, non-symptomatic infections. qPCR also enables the evaluation of stages of infection in plant tissues and the quantification of a fungal pathogen throughout the entire disease cycle (Sanzani *et al.*, 2014).

Coninck *et al.* (2012) developed a qPCR assay for detection and quantification of *Cercospora beticola* fungi in leaves of sugar beet. Moreover, Waalwijk and co-workers (2008), using a gene involved in fumonisin biosynthesis developed a qPCR

assay to quantify and detect fumonisin producing *Fusarium verticillioides* strains from maize obtained from South African subsistence farmers. These results were then compared to the fungal DNA content and with the fumonisin levels of the respective *F. verticillioides* strain. A qPCR assay was also developed by Nicolaisen *et al.* (2009) using *Ef1a* for quantification and detection of eleven *Fusarium* spp. isolated from field materials associated with wheat and maize. Similarly, Korsman and co-workers (2012), demonstrated the use of qPCR assay for detection and quantification of Grey leaf spot disease in maize leaves using cytochrome P450 reductase gene. These studies demonstrated the potential use of qPCR for detection and quantification of fungal pathogens and for probable selection of resistant plant cultivars in breeding lines. This also helps in understanding the processes involved in infection in a host-pathogen system and providing information on the bioecology (Demontis *et al.*, 2008; Covarelli *et al.*, 2012).

Mayer *et al.* (2003) and Jurado *et al.* (2006) used single copy mycotoxin biosynthetic genes to develop PCR assays for detecting of mycotoxigenic fungi. Assay sensitivity increased when ITS1 and ITS2 spacer regions were included as, these regions have sufficient variability to enable discrimination of closely related species in the genus *Aspergillus* (Edwards *et al.*, 2002). Subsequently, these regions have been successfully used for detection and identification of aflatoxigenic *Aspergillus* spp. (González-Salgado *et al.*, 2008; Sardiñas *et al.*, 2010).

The objective of this study was to develop a sensitive, specific qPCR assay for quantifying *A. flavus* biomass in infected maize tissues. The assay was used to measure, the sensitivity of two dry land African maize lines grown in Kenya KDV1 and GAF4, when infected with an aflatoxigenic isolate (KSM014). Similar studies have been done with other fungal species, but, this study is the first where the biomass of *A. flavus* from infected maize was detected and quantified with qPCR. This approach also could be used to discriminate between inbred maize lines that are sensitive or resistant to specific *A. flavus* strains and to help understand the mechanism of the maize defence response to *A. flavus*.

5.1 Materials and Methods

5.1.1 Cultures of fungi

The aflatoxigenic *A. flavus* KSM014 isolate was cultivated and maintained as described previously (Chapter 3, section 3.1.2) and thereafter stored as spore suspension in 15 % glycerol for short term storage at -20 ° C or for long term storage at -80 ° C prior to DNA/RNA extraction.

5.1.2 Maize cultivars

Kenya Dryland Varieties KDV1 and GAF4 were purchased from Kenya Agricultural and Livestock Research Organisation (KALRO), Nairobi, Kenya. The varieties were selected based on the agroecological region in which they were cultivated and their drought tolerance. KDV1 is an open-pollinated variety that is recommended for low to medium altitude. It matures early, is drought tolerant, and flowers between 45-52 days after germination. It is commonly grown in Makueni and Homa Bay (<http://drylandseed.com>). GAF4 is a *Striga* tolerant variety developed by the Kenya Agricultural Research Institute in Kibos, Kisumu county. It is grown in parts of western Kenya: Kisumu, Homa Bay and Busia (Sunda *et al.*, 2012).

5.1.3 Reagents and media preparation

Murashige and Skoog medium (MS), Phytigel, Glycine, Nicotinic acid, Thiamine hydrochloride, Pyridoxine hydrochloride, Myo-inositol, Potassium hydroxide were from Sigma-Aldrich (USA). MS vitamins; 250 mg nicotinic acid, 5 g myo-inositol, 500 mg pyridoxine-HCl, 100 mg glycine, and 500 mg Thiamine-HCl was prepared in sterile water, filter sterilised and stored at -20 ° C until use according to the manufacturer's instructions (Sigma-Aldrich, USA). The MS media was modified, briefly prepared by dissolving 2.15 g MS salts in sterile water, thereafter, 10 ml MS vitamins added and adjusted to pH 5.7 with 1 M KOH and volume adjusted to 1 litre with sterile water. Five grams of phytigel was added to MS media prepared and microwaved to dissolve the salts. The media (50 ml) was dispensed into tissue culture vessels, autoclaved and allowed to cool in biosafety cabinet (BSC) level 2 for approximately one hour before inoculations as described (Section 5.1.4).

5.1.4 Seed sterilisation and *Aspergillus flavus* infection

The seeds were sterilised in a biosafety cabinet, level 2 [Contained Air Solutions (CAS) BioMAT2, UK]. 20 ml of 95-100 % ethanol was used for sterilisation of viable seeds for 1 min with brief shaking for 15 s. Ethanol was discarded and replaced with 20 ml of 2.5 % sodium hypochlorite. It was then left to stand for 15 min thereafter shaken for 30 s and the liquid discarded. The seeds were washed 5 x with sterile water with intermittent shaking between each wash. The desired amount of sterile water was added and left to stand for 1 hour at room temperature. The water was replaced with 20 ml of 2 % Tween 20 and shaken for 30 s. The seeds were inoculated by conidia suspensions adjusted to 1×10^6 conidia ml⁻¹ using a haemocytometer. The tubes containing the seeds were sealed, para filmed and kept at 30 ° C for 30 min in a shaking incubator. Control seeds were treated with sterile water instead of conidia-spores and incubated under the same conditions. The inoculated seeds were left to dry in Petri dishes overlaid with Whatman No.1 filter paper overnight. Subsequently, the seeds were inoculated onto tissue culture media vessels and thereafter germinated in a Plant growth chamber, Conviron (Winnipeg, Manitoba, Canada) set at 28 ° C. The growth was monitored for a period of 14 days, plant tissues (shoots and roots) were harvested separately and stored at -80 ° C after flash frozen in liquid nitrogen prior to DNA/RNA extraction.

5.1.5 DNA extraction from *Aspergillus flavus* and maize tissues

DNA was extracted from 100 mg of each of the following samples: *A. flavus* KSM014 mycelia, infected and control healthy maize tissues following the method of Möller *et al.* (1992) with modifications. Briefly, 2 % SDS, 100 mM Tris pH 8.0, modified TES buffer, 10 mM EDTA, and 2 % (w/v) polyvinylpyrrolidone (PVP) was prepared. Four hundred and fifty microliters of TES buffer and 5 µl RNase (10 mg/ml) was added to a 2 ml microtube containing the tissues and homogenised with a microtube pestle or vortex for 15 min. 20 µl Proteinase K (1 µg/µl) was added, vortexed for 1 min, thereafter incubated at 60 ° C for 1 hour. 160 µl of 5 M NaCl (0.3 vol.), 70 µl 10 % CTAB (0.1 vol.) was then added and subsequently incubated for 10 minutes at 65 ° C. Chloroform/isoamyl-alcohol (24:1) (750 µl) was added, vortexed for 5 min and again incubated for 30 min on ice and then centrifuged for 10 min at 14,000 rpm. The aqueous phase was transferred carefully onto a new 2 ml microtube and 300–350 µl

isopropanol (0.55 vol.) added and then mixed gently for 30 s and left to stand at RT for 30 min. The mixture was centrifuged at 14,000 rpm for 10 min. Supernatant was discarded, the pellets rinsed twice with chilled 70 % ethanol (700 µl), gently mixed and centrifuged again for 2 min at 14,000 rpm. Ethanol was discarded, pellets air dried and dissolved in 40 µl TE buffer (10 mM Tris-Cl pH 8, 1 mM EDTA pH 8) or nuclease free water. DNA integrity was assessed on a 1 % agarose/EtBr gel and the concentration quantified on a Nano-Drop™ 1000 spectrophotometer (Nano Drop Technologies, USA). DNA was diluted to 10 ng/µl for further analysis.

5.1.6 Primer design

Three sets of primers (Table 5.1); *β-tubulin*, *Elongation factor 1 alpha (Ef1a)* and Membrane protein (MEP) were used in this study. *β-tubulin* was designed in Primer3 ver. 4.0 programme (Untergasser *et al.*, 2012) whereas, *Ef1a* and MEP were obtained from Dr. Shane Murray (pers. Comm). Potential secondary structure formation was assessed in DNAMAN software ver. 6.0 (Lynnon LLC., USA) and further verified in OligoAnalyzer Tool (Integrated DNA Technologies). The PCR and melt curve analysis were used to identify both specific and non-specific amplification.

Table 5.1 Specific primers used in the current study.

Primer name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)	Ta	Reference
Membrane Protein (MEP)	TGTA CT CGGCAATGCTCTTG	TTTGATGCTCCAGGCTTACC	203	64 °C	Manoli <i>et al.</i> , 2012
Elongation Factor 1 alpha (<i>EF1α</i>)	CGTTTCTGCCCTCTCCA	TGCTTGACACGTGACGATGA	102	62 °C	Nicolaisen <i>et al.</i> , 2009
<i>β-TubM</i>	TCTTCATGGTTGGCTTCGCT	CTTGGGT CGAACATCTGCT	118	62 °C	(Mitema <i>et al.</i> , 2018)

5.1.7 PCR amplification

Conventional PCR amplification was performed in volumes of 25 µl and consisted of 10 x reaction buffer with MgCl₂, 0.5 µl of 10 µM dNTPs (Bioline), 1 µl of 10 µM reverse and forward primers, 1 µl of 10 ng DNA template, 0.2 µl Kapa Taq and sterile water. Cycling conditions were performed according to the following protocol: 1 cycle at 94 ° C for 5 min followed by 35 x (at 94 ° C for 30 s, at 60 ° C for 45 s, at 72 ° C for 90 s). Elongation step was achieved at 72 ° C for 7 min and finally at 4 ° C for 1 min. The products of PCR were assessed on 2 % agarose/EtBr gel in TAE 1 X buffer

(Tris–acetate 40 mM and EDTA 1.0 mM). Fermentas (100 bp DNA ladder) was used as a molecular size marker.

5.1.8 Standard curves and fungal quantification

A ten-fold serial dilution of pooled 10 ng genomic DNA extracts from control plants and *A. flavus* were used to generate standard curves. For each dilution, the threshold cycle (Ct) values were plotted against the logarithm of the starting quantity of the template. Efficiencies of amplification were generated from the standard curves slopes according to the methods (Kubista *et al.*, 2006; Sardiñas *et al.*, 2011). Additionally, linear regression curves were drawn, and the qPCR efficiency was calculated as: $E = 10\left(\frac{-1}{\text{slope}}\right)$.

The amount of target DNA in an unknown sample was extrapolated from the respective standard curves.

Isolated DNA (10 ng) from healthy and infected maize shoots and roots respectively were used to test the specificity of the primers. To exclude false negative results, template DNA samples from fungi were tested for PCR amplification with primer pairs β -*Tub* and *EF1 α* . DNA extracted from control plant tissues and from pure fungal cultures (*A. flavus*) were pooled together, diluted to 10 ng/ μ l and used to estimate the amount of fungal DNA template in the infected plant tissue. The final fungal DNA template concentrations were 1, 5×10^{-1} , 2.5×10^{-1} , 1.25×10^{-1} , 6.25×10^{-2} , 3.125×10^{-2} ng/ μ l. These dilutions were used to determine the detection limits of the β -*Tub* and *EF1 α* primer pair in the infected plant tissues. A serial dilution of DNA extracted from healthy maize tissue also was prepared to measure the detection limits of the MEP. To normalise gene quantification between different samples, the amount of fungal DNA as calculated by the Ct value for β -*Tub* and/or *EF1 α* was divided by the amount of maize DNA as calculated by the Ct values for *MEP*. Rotor Gene 6000 2 plex HRM (Corbett Life Science Research, Australia) was used to evaluate the gene expression profiles. Master mix, Kapa SYBR Fast Kit (Kapa BioSystems, South Africa) containing DNA polymerase, dNTPs, reaction buffers and 3 mM MgCl₂ were used for each PCR reaction. Final concentrations of 1 X Kapa SYBR green, 10 μ M gene specific primers (0.2 μ l forward and 0.4 μ l reverse) and 1 μ l of gDNA template were

prepared to a total volume of 20 µl using nuclease free water. Primer sets of specific genes (Table 5.1) were used in separate reactions which were performed in triplicate.

For the integrity and quality of the isolated DNA, samples from control and infected tissues of the plant, and saprophytic fungi were subjected to PCR analysis with the reference genes under the following amplification conditions: 95 ° C for 10 min; 35 cycles of 95 ° C for 3 s, 64 ° C for 20 s, 72 ° C for 1 s for MEP and at aT 62 ° C for both *β-tubulin* and *Ef1a*.

5.1.9 Statistical analysis

The statistical analysis was performed as previously described (Chapter 4, sections 4.1.7-4.1.8).

5.2 Results and Discussion

5.2.1 Gene specificity and qPCR assays

To our knowledge, a qPCR assay for the detection and quantification of *A. flavus* biomass using extracted fungal DNA from control or infected maize tissues has not been previously reported. Since this is the first report, our discussion will be in comparison with reports for *Fusarium* spp. and related fungi where this assay is more commonly used.

In this study, the qPCR assay was developed to specifically detect and quantify *A. flavus* gDNA in maize tissues. Primers were designed, and their specificity was confirmed by testing against control and infected tissues (Fig.5.1). The fungal biomass in the co-infected shoots differed from the fungal biomass in the roots according to 1-way ANOVA analysis and TMCT test ($P < 0.05$).

Amplification of the MEP gene (203 bp) was used to detect maize DNA, while amplification of *β-tubulin* (118 bp) and *Ef1a* (102 bp) were used to detect *A. flavus* DNA (Table 5.1; Fig.5.1). The specificity of the primer pairs was determined by conventional PCR (Fig.5.1) after *A. flavus* KSM014 infection of GAF4 and KDV1 maize lines. *A. flavus* DNA extracted from infected maize plant tissues, for both lines, gave an amplification product for both *β-tubulin* (118 bp) and *Ef1a* (102 bp) (Fig.5.1). However, there was amplification product for *Ef1a*

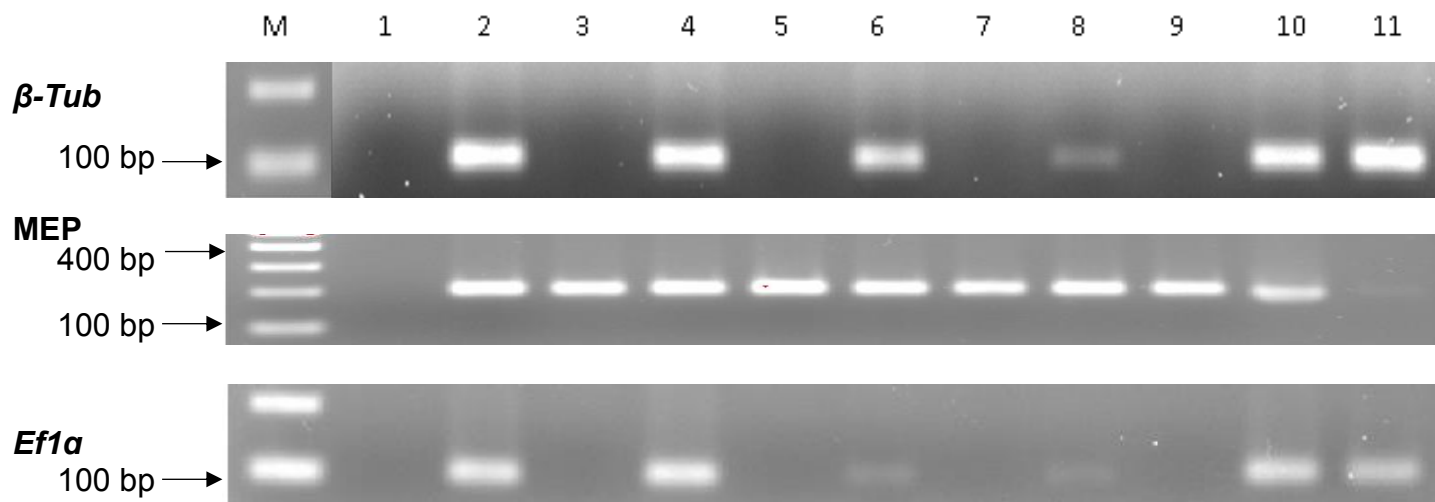


Figure 5.1 Gel electrophoresis images of qPCR amplicon sizes for *Aspergillus flavus* marker genes (*β-Tub*, *Ef1a*) and maize marker gene (MEP) assessed on 2 % agarose/EtBr gel run at 80 volts for 45 min. **M.** 100 bp ladder; **1.** NTC; **2.** Pooled samples (Pure fungal gDNA and Maize gDNA); **3.** GAF4 (control roots); **4.** GAF4 (infected roots) **5.** GAF4 (control shoots); **6.** GAF4 (infected shoots); **7.** KDV1 (control shoots); **8.** KDV1 (infected shoots); **9.** KDV1 (control roots); **10.** KDV1 (infected roots); **11.** KSM014 (Positive control).

than there was for to *β-tubulin* (Fig.5.1), especially in the roots. The MEP gene (203 bp) was amplified in both control and infected maize plants for both lines (Fig.5.1). MEP amplification was plant specific and *β-Tub* and *Ef1a* were fungal specific. Based on these results, *β-Tub* is a better marker for detecting *A. flavus* in infected maize tissues than was *Ef1a* (Fig.5.1), and was used for fungal biomass determination.

5.2.2 Colonisation of plant tissues by *A. flavus*

Aspergillus flavus KSM014 infection of both maize lines resulted in changes in maize phenotype with the KDV1 showing more severe symptoms than GAF4 (Fig.5.1, Supplementary data, Table S5.1). After 3-14 days post infection, the infected kernels for both maize lines showed stunted growth compared to control kernels (Figs.5.1). Additionally, the shoots and roots exhibited minimal growth with the *A. flavus* fungi colonising the kernels and this could possibly explain the reason for stunted growth or germination (Supplementary data, Table S5.1). The phenotypic observations suggest that KDV1 maize line grown in Makeuni is more susceptible to fungal infection (*A. flavus*), whereas GAF4, grown in Kisumu and Homa bay appeared more resistant to the infection (Figs.5.2; Supplementary data, Table S5.1).

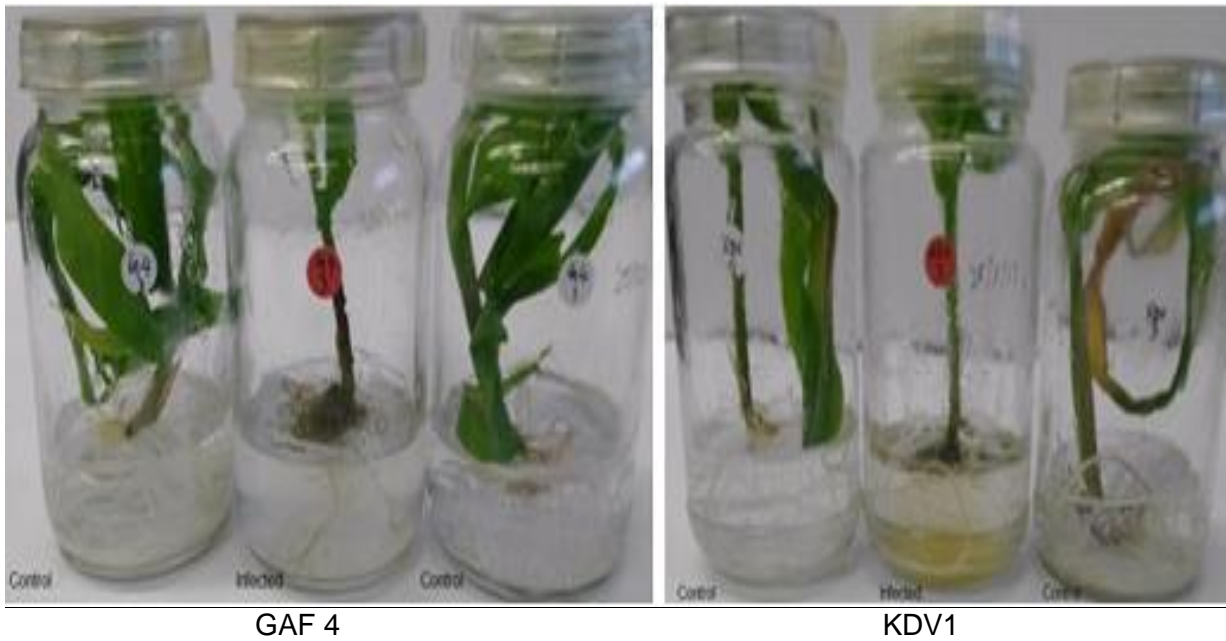


Figure 5.2 The GAF4 and KDV1 maize lines after 14 days of growth with and without *Aspergillus flavus* KSM014 infection. The red sticker shows infected maize plants while the white stickers are the control, uninfected maize plants.

The observed phenotypic characteristics were further supported by the detection and quantification of fungal biomass load in gDNA extracted from infected and control plant tissues as revealed by the qPCR assay (Fig.5.3).

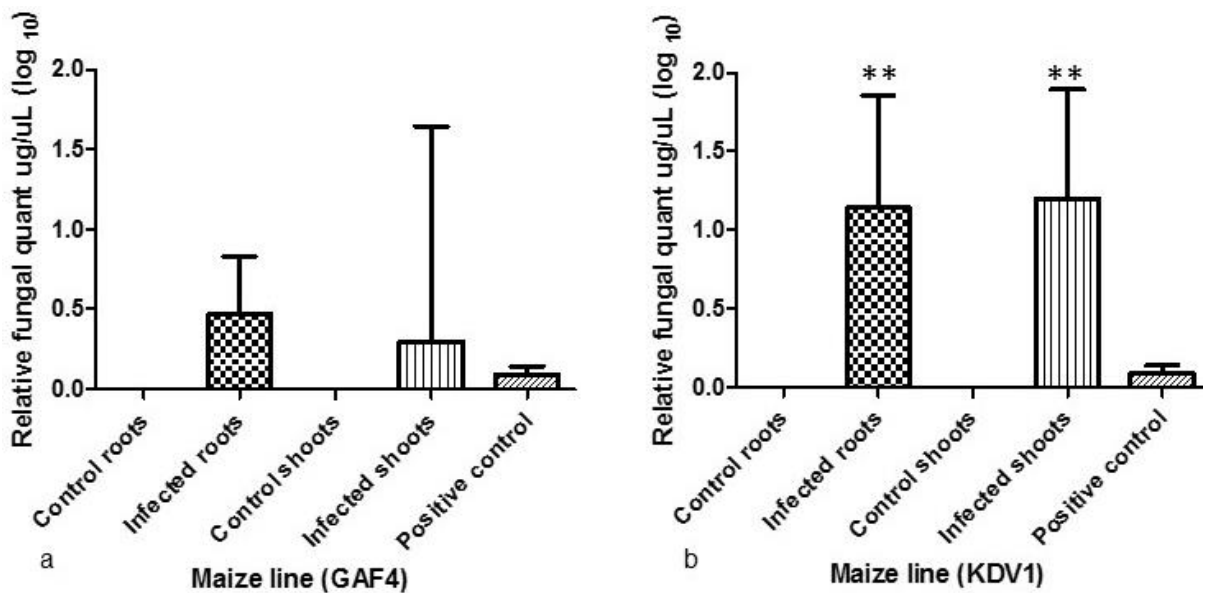


Figure 5.3 qPCR analysis showing fungal load of *A. flavus* KSM014 in the root and shoot tissue of GAF4 and KDV1 maize lines respectively. Fungal biomass was measured in infected and non-infected (control) GAF4 (a) and KDV1 (b) maize lines after 14 days where the *A. flavus* β -*Tub* gene was used for fungal quantification against the maize MEP gene. A one-way ANOVA and Tukey's Multiple Comparison Test; $P < 0.05$), was done where the asterisks indicate significance and the error bars shows standard mean deviation.

No significant difference was seen in fungal biomass between the control and infected plant tissues for the GAF4 maize line (Fig.5.3a). In contrast, significant differences in fungal biomass for the KDV1 maize line was observed upon infection ($p < 0.05$) for both the root and shoot tissue (Fig.5.3b). The level of fungal gDNA was lower in the infected tissue of the GAF4 maize line compared to KDV1 suggesting that GAF4 was more resistant to *A. flavus* KSM014 infection than KDV1 (Fig.5.3).

The fungal biomass of *Alternaria dauci* was observed to be equivalent in two carrot cultivars between 1-15 days of post-inoculation, whereas it was found to be four-fold higher in the more susceptible cultivar between 21-25 days post-inoculation (Boedo *et al.*, 2008). This suggests that fungal pathogens may colonise both susceptible and resistant cultivars in a similar manner during the first stages of the interaction, but fungal development is subsequently restricted in the partially resistant cultivar due to putative plant defence mechanisms (Boedo *et al.*, 2008).

It must be noted that we measured fungal biomass fourteen days after infection when symptoms of the infection was phenotypically visible. However, other fungal biomass studies have shown that specific fungi could be detected even before symptom development. Debode *et al.* (2009) detected the presence of *Colletotrichum acutatum* by qPCR in strawberry leaves two hours post-inoculation whereas the first symptoms of the disease only appeared after 96 hours. Similarly, Divon & Razzaghian, (2012), accurately measured *Fusarium langsethiae* gDNA in oats independently from disease symptoms. These findings show the specificity and efficiency of the qPCR assay for the detection and quantification of fungal pathogens upon infection at early stages, before symptomatic appearances.

GAF4 is a *Striga* spp. resistant maize line cultivated in Kisumu, Kibos, Homa Bay and some parts of Nandi, while KDV1 is an open pollinated maize variety cultivated in Makueni and the neighbouring counties. The observation that KDV1 maize line as more susceptible to aflatoxigenic *A. flavus* (KSM014) infection could be one of the contributing factors to why Makueni and the neighbouring regions are more prone to frequent aflatoxicosis outbreak and high levels of aflatoxin contamination of the maize used for consumption.

The current study relates to the previous findings on Makueni maize samples (Mitema *et al.*, 2018); Chapter 3: section 3.2.3) where we screened the strains of *A.*

flavus isolated from maize kernels obtained from Makueni region on CAM media and found that there was significant variation in production of blue (toxigenic) and green (atoxigenic) fluorescence by most isolates. Seventy eight percent of the isolates from Makueni were observed to produce high amounts of aflatoxin AFB1, AFB2, the most potent carcinogen compared to other regions under study (Chapter 3; Fig.3.6d; (Mitema *et al.*, 2018). Additionally, studies conducted by Probst *et al.* (2010) in eastern Kenya, revealed a similar result where they performed culture-based methods to monitor and describe the population structures of aflatoxigenic fungi and its closely associated strains on maize kernels. Moreover, a related study by Lewis *et al.* (2005) and Klich, (2007) observed that in sub-Saharan Africa, products from subsistence farmers may reach the final consumer without the appropriated monitoring, resulting in critical risks for human health.

Moreover, the current study developed a qPCR assay using *A. flavus* gDNA and the β -tubulin gene for the quantification of *A. flavus* in maize tissue. Due to its high sensitivity and specificity, qPCR has been incorporated in official protocols of the European Plant Protection Organization ([http:// archives.eppo.org/index.htm](http://archives.eppo.org/index.htm)) for the production, certification and assessment of healthy plant materials (Blanco-meneses *et al.*, 2011; Boutigny *et al.*, 2012). This could therefore, in future, provide a screening strategy for finding African maize cultivars that are resistant to *A. flavus* infection or as an assessment of healthy maize plants. Zhao *et al.* (2013) developed a qPCR assay for the detection of *Magnaporthe poae* resistant *Poa pratensis* (Kentucky bluegrass turf), which typically needed three weeks to detect using conventional culture-based methods. Further, Montes-Borrego *et al.* (2011) demonstrated that fungal presence can be detected earlier, enabling the selection of resistant plants even when samples are indistinguishable based on visual assessment. Lastly, the early detection of latent infections of rust on leaves of cereals was used to estimate infection levels before the appearance of the disease (Sanzani *et al.*, 2014).

The genomic DNA extracted from the co-infected shoots of both maize lines showed varied concentrations of fungal biomass load compared to the roots according to analysis using 1-way ANOVA and TMCT test ($P < 0.05$). The quantification of *Verticillium dahliae* gDNA in different tomato cultivars also revealed the concentration of pathogen DNA in plant tissues increased and decreased in susceptible and

resistant cultivars, respectively (Gayoso & Ilarduya, 2007). Similarly, significant differences were found in the amount of *F. oxysporum* DNA in roots of different chickpea cultivars (Jiménez-Fernández *et al.*, 2010), while the detection of *Phomopsis sclerotioides* in pumpkin, melon, cucumber and watermelon showed that infection and rate of disease development of this polyphagous pathogen may vary according to the host (Shishido & Kubota, 2013). In general, (Vandemark & Barker, 2003), concluded that low levels of pathogen DNA in resistant plants is indicative of a mechanism that inhibits pathogen growth, whereas, the presence of a relatively high amount of pathogen DNA in asymptomatic plants indicates a resistance mechanism based on tolerance rather than on true resistance.

5.3 Conclusion

The study demonstrated that KDV1 maize line was more susceptible to *A. flavus* infection when compared to GAF4. This also implies that a possible reason for the frequent cases of aflatoxicosis in Makeuni county is the fact that the KDV1 maize line is grown in that region is more susceptible to *A. flavus* infection.

The β -Tubulin gene is a potential marker for quantification of the *A. flavus* biomass load in maize plants compared to *Ef1 α* . The MEP gene for maize gDNA was also found to be plant specific by the absence of cross-reaction with fungal gDNA. The specificity of the qPCR assay for *A. flavus* biomass quantification makes it a useful tool in other areas such as screening of *A. flavus* resistant maize lines for breeding, determining possible asymptomatic infection and in plant-pathogen interaction studies.

The next chapter will focus on in vitro biocontrol approach in aflatoxin mitigation and bio-analytical approaches to detect and quantify aflatoxins. The aim is to determine whether biocontrol can minimise aflatoxin production and to find important metabolites that are produced by specific *A. flavus* isolates.

CHAPTER SIX

Metabolomics and *in-vitro* biocontrol strategies for *Aspergillus flavus* mitigation

6.0 Introduction

The previous chapters focussed on the characterisation of *A. flavus* isolates from four climatic regions of Kenya with respect to their morphology, VCG diversity, phylogeny and expression of genes involved in aflatoxin biosynthesis. The sensitivity of two Kenyan maize lines to *A. flavus* isolate KSM014 was evaluated by using quantitative RT-PCR.

The current chapter focuses on, firstly, optimisation and validation of bioanalytical techniques for the detection of aflatoxins in *A. flavus* isolates. Secondly, to gauge whether the optimised method can be useful in differentiation of atoxigenic and aflatoxigenic strains of *A. flavus* and lastly, to investigate whether a laboratory based biocontrol strategy could have potential in aflatoxin mitigation.

The levels of aflatoxin in foods and feeds are strictly regulated and can limit access to foreign markets depending on the level of contamination (Payne & Yu 2010; Wu & Khlangwiset, 2010). It is therefore important to have rapid, sensitive, quantitative and relatively easy techniques for aflatoxin detection at various stages in the food chain (Wacoo *et al.*, 2014a). Molecular techniques based on PCR or culture-based methods are used primarily to differentiate aflatoxigenic and atoxigenic *Aspergillus* strains (Hayat *et al.*, 2012; Zhao *et al.*, 2014; Sulyok *et al.*, 2007). The methods used to detect and quantify aflatoxins can be chromatographic, spectroscopic or immunochemical, with each having advantages and limitations depending on the application (Wacoo *et al.*, 2014a).

Chromatographic methods, such as thin layer chromatography (TLC) and high-pressure liquid chromatography (HPLC), are the most widely used in aflatoxin detection and are considered the gold standard for aflatoxin detection (Wacoo *et al.*, 2014a). Filtenborg *et al.* (1983) demonstrated the detection of mycotoxins in agar plugs of pure *Aspergillus* section *Flavi* cultures using TLC. Aflatoxins are well suited for analysis by TLC since most of the compounds fluoresce strongly under long-wavelength UV light. HPLC coupled with a variety of detectors, such as a fluorescent detector (FLD), on ultraviolet (UV) detector, or a diode array detector (DAD) also is

commonly used for aflatoxin detection and identification in foods and feeds (Songsermsakul, 2008; Malachová *et al.*, 2014a). Recently, HPLC coupled with tandem mass spectrometry (HPLC-MS/MS) has become the method of choice due to its high sensitivity and selectivity which allows the determination of multiple mycotoxins in one run (Zöllner & Mayer-Helm 2006; Malachová *et al.*, 2014a). In HPLC-MS/MS analysis for purification of the plant crude extract is not needed and the crude extract can be injected directly (Sulyok *et al.*, 2010; Malachová *et al.*, 2014a).

A very broad definition of biocontrol is the reduction and/or suppression of growth of a destructive organism by the use of another organism which are either predators or competitors of the destructive organism (Dorner, 2004; Cotty, 2006). With respect to biocontrol of *A. flavus*, this process usually involves atoxigenic strains of *A. flavus* with registered atoxigenic strains of *A. flavus* commercially available (Dorner, 2004; Cotty, 2006; Mehl & Cotty, 2008; Atehnkeng *et al.*, 2016a; Bandyopadhyay *et al.*, 2016). The atoxigenic *A. flavus* (AF36) and *Afla-Guard* have both been approved by the USA Environmental Protection Agency with *A. flavus* strain AF36 shown to successfully suppress aflatoxin producers on cottonseed in United States of America (Cotty & Jaime-Garcia, 2007). Each atoxigenic strain is in a distinct VCG and affects aflatoxin levels by competitively excluding aflatoxin producers (Cotty, 2006). In addition, *Aflasafe*TM NG, with four atoxigenic strains of *A. flavus* have been provisionally registered for commercial use on maize in Nigeria by the National Agency for Food and Drug Administration and Control (Bandyopadhyay *et al.*, 2016b).

The use of atoxigenic *A. flavus* for biocontrol is directly connected with bioanalytical methods to detect aflatoxin detection as it is necessary to confirm that the biocontrol agent reduces aflatoxin contamination. The present study was therefore divided into three parts, where the first was to optimise bioanalytical approaches for detection of aflatoxin metabolites. Secondly, to evaluate whether the selected method can be used successfully in differentiation of atoxigenic and aflatoxigenic isolates of *A. flavus*. Lastly, to perform *in-vivo* assays on maize kernels using atoxigenic *A. flavus* strain; KSM012; as a biocontrol agent to minimise aflatoxin contamination by the toxigenic *A. flavus* strain; KSM014.

6.1 Materials and methods

6.1.1 Culturing of *A. flavus* isolates

The *A. flavus* isolates used were obtained from previous studies (Chapter 2; sections 2.1.2-2.4). Briefly, five *A. flavus* isolates (KSM012, KSM014, HB021, HB026 and HB027) were grown on both aflatoxin inducing [Yeast Extract Sucrose (YES)] and non-inducing [Yeast Extract Peptone (YEP)] medium and incubated in the dark for seven days at 30 ° C. Conidia were harvested from the culture surfaces with cotton swabs and suspended in aqueous Tween 20 (0.2 %). The working concentration was adjusted to 1×10^6 conidia/ml using a haemocytometer. Thereafter, the inoculum was stored at 4 ° C and used for further studies within 1 week or stored in 15 % glycerol at -20 ° C for 1-3 months or longer at -80 ° C.

6.1.2 Chemicals and reagents

Potato dextrose agar, yeast extract, sodium chloride, ammonium acetate, tryptone, mycological peptone, malt extract agar, agar, chloroform, acetone, ethanol, methanol, dichloromethane, acetonitrile, formic acid (> 98 %) and trifluoroacetic acid (99.8 %) were from Sigma-Aldrich (USA). Mycotoxin reference standards of aflatoxin B + G mixture dry concentrate containing 5.8 µg AFB₁, AFG₁ and 1.7 µg AFB₂, AFG₂/ml were from Sigma Aldrich (Germany). Coconut cream was purchased from Pick n Pay supermarket (Pick n Pay, Cape Town, South Africa). Pure and ultrapure grade water was processed by Milli Q water purification system in Molecular and Cell Biology department (Millipore LTD, Bedford, MA, USA).

6.1.3 Metabolite extraction

Fungal metabolites were extracted from *A. flavus* strains using different solvents. Briefly, fresh mycelia (200-400 mg) was scraped off the respective YES and YEP culture plates and placed into screw capped disposable vials containing, and/or not approximately four glass beads of 4 mm in diameter (Assistant, Germany). Thereafter, aflatoxin and other metabolites were extracted ultrasonically for 15 min using, 2-10 ml of extraction solvent consisting of methanol dichloromethane, ethyl acetate [MeOH: DCM: EtOAc (1:2:3)] in 1 % formic acid. The extracts were centrifuged at 14000 rpm for 15 minutes at 4 ° C and 500 µl transferred to sterile 2 ml tubes. Samples were dried using a Savant SpeedVac Plus SC210A Concentrator (Thermo

Scientific, USA) for 12 hours. The residue was reconstituted in 400 µl methanol made up of 0.6 % (v/v) FA, 0.02 % (v/v) HCl and 2.5 % (v/v) water. The reconstitution was carried out in ultrasonic bath sonicator for ten minutes at room temperature. Samples were finally centrifuged at 14000 rpm for 15 minutes at 4 ° C and 250 µl transferred to glass vials for TLC, HPLC and LC-MS/MS analysis.

6.1.4 Preparation of aflatoxin standards

The mycotoxin reference standards; aflatoxin B + G mixture dry concentrate (Sigma Aldrich, Germany) was dissolved in 5 ml HPLC grade methanol (Sigma Aldrich, USA) to make a working stock solution and stored in a freezer at -20 ° C according to manufactures recommendations (Sigma Aldrich, Germany). A working stock solution for bioanalytical analysis was prepared in a one-fold dilution containing 200 µg l⁻¹ AFB1, 50 µg l⁻¹ AFB2, 200 µg l⁻¹ AFG1 and 50 µg l⁻¹ AFG2 and intermediate solutions stored in amber bottles at -20 ° C for three months and/or -80 ° C for longer storage.

6.1.5 Thin layer chromatography

Thin Layer Chromatography (TLC) was carried out on a TLC silica gel 60 plate 20 × 20 cm (Merck, KGaA, Darmstadt, Germany). Acetonitrile/methanol/formic acid (9:1:0.2 v/v) was used as a mobile phase for mycotoxins. Ten microliters of aflatoxins standard mix with certificated concentration of 5.86 µg/ml for AFB1 and AFG1, and 1.70 µg/ml for AFB2 and AFG2 and 20 µl of test samples were spotted on TLC plates and run for approximately 70-90 min in a TLC tank at RT. Subsequently, the plates were left to air dry in the fume hood at RT for approximately 30 min. Additionally, the plates were either observed under UV light wavelength (254 and 366 nm), or sprayed with *p*-anisaldehyde solution, vanillin solution or subjected to iodine vapour. The intensity of the sample spots against the standard aflatoxins were compared and aflatoxins concentrations calculated based on the generated formula equation:

$$E = \frac{SCV}{WZ}$$

Where;

E = aflatoxins (µg/kg)

S = µl of aflatoxin standard equal in fluorescence to sample spot

C = aflatoxin standard concentration in µg/mL

V = final dilution of the sample extract (μl)

Z = sample matching the standards (μl)

W = sample extract weight (mg)

6.1.6 High Performance Liquid Chromatography and optimisation

Four sets of optimisations were carried out (Supplementary data, Table S6.1). Optimisation and chromatographic separations were achieved on an Agilent HPLC 1200 system comprised of a binary pump equipped with micro vacuum degasser, thermostatic autosampler, column compartment and Diode Array Detector (Agilent Technologies, Waldbronn, Germany). Fluorescence detection was performed at excitation and emission wavelengths from 200 nm to 410 nm. Separations were performed on Agilent Zorbax Eclipse XDB-C₁₈ column, 4.6 X 150 mm I.D., particle size 5 μm (Agilent Technologies, Waldbronn, Germany), maintained at 40 ° C operating at a flow rate of 1.0 ml min⁻¹. Water and acetonitrile, both containing 0.005 % trifluoro acetic acid (TFA) were used as mobile phases. A gradient starting from 85 % water and 15 % acetonitrile went to 100 % acetonitrile for 20 min, maintained at 100 % acetonitrile for 23 min and final 15 % acetonitrile for 27 min (Supplementary data, Table S6.1). Sample injection volume was 15 μl . All chemicals used were HPLC grade. UV wavelength signals were set at 200, 210, 230, 270, 280, 320, 350 and 410 nm. Aflatoxins in the sample solution were identified by comparison of their retention times and peak height/area with corresponding standards in the standard solution.

6.1.7 TOF/Q-TOF Mass Spectrometer

Aflatoxin metabolites pilot experiment detection and quantification was attempted with a Mass Spectrometer quadrupole time-of-flight (MS Q-TOF) component model G6530A system (Agilent Technologies, Waldbronn, Germany). The liquid chromatography instrument was an Agilent 1200 Series, consisting of an auto sampler HiP model G4226A, two isocratic high pressure mixing pumps model G4220A, a vacuum degasser unit, diode array detector (DAD) model G4212A and a chromatographic oven. Chromatographic separation was performed at 25 ° C on an Eclipse-Plus Agilent ZORBAX RRHD SB-C₁₈-column, 50 x 2.1 mm i.d., 1.8 μm particle size, equipped with a C₁₈ security guard cartridge, 4 x 3 mm i.d. (Phenomenex, Torrance, CA, USA). Ultrapure water (eluent A) and 100 % acetonitrile (eluent B) both contained 0.1 % FA (Supplementary data, Table S6.2).

The initial mobile phase composition of 97 % A and 3 % B was held for 1 minute, before % B was increased linearly to 97 % over 13 minutes (Supplementary data, Table S6.2). The % B was kept at 97 for 2 minutes before decreasing to the starting conditions over 2 minutes (Supplementary data, Table S6.2). The mobile phase flow rate was 0.3 ml min⁻¹, the injection volume for standards and sample extracts was 20 µl. All chemicals used were HPLC grade and UV wavelength signals were set at 200, 210, 230, 270, 280, 320, 350 and 410 nm. Aflatoxins in the sample solution were identified by comparison if their retention times, targeted mass and peak height with the corresponding standards in the standard solution.

Nitrogen (99.99 %) was used as a nebulising (45 psig) and drying gas (300 °C, 8 L min⁻¹) in the Electrospray ionisation (ESI) source. The Q-TOF instrument worked in the 2 GHz Extended Dynamic Range resolution mode (mass resolution 5000 at m/z values of 120) and compounds were ionized in positive ESI by applying a capillary voltage of 3500 V. Agilent Mass Hunter Qualitative Analysis Workstation software ver. B.05.00 was used to control the LC-ESI-QTOF-MS system and to process the obtained data (Agilent Technologies, Waldbronn, Germany).

Precursor [M+H]⁺ ions for targeted compounds were obtained by using a fragmentor voltage of 120 V. Collision energies were optimized with the aim of generating several products from each precursor. Accurate production scan (MS/MS) spectra were acquired in the range of m/z values from 60 to 750 units, considering a time window of 1 min centred in the retention time of each analyte. Full scan MS spectra (m/z range 100–1000 units) were simultaneously acquired with the MS/MS spectra. Acquisition rates in MS and MS/MS modes were set at 1 spectra s⁻¹, with each spectrum being the combination of 9600 transients. Selective LC–MS and LC–MS/MS chromatograms were extracted with a mass window of 50 ppm around the [M+H]⁺ and the most intense product ion of each metabolite, respectively. The MS/MS mode was employed for quantification purposes whereas, LC–MS chromatograms were used in the post target analysis and to screen for the presence of additional metabolites.

6.1.8 HPLC and LC-MS/MS method validation

The optimised parameters (Section 6.1.6; 6.1.7) were used to validate the method; accuracy, specificity, limits of detection (LOD) and limits of quantification

(LOQ). These parameters were used to confirm aflatoxin presence or absence in a sample based on European Commission regulations for performance of analytical methods (European commission 657/EC 2002). Linear regression analysis was carried out for the aflatoxins mixture (AFB1, AFB2, AFG1 and AFG2). The five-point calibration curves for aflatoxins were made in the concentration ranges of 0.05–1.17 µg/mL for (AFB1, AFB2, AFG1, AFG2) for HPLC. The linear regression was used to plot the peak area ratio of each mycotoxin against its concentration. Accuracy and precision were determined by extraction efficiency experiments carried out in triplicates. LOQ and LOD were estimated for a signal to noise ratio of 10 and 3 respectively from the chromatograms of the samples at the lowest level validated. The detection level of the target compounds was close to the assumed LODs and LOQs based on the preliminary experiments. Of these measurements, calibration curves for each analyte were established and then utilised to calculate the LOD and LOQ. Aflatoxins in the sample solution were identified by comparison of their retention times and peak area for HPLC or MS spectra (MRM mode) with the corresponding standard in the standard solution.

6.1.9 Maize cultivars

The maize lines used in the present study were as described previously (Chapter 5, section 5.1.2).

6.1.10 *In vitro* co-infection of maize lines and Biocontrol strategy

The schematic flow diagram (Fig.6.1) outlines the steps in the biocontrol approach from maize kernel sterilisation, through co-infection with aflatoxigenic or atoxigenic isolates and finally aflatoxin detection.

Undamaged kernels from KDV1 and GAF4 maize lines were surface sterilised in hot water for 45 s at 80 ° C previously described (Mehl & Cotty, 2010). Briefly, maize moisture content was quantified with a HB43 Halogen Moisture Analyzer (Mettler Toledo, Columbus, OH), and adjusted to 25 % by soaking the kernels in sterile water for 30-60 minutes as described by Abbas *et al.*, (2006).

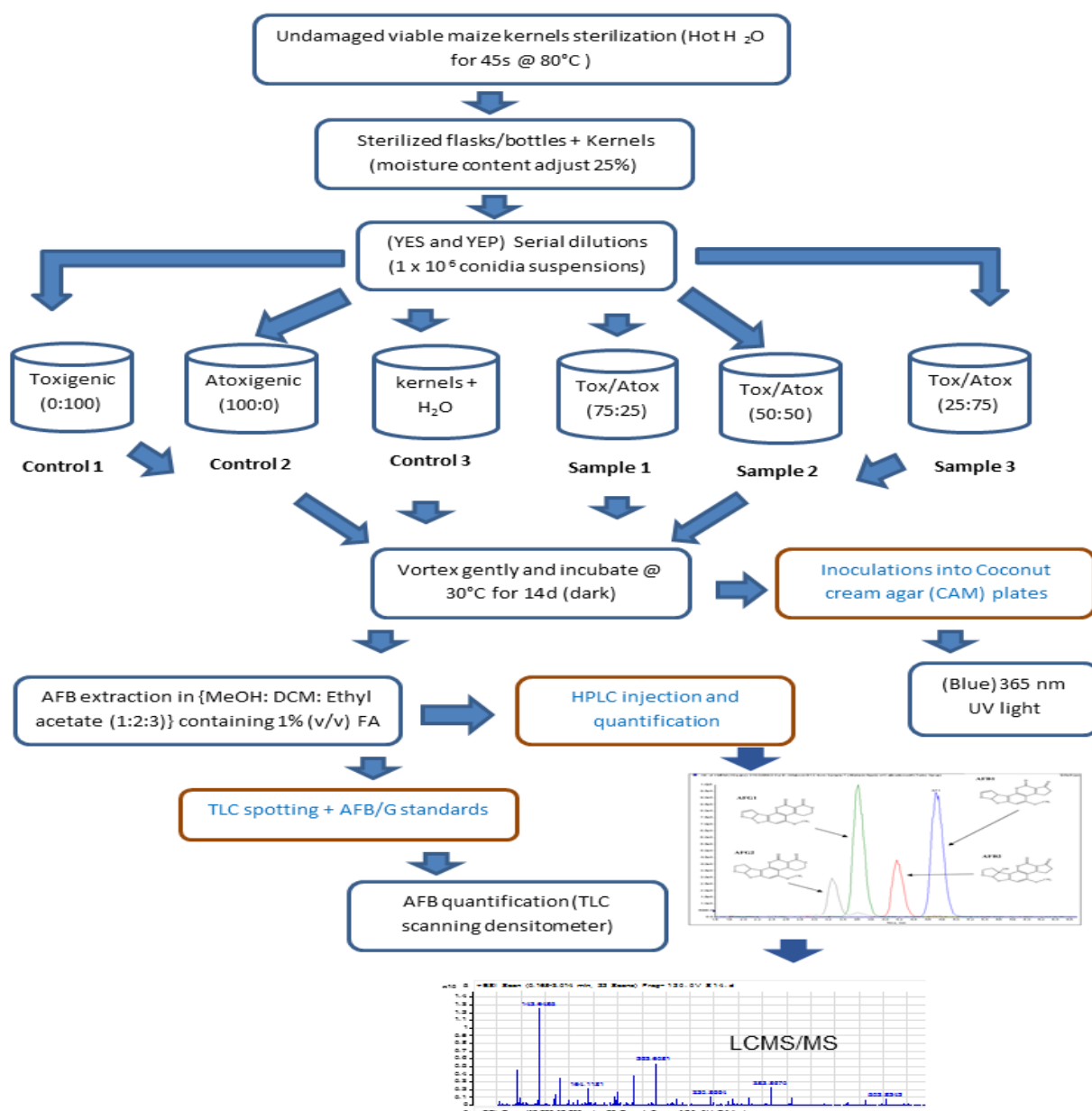


Figure 6.1 Experimental flow diagram for *in-vitro* biocontrol study of aflatoxin mitigation (micro-laboratory based) and metabolite determination using bioanalytical techniques. Undamaged maize kernels were surface sterilized and co-infected with a known concentration of atoxigenic isolate KSM012 and/or aflatoxigenic isolates KSM014 of *A. flavus* at varying proportions prior to metabolite extraction for thin layer chromatography screening and liquid chromatography with tandem mass spectrometry analysis. The isolates were cultured on inducing and noninducing media (YES: yeast extract sucrose agar; YEP: yeast extract peptone agar; Tox: toxigenic and Atox: atoxigenic respectively; TLC: thin layer chromatography; LCMS/MS: liquid chromatography coupled to mass spectrometry).

Approximately 30 sterilized grains were seeded with aliquots of spore suspensions of 1000 μ l (1 x 10⁶ conidia/ml) in a biosafety cabinet (Contained Air Solutions (CAS) BioMAT2, UK) with respective fungal isolates (atoxigenic and aflatoxigenic) at different ratios (0:100, 25:75, 50:50, 75:25, 100:0) in sterile vials. Controls were inoculated with

1000 µl sterile water instead of fungal spore suspensions. Both infected and control vial contents were shaken for 30 s in a vortex mixer (SciQuip Ltd, UK) to ensure complete and uniform coating of kernels with inoculum. Vial lids were loosened briefly, to enable gas exchange and incubated at 30 ° C for 14 days in the dark. At the end of the incubation period, fungal activity was discontinued by addition of 50 ml, 80 % methanol or halted by oven drying at 45 ° C for one day and the contents then prepared for aflatoxin extraction and further analysis. Three biological replicates of the experiments were performed twice. The efficiency of surface sterilisation and ability of kernels to germinate were monitored by plating five randomly selected kernels from each vial onto a selective inducing agar medium (YES), and non-inducing medium (YEP), followed by incubation at 30 ° C in the dark for 14 days. Approximately, 99 % of the kernels germinated and fungal contaminants were not observed at the end of the incubation period.

6.1.11 Statistical analyses

A randomized design with three biological technical replicates was used in all experiments. Aflatoxin concentration and percentage reduction were log transformed prior to analyses using GraphPad Prism, One-way analysis of variance (ANOVA), Tukey's Multiple Comparison Test (TMCT) and Post-test for linear trend analysis. Mean differences in aflatoxin levels (percent difference between inoculated maize and control maize treatments) were calculated as:

$$1 - \frac{\textit{TACM co-inoculted with both atox and aflatox isolates of A. flavus}}{\textit{TACM inoculated with the aflatoxigenic isolate alone}} \times 100$$

Where:

TACM is total aflatoxin content in maize;

atox: atoxigenic and

aflatox: aflatoxigenic.

Standard deviations of mean differences in aflatoxin levels were calculated as a measure of variability in efficacy. The efficiency (E) of each isolate was calculated as:

$$E = \frac{R}{\frac{A}{A+T}}$$

where R is the percentage of aflatoxin reduction and the denominator is the percentage of the total *A. flavus* inoculum made up by the atoxigenic isolate (A). 'A' is

the quantity of atoxigenic strain and 'T' is the quantity of aflatoxin-producer. All analyses and calculations were performed in GraphPad Prism software ver. 5.0.2.

6.2 Results and Discussion

6.2.1 Metabolite extraction and efficiency

Metabolites were extracted from five *A. flavus* isolates (KSM012, KSM014, HBO21, HB026 and HB027) grown on YES/YEP medium. The extraction methods were tested and optimised at different temperatures, sonication times, with or without bead beating and solvent proportions to ascertain the best method for good, consistent metabolite yields (Table 6.1).

Table 6.1 Percentage extraction yield at varying parameters (solvent proportions, bead beating, time frame and sonication).

Extraction solvent	Starting material (mg)	Sonication time	% yield without bead beating	% yield with bead beating
MeOH: DCM: EtOAc (1:2:3) and 1 % (v/v) FA	700	1 hr at 65 ° C	0.20 %	15 %~105 mg
MeOH: DCM: EtOAc (1:2:3)	700	1 hr at 65 ° C	0.00 %	<0.01 %
MeOH: DCM: EtOAc (1:2:3) and 1 % (v/v) FA	700	15 min at 30 ° C	1.29 %	23 %~161 mg
MeOH: DCM: EtOAc (1:2:3)	700	15 min at 30 ° C	0.00 %	<0.01 %
70 % MeOH	700	30 min at 40 ° C	0.11 %	12 %~83 mg
90 % ACN in H ₂ O	700	30 min at 40 ° C	0.01 %	0.24 %~1.7 mg

N/B. Solvents; MeOH: methanol; DCM: dichloromethane; ACN: acetonitrile; FA: formic acid; EtOAc: ethyl acetate.

Varying proportions of solvents: MeOH, DCM, EtOAc, ACN with or without FA, ultrasonic extraction, bead beating/bashing methods were tested at different temperatures and compared to each other based on the same starting material. A 12 % yield of end product was obtained with 70 % MeOH while extraction in ACN under the same conditions resulted in only a yield of 0.24 % (Table 6.1). The MeOH/DCM/EtOAc (1:2:3) extraction solvent in FA yielded 15 % when sonicated for one hour at 65 ° C compared to a yield of 23 % when sonicated for 15 min at 30 ° C (Table 6.1). Extraction without bead beating exhibited no significant yield (Table 6.1) and this could be attributed to possibly the tough cell wall of the fungi *A. flavus* which requires thorough disruption. Raeder & Broda, (1985) demonstrated that filamentous fungi have a sturdy cell wall which is resistant to standard extraction procedures and similar result was also observed in the contemporary study.

Arranz *et al.* (2006) and Gallo *et al.* (2010), evaluated extraction efficiency of various organic aqueous solvents on contaminated matrices. They demonstrated that acetone-water (85:15 or 8:2) had better extraction efficiency for AFB1 than a mixture of methanol-water, but less data was observed for other mycotoxins. Additionally, Möller & Nyberg, (2004) reported that acetonitrile–water (6:4v/v) and chloroform–water (10:1v/v) gave better analytical results for aflatoxins extracted from peanut meal with aqueous acetone and acetonitrile exhibiting greater extraction efficiency compared to aqueous methanol. Their findings were similar to the current, though the use of beads were incorporated and sonication water bath. Detection and quantification of aflatoxins in contaminated samples require an efficient extraction step (Taylor *et al.*, 1993; Bertuzzi *et al.*, 2012; Wacoo *et al.*, 2014b). Additionally, aflatoxins were observed to be soluble in polar protic solvents (methanol, acetone, chloroform, and acetonitrile) mixed in different proportions with small amounts of sterile water (Taylor *et al.*, 1993; Bertuzzi *et al.*, 2012; Wacoo *et al.*, 2014b).

The extracted metabolites were subjected to further analysis using bioanalytical methods (TLC and HPLC) to ascertain the Limit of detection (LOD) and Limit of quantification (LOQ).

6.2.2 Thin Layer Chromatography

TLC has been widely used in the determination of aflatoxins in different foods as low as 1–20 ppb (Younis & Malik, 2003) and also regarded as the method of choice for detection (Helrich, 1990).

The study revealed some compounds adhering to the silica gel and moved short distances on the plate (polar), whereas other compounds moved further on the plate by the mobile phase (non-polar). Wacoo *et al.* (2014a), demonstrated that in TLC, the distribution of aflatoxins between the mobile and stationary phases is based primarily on differences in solubility of the analytes in the two phases. Different analytes, depending on their molecular structures and interaction with the stationary and mobile phases, either adhere more to the stationary phase or remain in the mobile phase, thereby allowing for quick and effective separation.

Diethyl ether/methanol/water (96:3:1); water/acetonitrile/methanol (6:2:2); water/acetonitrile/methanol (1:2:2), did not result in clear separations (data not

shown). The polar metabolites moved with the solvent front solvent combinations displaying promising separations including: chloroform/acetone (9:1); acetonitrile/methanol (9:1); chloroform/acetone/isopropanol (85:12.5:2.5) and toluene/isoamyl alcohol/methanol (90:32:3). Based on the availability of the chemicals, cost and carcinogenicity effect, the use of acetonitrile/methanol/formic acid (9:1:0.2 v/v) as a mobile phase was adopted. It was also found to give better separation of the mycotoxins in the current study (Fig.6.2).

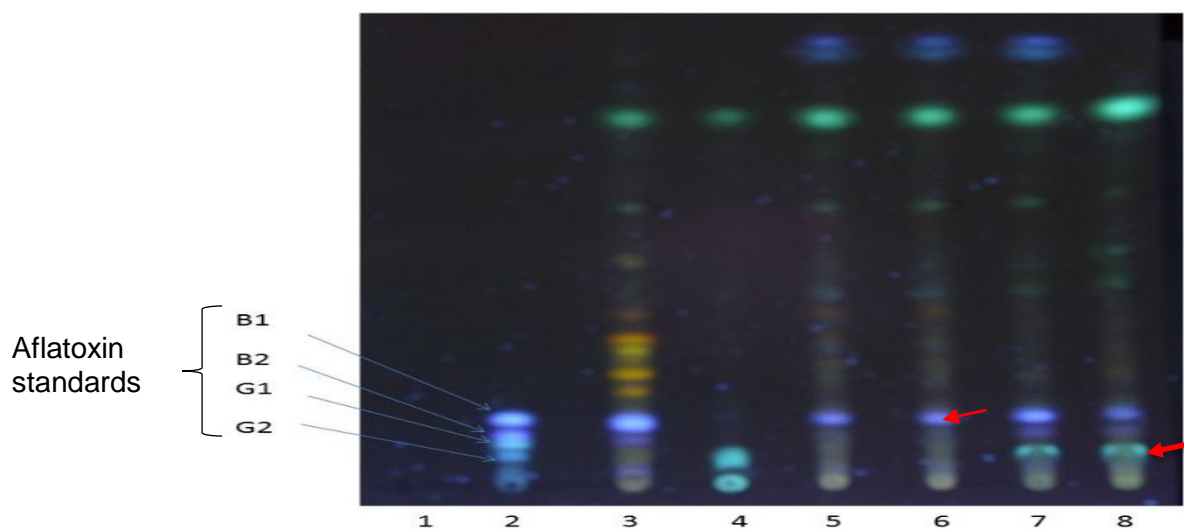


Figure 6.2 Thin layer chromatography plate showing suspected mycotoxin presence or absence from the isolates in comparison with the aflatoxin standards. Lanes: (1) Blank; (2) aflatoxin standards mix (B1: aflatoxin AFB1; B2: aflatoxin AFB2; G1: aflatoxin AFG1; G2: AFG2); (3) Isolate KC2912; (4) Non-toxic isolate KSM012; (5-8) Toxic isolates (KSM014, HB027, KSM015 and MC040 respectively). The red arrows show blue and green fluorescence respectively.

Moreover, in non-polar solvents like pentane and hexane, it was observed that most polar compounds did not move far, whereas non-polar compounds were seen to travel some distance up the plate. In contrast, polar solvents have been observed to usually move non-polar compounds to the solvent front and push the polar compounds off of the baseline. In practice, the mobile phase that separates the compounds in a mixture the best should be used.

TLC plates visualised in the UV-cabinet chamber at wavelength 365 nm confirmed the presence or absence of aflatoxins (Fig.6.2). Visible spots with green and blue fluorescence (red arrows; Fig.6.2) for aflatoxins matching with the corresponding aflatoxin standards were observed (Fig.6.2). Isolates KSM014 and HB027 showed the presence of AFB1 (Fig.6.2; lanes: 5, 6) which correlated with the

aflatoxin standards, lane 2. Additionally, both isolates KSM015 and MC040 exhibited the presence of three aflatoxins (AFB₁, AFB₂, AFG₁) (Fig.6.2; lanes: 7, 8). Isolate KC2912, an aflatoxin producing strain showed the presence of AFB₁, AFB₂ with some orange/yellow spots of unknown metabolites (Fig.6.2; lanes: 2, 3). The non-toxin producing strain (isolate KSM012) exhibited no blue fluorescence (Fig.6.2, lane 4) indicating the strains inability to produce aflatoxins, AFB₁ and AFB₂ (Fig.6.2), which confirmed the previous real-time qPCR gene expression data (Chapter 4, section 4.2.2, Figs.4.4, 4.5).

However, though TLC can detect several types of mycotoxins in a single test sample (Trucksess *et al.*, 1984), it requires skilled technical personnel, sample pre-treatment, and in addition, TLC lacks precision due to accumulated errors during sample application, plate development and interpretation (Stroka & Anklam, 1999; Papp *et al.*, 2002).

The TLC results were validated with HPLC to quantify the level or limit of detection of aflatoxins present after co-infection.

6.2.3 HPLC method optimisation and validation

Different mobile phases, sample injection volumes, columns and set parameters were experimented and gave varying results. The best separation method for aflatoxins was adopted after four sets of optimisations were evaluated (Section 6.1.4; Supplementary data, Table S6.1). The first HPLC optimisation showed no peaks or HPLC signals. The observation could possibly be due to the mobile phase used; H₂O/MeOH/ACN (3:1:1) with different wavelength signals which were set at 230, 254, 280, 365, 450 nm and injection of low volume (5 µl) at a flow rate of 1 ml min⁻¹. The injection volume probably was possibly so low such that no signals could be detected (data not shown). The second optimisation exhibited peaks which were observed to emerge between retention time 17-25 min and found to be negligible with negative absorbance (data not shown). The negative absorbance could be attributed probably due to low concentrations of *A. flavus* metabolites and aflatoxins. Peaks were observed emerging between retention time 5-24 min for the third optimisation but were not quantifiable (data not shown).

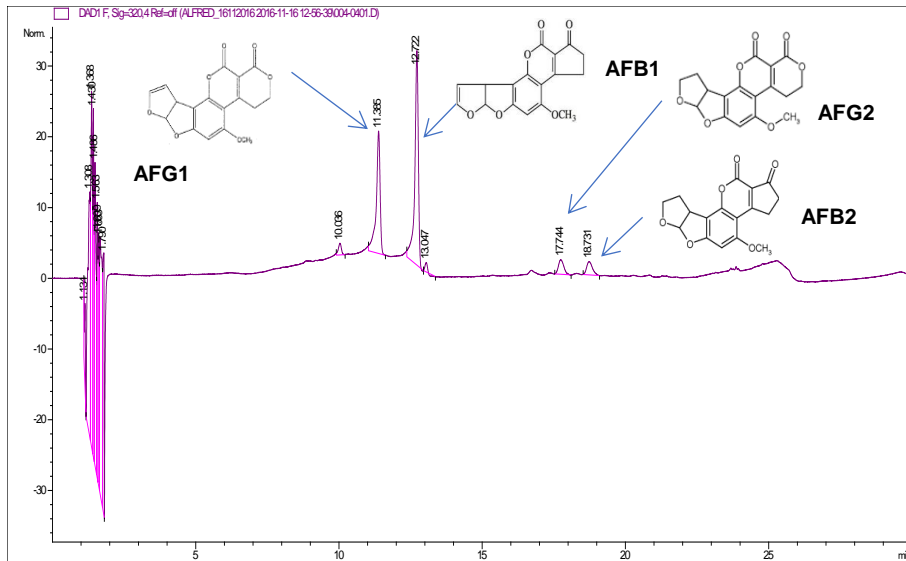
Kok, (1986), observed that chemical derivatisation of aflatoxins B1 and G1 is of potential in enhancing sensitivity of HPLC during analysis since the natural fluorescence of these aflatoxins may not be high enough to reach the required detection limit. Similarly, Nielsen & Smedsgaard, (2003) demonstrated that derivatisation by trifluoro-acetic acid in the acetonitrile makes the gradient more suitable for positive electrospray ionisation, and thus improves the metabolite resolution and to stay within the optimal flow range of the Z-spray source. Kok, (1986) and Nielsen & Smedsgaard, (2003) observations could explain the current findings where in the first two optimisations, no signals were observed probably due to lack of derivatisation and/or low sample injection volume. With this information, the fourth optimisation consisted of derivatisation using trifluoro-acetic acid (0.005 %) in both H₂O and 100 % ACN. The UV wavelength signals were set at 200, 210, 230, 270, 280, 320, 365 and 410 nm. The column temperature was maintained at 40 ° C and injection volume was adjusted to 15 µl at a flow rate 1 ml min⁻¹ and the gradient was set as indicated previously (Supplementary data, Table S6.1). Chromatograms were generated and were observed to exhibit satisfactory separation with peaks detected between retention time 9-20 min (Fig.6.3). The fourth optimisation method was therefore adopted and considered as the best mobile phase in the current study.

HPLC chromatograms generated for the isolates exhibited retention times for aflatoxins [AFG1 (11.39-11.68); AFB1 (12.72-12.84); AFG2 (17.71-17.80) and AFB2 (18.73-18.91)] based on the aflatoxins standards (Fig.6.3). The non-toxin producing strain (isolate KSM012) exhibited no HPLC peaks and no blue fluorescence on TLC plate confirming the probability that it is non-toxicogenic (Figs.6.2, lane 4; 6.3c). Isolate KSM104 had similar retention time as the aflatoxin standards, AFB1 and AFB2, confirming that this isolate was aflatoxicogenic, S-morphotype (refer to previous chapter). The interesting isolate KSM015, produced AFB1 and AFB2 in addition to AFG1 and AFG2 respectively (Fig.6.3b, d) confirming its identity as possibly an S_{BG} morphotype (refer to previous chapter).

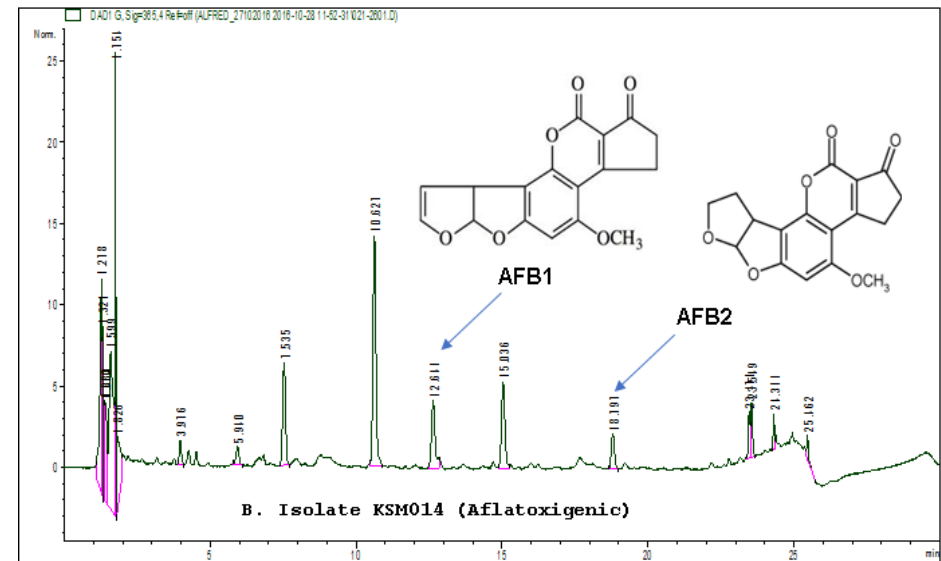
The detection limits and sensitivity including linearity showed that the method developed was acceptable for mycotoxin determination from cultures of *A. flavus* mycelia (Figs.6.3; supplementary data, S6.1). The LOD and LOQ ranged from 0.01–6.8 µg/ml and 0.02–35.81 µg/ml respectively (Fig.6.3; supplementary data, S6.1). The

contemporary findings exhibited similar trends as observed by Gallo *et al.* (2010) and Malachová *et al.* (2014a), who obtained LOD range 0.6-1.9 µg/kg and LOQ range 0.02-0.05 mg/kg respectively for aflatoxins extracted on highly contaminated animal feedstuff.

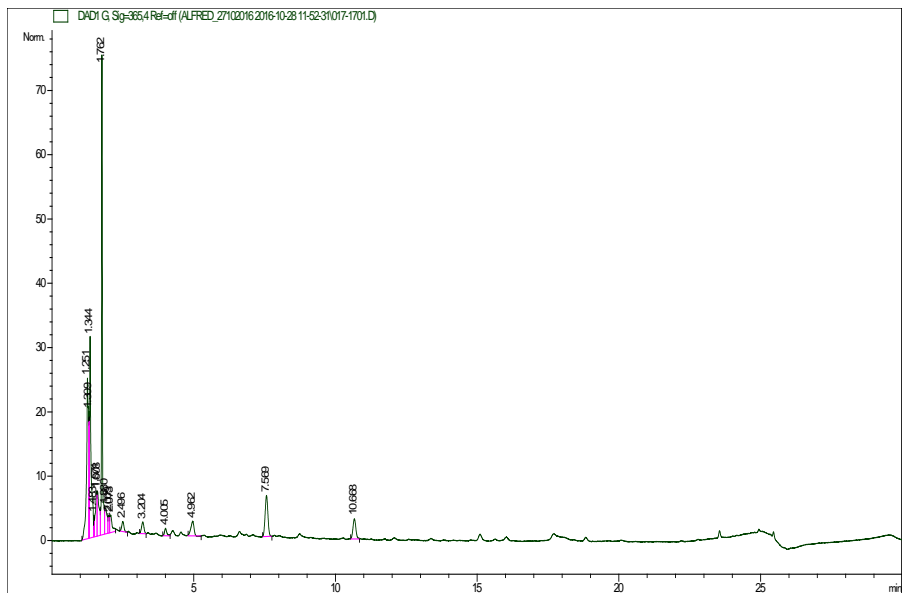
Herzallah (2009) noted that HPLC provides fast, accurate and sensitivity of aflatoxins detection results as low as 0.1 ng/Kg using fluorescent detector (FLD) within a short time which were in agreement with the current optimised method. The results were also found to be accurate and precise for both the UV and fluorescent detector (Figs.6.3; supplementary data, S6.1).



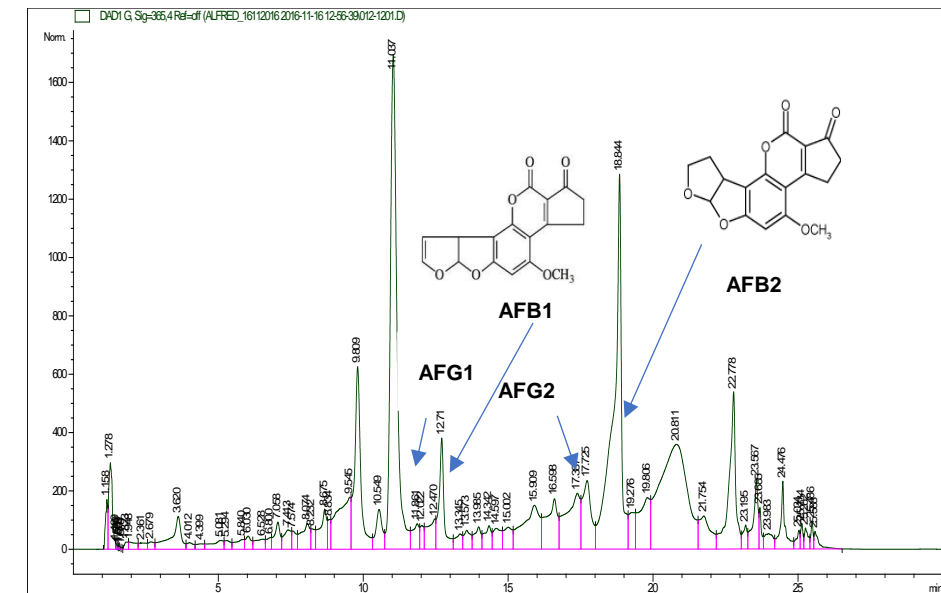
a. Aflatoxins standards (AFG1, AFB1, AFG2 and AFB2)



b. Isolate KSM014 (aflatoxigenic)



c. Isolate KSM012 (non-toxicogenic)



d. Isolate KSM015 (S_{BG})

Figure 6.3 HPLC chromatographic separations (a): Standard chromatogram obtained after the analysis of aflatoxins standard mix with certificated concentration of 5.86 $\mu\text{g/ml}$ for AFB1 and AFG1, and 1.70 $\mu\text{g/ml}$ for AFB2 and AFG2; (b): chromatograms of isolate KSM014 (aflatoxigenic); (c): Isolate KSM012 (non-toxicogenic); and (d): Isolate KSM015 suspected to be taxon S_{BG} respectively. (AFB1: aflatoxin B1; AFB2: aflatoxin B2; AFG1: aflatoxin G1; AFG2: aflatoxin B2; KSM: Kisumu).

HPLC analyses findings were found to correlate with the TLC method and therefore both approaches could detect the presence or absence of aflatoxin in a sample. Though TLC technique could detect the presence or absence of aflatoxins (Fig.6.2), it was not able to measure the LOD and LOQ as opposed to HPLC analysis (Figs.6.3; supplementary data, S6.1). However, HPLC requires advanced technical skills, trained personnel to operate the instrument and analyse the data and is more expensive in terms of overall cost. Additionally, samples for HPLC analyses requires rigorous sample purification and tedious pre- and post-column derivatisation processes to improve the detection limits of AFB1 and AFG1 (Li *et al.*, 2009; Li *et al.*, 2011). Despite these drawbacks, HPLC provides accurate and sensitive detection of aflatoxins within a short time compared to TLC.

6.2.4 LC–MS/MS and data analysis

Metabolite profiling (or metabolome analysis) represents a tool that finds common application in all aspects of discovery, understanding and utilization, and hence it represents a focal point in studies of fungal taxonomy and physiology. The analytical methodologies for metabolite profiling have been extensively discussed in the literature and reviewed in several papers and books (Pramanik *et al.*, 2002; Villasbo *et al.*, 2005).

Currently, no standard guidelines has been established for the validation of analytical methods for the determination of multiple mycotoxins or for multiple analytes (Malachová *et al.*, 2014a). The only available guideline, the Commission decision 2002/657/EC (European commission 657/EC 2002), provides some requirements and recommendations concerning the performance of analytical methods for official control and the interpretation of results. However, the guidance provided is insufficient for multianalyte methods (Malachová *et al.*, 2014a). A definition of matrix effects and their evaluation has not been defined and the term recovery is not exactly specified (whether it is extraction efficiency or apparent recovery), and the determination of LOD and LOQ by spiking of samples at one level for each matrix is not feasible for hundreds of analytes due to possibly the costs of analytical standards (Malachová *et al.*, 2014b). Based on the observations, validation of the current findings were according to SANCO protocol No. 12495/2011 (European Commission, 2011).

The development of LC–MS/MS based multi-mycotoxin methods tries to overcome the need for clean-up and/or multiple analytical techniques when dealing with the chemical diversity of mycotoxins (Capriotti *et al.*, 2011). Studies on generic extraction methods for multiple contaminants in different food and feed matrices have demonstrated that mixtures of acidified water with organic solvents such as methanol, acetonitrile or acetone are suitable extraction solvents (Mol *et al.*, 2008; Lacina *et al.*, 2012).

The pilot experimental study with aflatoxin metabolites consisted of different outputs that measured chromatographic responses for optimisation such as peak area and signal to noise ratio. Mycotoxins have a wide range of polarity strengths and therefore, a mobile phase with a variable degree of hydrophobicity over the time course of each analysis was considered. The chromatography system was thus started with a more polar mobile phase composition for the elution of the more polar metabolites, and the polarity was gradually decreased to elute the less polar compounds within a reasonable time frame (see Supplementary data, Tables S6.1; S6.2). The best chromatograms of the mycotoxins with lowest noise was obtained using a mobile phase consisting of 100 % acetonitrile and ultra-pure water spiked with 0.1 % formic acid at a flow rate of 0.3 ml/min with a gradient elution programme (Supplementary data, Table S6.2).

The best peak shapes with the lowest noise were obtained under LC conditions (Section 6.1.5). Twenty microliter injection volume provided the lowest noise signal in chromatograms (Figs.6.4; 6.5). Figures 6.4; 6.5 further, shows LC–MS/MS chromatogram of selected mycotoxins in selective reaction mode (SRM). Different collision energies were used to determine the most abundant product ions.

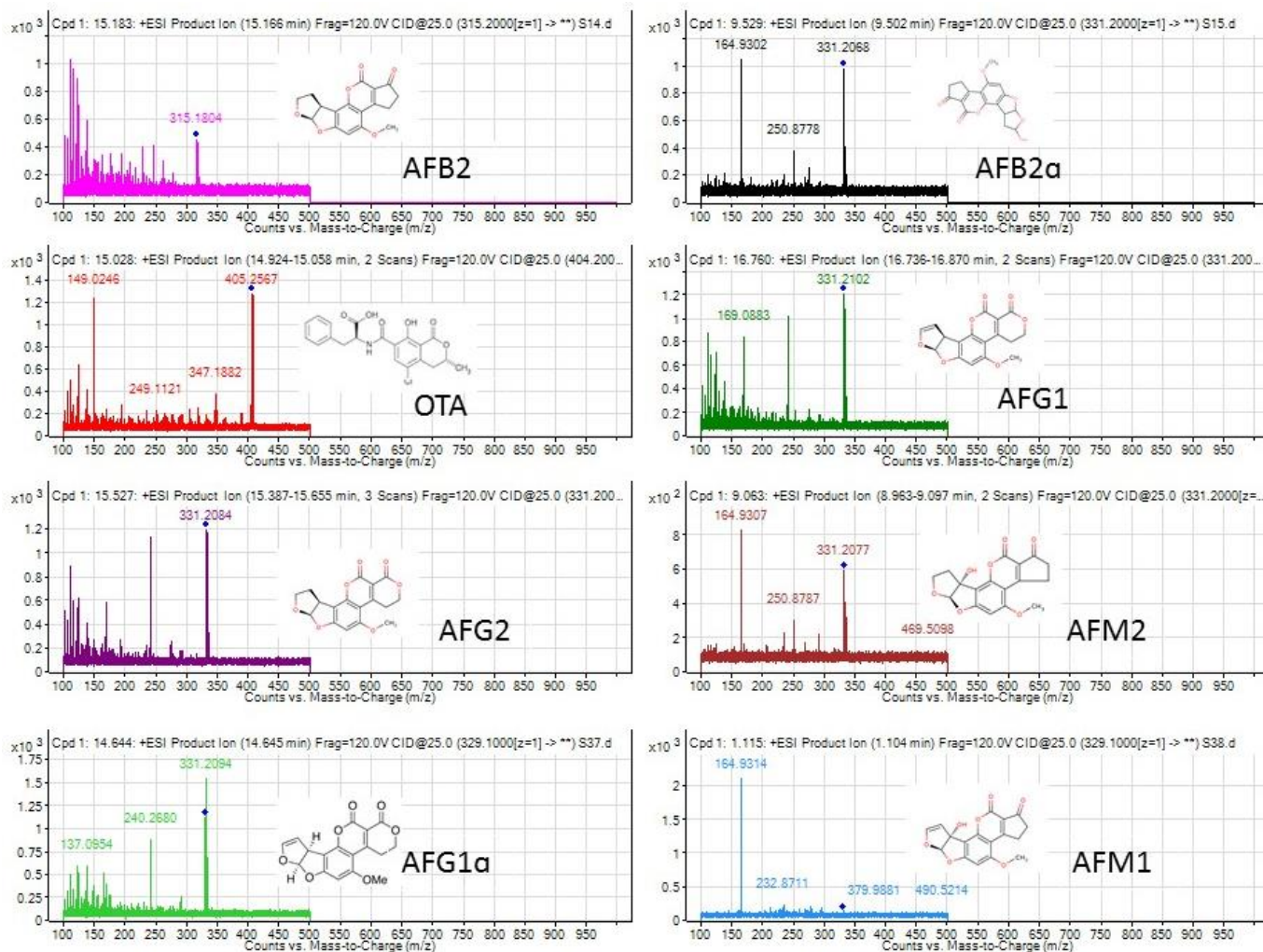


Figure 6.4 LC-MS/MS chromatograms showing mycotoxin metabolites and their derivatives identified from eight different samples (KSM014, KSM015, KSM018, KSM019, HB023, MC033, MC037, MC038) isolated from four different climatic regions of Kenya based on electrospray ionisation mass-to-charge (m/z) ratio and targeted mass. The mycotoxins identified (AFB2: aflatoxin B2; AFG1: aflatoxin G1; AFG2: aflatoxin G2; AFM1: aflatoxin M1; AFM2: aflatoxin M2 and OTA: ochratoxin A). (KSM: Kisumu; HB: Homa Bay; MC: Makueni respectively).

Application of two positive and negative ion modes with electrospray ionisation (ESI) showed that mycotoxins exhibited better fragmentation patterns in positive mode (Fig.6.5).

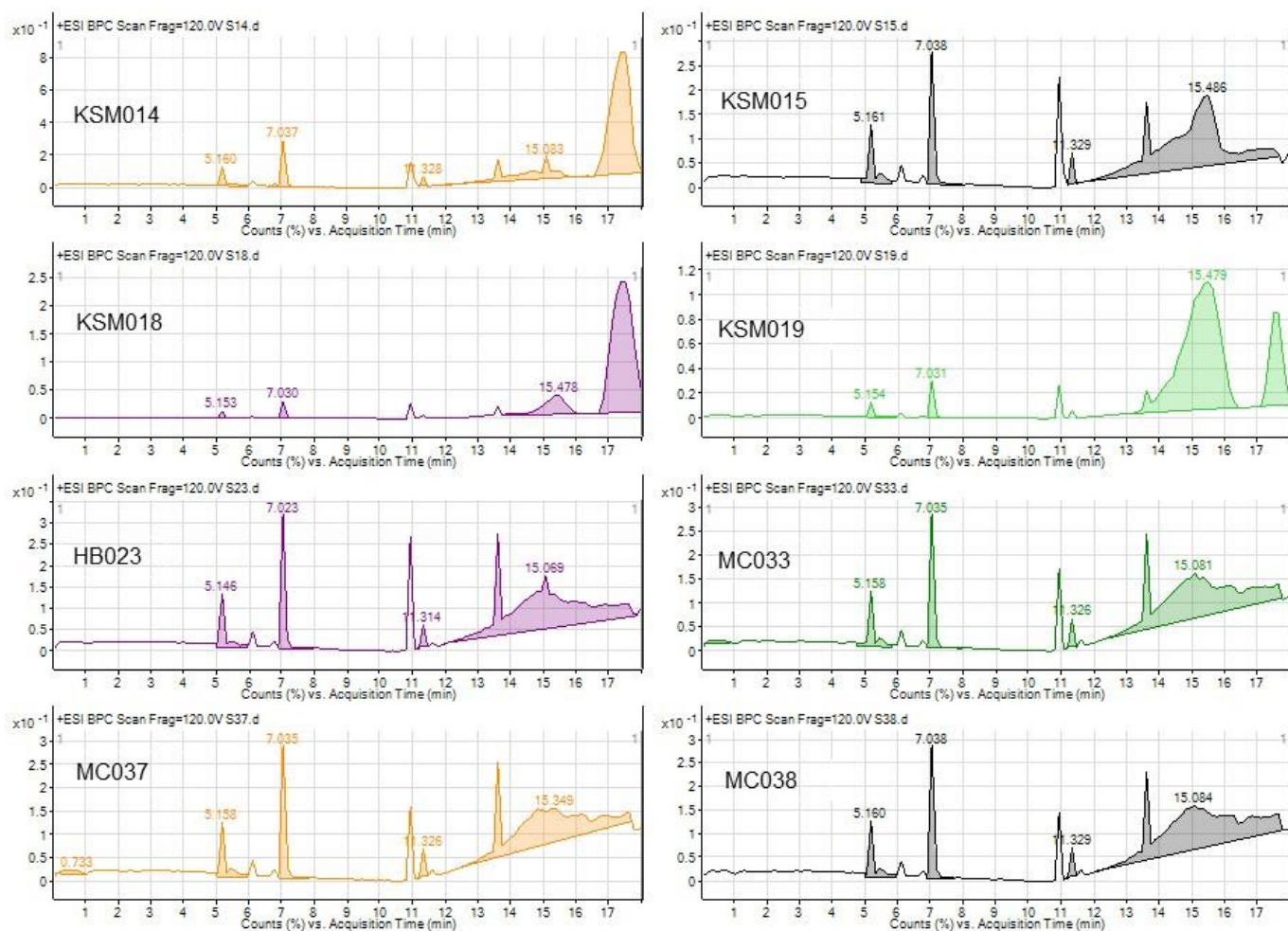


Figure 6.5 Base peak chromatograms ESI BPC counts (%) vs, acquisition time generated from selected *A. flavus* strains isolated from maize kernels from four climatic regions of Kenya exhibiting different intensity of the analytes present in the samples.

Subsequently, the optimised multiple reaction monitoring (MRM) parameters were obtained in both positive and negative ion modes (Figs.6.4; 6.5). The aflatoxin metabolites were identified based on targeted formula mass and mass spectra (Tables 6.2-6.3).

Table 6.2 Metabolites identified by formula mass, possible compounds, monoisotopic masses and other chromatographic properties.

Base peak (m/z)	Type of adduct	Neutral molecule (Da)	Suggested elemental composition	Possible compound	Monoisotopic mass in literature (Da) <1>	Mass deviation between base peak and reported literature value (Da) *	Base peak found in samples
183.1021	M+H	182.0943	C ₁₀ H ₁₄ O ₃	Barnol	182.0943	0.0000	NC04
233.1164	M+H	232.1086	C ₁₄ H ₁₆ O ₃	Sorbicillin	232.1099	0,0007 to 0,0013	NC05, NC08, KSM014, HB022, HB023, HB026
235.1312	M+Na	212.1414	C ₁₂ H ₂₀ O ₃	Dihydrojasmonic acid	212.1412	0,0002 to 0,0003	KSM018, HB021, MC037
235.1331	M+H	234.1253	C ₁₄ H ₁₈ O ₃	2',3'-Dihydrosorbicillin	234.1256	0,0001 to 0,0008	NC010, KSM012, KSM013, KSM020, HB029, HB030, MC040
251.0920	M+H	250.0842	C ₁₃ H ₁₄ O ₅	Citrinin	250.0841	0.0001	HB029
251.1278	M+H	250.1200	C ₁₄ H ₁₈ O ₄	Vertinolide	250.1205	0.0005	HB021
275.1612	M+Na	252.1714	C ₁₅ H ₂₄ O ₃	Walleminone	252.1725	0.0011	KSM012
277.1060	M+H	276.0982	C ₁₅ H ₁₆ O ₅	Ascochitine	276.0998	0.0016	NC05
279.1250	M+K	240.1618	C ₁₆ H ₂₀ N ₂	Pyroclavine, costaclavine or secoclavine	240.1626	0.0008	HB025

<1> (Nielsen & Smedsgaard, 2003)

Ranges indicate the highest and lowest mass difference found within the different samples

* Absolute values reported

N/B: NC: Nandi county; KSM: Kisumu county; HB: Homa Bay and MC: Makueni county

Table 6.3 Metabolites identified by targeted mass algorithm

Sample ID	Monoisotopic mass (Da)	Base peak (m/z)	Type of adduct	Retention time (min)	Algorithm	Suggested elemental composition	Possible compound
KSMO14	314.1845	315.2	M+H	15.183	Targeted MS/MS	C ₁₇ H ₁₄ O ₆	Aflatoxin B2
KSM015	330.1896	331.2	M+H	9.529	"	C ₁₇ H ₁₄ O ₇	Aflatoxin M2
KSM018	403.2463	404.2	(M+H+Na)+2	15.028	"	C ₂₀ H ₁₈ ClNO ₆	Ochratoxin A
KSM019	330.1991	331.2	M+H	16.76	"	C ₁₇ H ₁₄ O ₇	Aflatoxin G2
HB023	330.2024	331.2	M+H	15.527	"	C ₁₇ H ₁₄ O ₇ C ₁₇ H ₁₂ O ₆	Aflatoxin G2 α
MC033	330.1895	331.2	M+H	9.063	"	C ₁₇ H ₁₄ O ₇	Aflatoxin M2
MC037	328.0927	329.1	M+H	14.644	"	C ₁₇ H ₁₂ O ₇	Aflatoxin G1
MC038	328.1138	329.1	M+H	1.115	"	C ₁₇ H ₁₂ O ₇	Aflatoxin M1

N/B:

KSM: Kisumu; HB: Homa Bay and MC: Makueni county

Retention times for each aflatoxin derivative played a significant role in the identification of the metabolites. The major aflatoxin derivatives identified by targeted masses were aflatoxin AFG1, aflatoxin AFG2, aflatoxin AFG2 α , aflatoxin AFB2, aflatoxin AFM1 and aflatoxin AFM2. Other metabolites identified were barnol, sorbicillin, dihydrojasmonic acid, citrinin, vertinolide, walleminone, ascochitine, pyroclavine, costaclavine or secoclavine and ochratoxin A (Tables 6.4-6.5). The findings were similar to (Nielsen & Smedsgaard, 2003) who studied and presented an updated method utilising LC–UV–MS for dereplication of metabolites in fungal extracts supplemented with data from analysis of approximately 474 fungal metabolites and mycotoxins. Some of the metabolites and aflatoxins revealed by this study were also identified by Malachová *et al.* (2014b). Similarly, Malachová *et al.* (2014b), optimised and evaluated the performance of a multianalyte method for mycotoxins and other fungal metabolites. The targeted mycotoxins were detected between retention time 1-18 min in the current study; however, co-elution of some mycotoxins was observed; AFG2 was eluted at approximately 15.53 min whereas, OTA after 15.028 min (Table 6.5).

Compared with plants, *A. flavus* has a complex metabolism and produces a broad range of secondary metabolites (Nielsen & Smedsgaard, 2003; Frisvad *et al.*, 2004) and extracellular enzymes. This very high metabolic diversity has been actively exploited for many years and many metabolites produced by filamentous fungi *A. flavus* are bioactive compounds used as antibiotics, cholesterol lowering agents, anti-tumour agents, and immune-suppressors (Newman *et al.*, 2003).

The pilot, multianalyte mycotoxin study did not reveal more information due to time and experimental constraints. Despite the drawbacks, the pilot study managed to identify nine aflatoxin metabolites by formula mass and eight major mycotoxins by targeted mass algorithm (Tables 6.2; 6.3). From the preliminary findings, it could be suggested and concluded that LC-MS/MS could be suitable for multi-mycotoxin and metabolite identification. However, there is a possibility that it might give conflicting information due to formula mass overlap which is a common feature in closely related compounds or elements. This overlap could possibly be resolved by use of Nuclear magnetic resonance spectroscopy (NMR) which identifies and gives the structure of

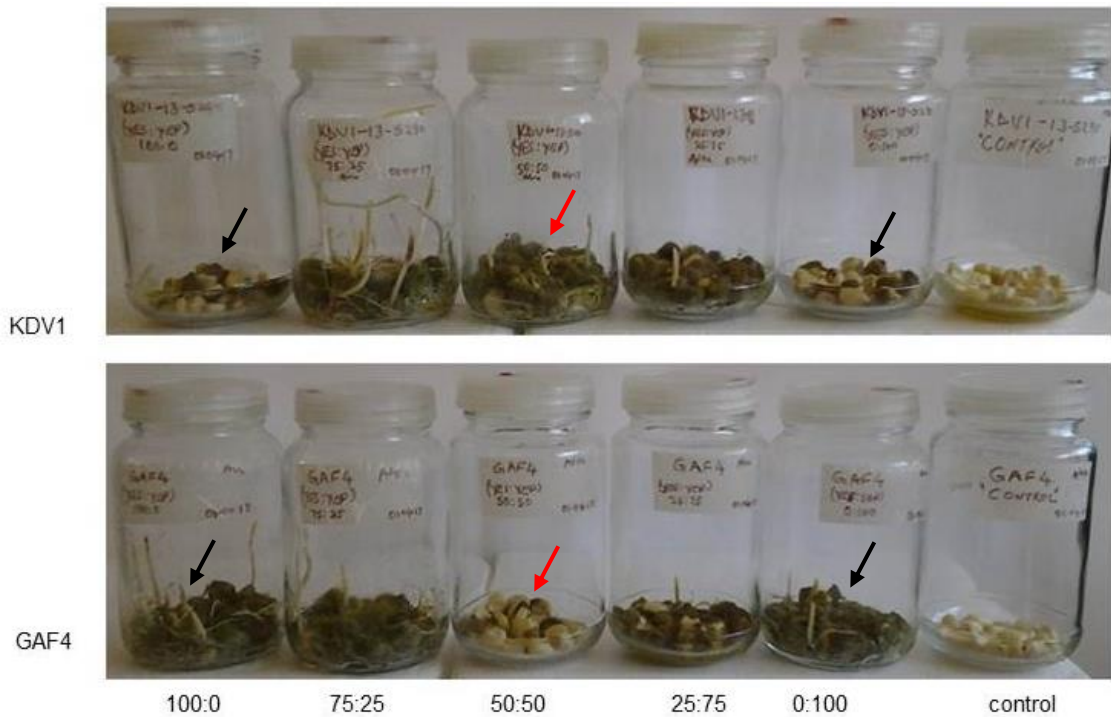
the compound. Thus, a combined LC-MS/MS and NMR approach may be best for clear metabolite distinction.

6.2.5 *In-vitro* biocontrol strategies in aflatoxin management and *Aspergillus flavus*.

Two maize lines (GAF4 and KDV1) suspected to be resistant and sensitive to *A. flavus* and aflatoxins respectively were subjected to co-infection by aflatoxigenic strain (KSM014) and atoxigenic strain (KSM012) in the laboratory. The main objective was to test whether non-toxin producing strain (atoxigenic) could be deployed in aflatoxin mitigation or inhibition or reduction of aflatoxin production by their counterpart, aflatoxigenic strains.

Bioanalytical techniques (TLC and HPLC) was used to quantify the limit of detection and limit of quantification of aflatoxin present in both maize lines following co-infection. The findings could possibly provide or give an insight in possible biocontrol system approaches in aflatoxin and *A. flavus* mitigation at laboratory level.

The co-infected two maize lines (KDV1 and GAF4) exhibited different rates of fungal colonisation upon co-inoculation with atoxigenic (KSM012) and aflatoxigenic (KSM014) strains of *A. flavus* (Fig.6.6). KDV1 maize line exhibited high fungal colonisation in comparison to GAF4 at equal co-infection ratio 50:50 (Fig.6.6, 50:50) (see red arrow). In addition, minimal fungal growth was observed when the kernels were inoculated with individual strains of *A. flavus* for KDV1, and high fungal colonisation for GAF4 (Fig.6.6, 100:0; 0:100) (see dark arrow). Moreover, controls showed no fungal growth, an indication of no contamination with either fungal spores during co-inoculations (Fig.6.6, control). The *A. flavus* fungal cultures on the kernels were scrapped off and thereafter, subjected to metabolite extraction and further bioanalytical analysis (TLC and HPLC) to compare the presence or absence of aflatoxins and to evaluate the LOD and LOQ of mycotoxins in a sample at different co-inoculation ratios.



Ratio of atoxigenic/aflatoxigenic *Aspergillus flavus* strains

Figure 6.6 Biocontrol approach using locally identified atoxigenic (KSM012) and aflatoxigenic (KSM014) strains of *Aspergillus flavus* to mitigate aflatoxin production. The kernels for resistance (GAF4 maize line) and sensitive (KDV1 maize line) were co-infected at different ratios (0:100; 25:75; 50:50; 75:25; 100:0) with atoxigenic and aflatoxigenic strains respectively.

6.2.6 TLC plates and aflatoxins

The TLC plates examined showed significant reduction in the aflatoxin levels from plants inoculated with a 50:50 ratio of atoxigenic/aflatoxigenic strains of *A. flavus* (Fig.6.7, lane 5).

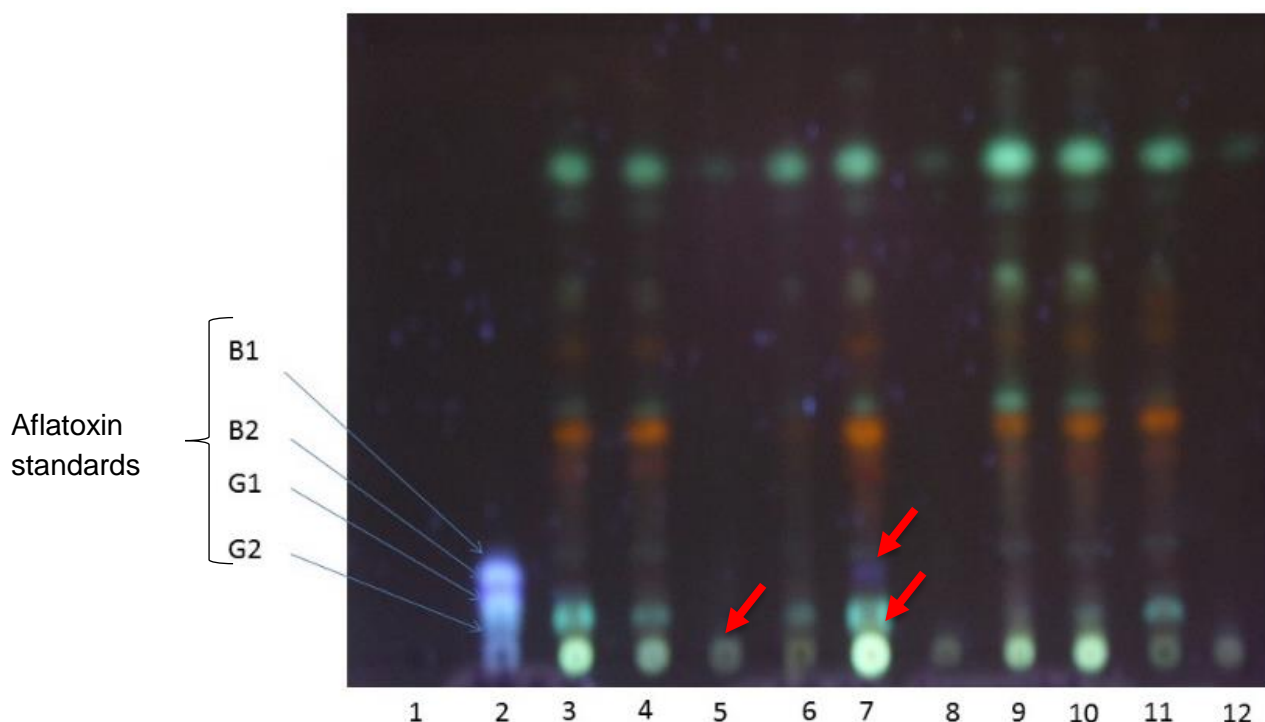


Figure 6.7 Thin layer chromatography plates showing the presence or absence of mycotoxins from the isolates in comparison with the aflatoxin standards at long wavelength, 365 nm. Lanes: (1) Blank; (2) Standard; (3) G100/0; (4) G75/25; (5) G50/50; (6) G25/75; (7) G0/100; (8) K100/0; (9) K75/25; (10) K50/50; (11) K25/75; (12) K0/100. (atoxicogenic/toxigenic. (B1: aflatoxin AFB1; B2: aflatoxin AFB2; G1: aflatoxin AFG1; G2: aflatoxin AFG2).

At 365 nm, visible spots with blue and green fluorescence for aflatoxins matching with the corresponding aflatoxin standards were observed (Fig.6.7, red and blue arrows). It should be noted that at short wavelength (254 nm), there was no observable blue or green fluorescence for mycotoxins (data not shown). This observation shows that the presence or absence of aflatoxins and their derivatives in a sample could be visualised and identified at long wavelength according to the present study. Staining with *p*-anisaldehyde, vanillin in phosphoric acid or iodine vapour did not yield any results (data not shown). The stains washed the spots on the silica gel plates leaving no observable spots.

6.2.7 LOD and LOQ of the metabolites

HPLC analysis of the metabolites extracted from the co-infected cultures was also performed to identify the peaks associated with aflatoxins (Fig.6.8 A-B).

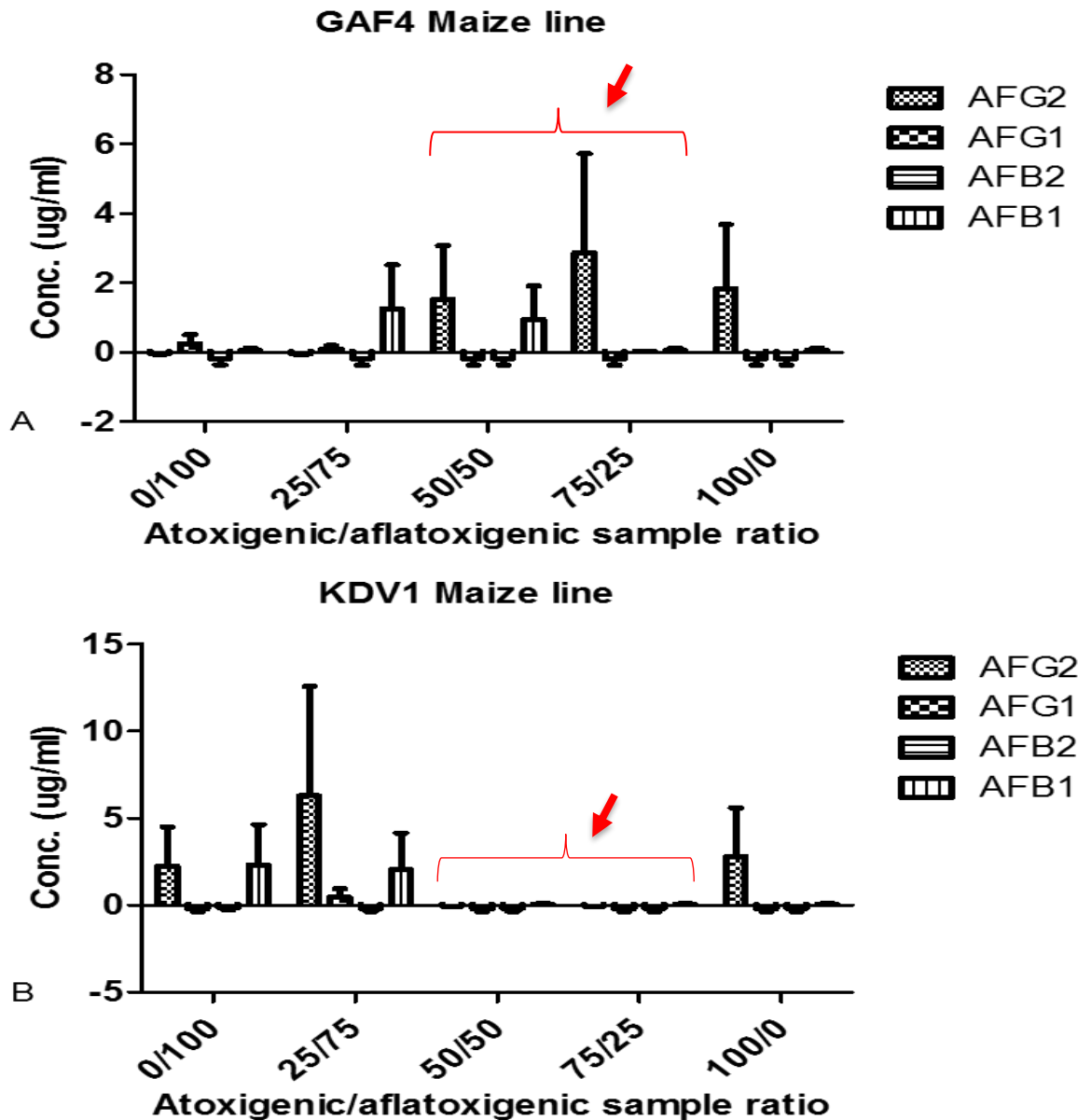


Figure 6.8 High Performance Liquid Chromatography analysis highlighting biocontrol strategy exhibited when two maize lines (GAF4 and KDV1) were co-infected with atoxigenic (KSM012) and aflatoxigenic (KSM014) strains of *Aspergillus flavus*. At co-infection ratio (A. indicated by red arrow: 50:50; aflatoxin AFG2, AFB1 and at 75:25; AFG2 were detected whereas AFG1, AFB1 and AFB2 were not detected for GAF4 maize line respectively); (B. at 50:50 and 75:25) there was significant decrease in presence of both aflatoxins or no signals detected by HPLC on KDV1 maize line, an indication of probably complete inhibition of the aflatoxigenic isolate by atoxigenic counterpart. GAF4 maize appeared less susceptible to *Aspergillus flavus* colonisation compared to KDV1. (AFB1: aflatoxin B1; AFB2: aflatoxin B2; AFG1: aflatoxin G1; AFG2: aflatoxin G2).

The quantity of aflatoxins AFB1 and AFB2 presence in the maize tissues was significantly decreased in both maize lines at co-infection ratio (50 atoxigenic:50 aflatoxigenic) (Fig.6.8). This observation suggests that aflatoxin production by KSM014 might have been suppressed by KSM012. Interestingly, the detection of

AFB1 and AFG2 was significantly higher in GAF4 maize line (Fig.6.8A) compared to possibly sensitive KDV1. Additionally, significant reduction of both aflatoxins at co-infection 50:50 and 75:25 (atoxigenic/ aflatoxigenic) were noted in KDV1 maize line suspected to be sensitive to *A. flavus* infection and aflatoxins (Fig.6.8B). The observation was believed to be a potential complete inhibition of aflatoxin production by their counterpart, atoxigenic strain.

The observations above probably indicate that upon colonisation of kernels by aflatoxigenic isolate, atoxigenic strain might have had a potential to limit colonisation of the former strain and thereby inhibited and reduced toxin levels. Consequently, this was observed as a probable confirmation of possible complete inhibition of aflatoxin production. These findings were found to be promising and might be suitable for development of biocontrol system appropriate for aflatoxin mitigation and *A. flavus*.

Bandyopadhyay *et al.* (2016a), demonstrated that biocontrol of aflatoxins is a cost-effective method for managing aflatoxins with the potential for a long-term solution to aflatoxin contamination in some parts of the world and sub-Saharan Africa. However, for biocontrol to reach its full potential in relieving the burden of aflatoxin contamination in Africa, management programs that optimise both biocontrol's long-term and area-wide benefits are required (Bandyopadhyay *et al.*, 2016a).

Biocontrol experiments (Atehnkeng *et al.*, 2016a; Bandyopadhyay *et al.*, 2016a), showed a reduction in aflatoxin contamination on peanuts and corn fields between 74.3 % to approximately 99.9 % on wheat coated with atoxigenic *A. flavus* strains. Their observations were similar to the contemporary findings where at co-inoculation ratio of atoxigenic to aflatoxigenic isolates (50:50 and 75:25) (Fig.6.8B), there was insignificant presence of aflatoxins or no signals observed on HPLC. The findings suggest that atoxigenic isolate might competitively excluded aflatoxigenic producer and, or displaced the aflatoxigenic counterpart hence the reduction in toxin production (Fig.6.8B). For biocontrol and biosafety application of atoxigenic *A. flavus* isolates in the field as a potential biological control agent, the atoxigenic *A. flavus* isolates should ideally be indigenous, genetically stable and must belong to a VCG that does not contain aflatoxigenic members (Cotty, 2006; Mehl & Cotty, 2008; Mehl *et al.*, 2012).

Atehnkeng *et al.* (2016a) identified atoxigenic isolates (La3279, Og0222, Og0437 and Ka16127) as potential candidates for biocontrol strategies in corn fields in Nigeria. From the study, it can be suggested that atoxigenic isolate (KSM012) might be a possible potential candidate for aflatoxin and *A. flavus* mitigation thus, a recommendation for possible field trial is required.

Studies by Medina *et al.* (2015); Lima *et al.* (2015) and Stevenson *et al.* (2015) conducted under laboratory conditions showed that abiotic stress-related factors such as temperature, humidity, water activity and solutes can impact growth and aflatoxin production in *Aspergillus* spp. An improved understanding of the impact of these environmental factors on the abiotic stress of *A. flavus* under field conditions could be potential in improving the efficacy of biocontrol of aflatoxigenic isolates (Stevenson *et al.*, 2015).

The mechanism for biocontrol may be the displacement of aflatoxigenic isolates from host plants through initiation effects and a discrepancy in sporulation on substrates (Mehl *et al.*, 2012). Horn & Greene, (1995) observed that atoxigenic isolates compete with aflatoxigenic isolates during co-infection and could interfere with aflatoxin contamination. Competitive exclusion by atoxigenic isolates elucidates aflatoxin reduction during co-infection (Hruska *et al.*, 2014), a process aided by primary host contact (Mehl *et al.*, 2011). Huang *et al.* (2011) showed that the potential mechanisms for the latter includes thigmo-downregulation of aflatoxin biosynthesis and variance in ability among isolates in nutrient resource use (Mehl & Cotty, 2013).

6.3 Conclusion

Bioanalytical methods developed for metabolite extraction, fungal detection and quantification were validated and found to be appropriate for diagnosis of aflatoxin presence or absence in *A. flavus* isolates and in co-infection studies on maize. TLC approach was found to be simple, robust and non-quantitative method for aflatoxin detection. HPLC and LC-MS/MS was able to precisely determine and quantify aflatoxins with the latter able to determine a broad range of other metabolites in the *A. flavus* isolates.

The KDV1 maize line, which is cultivated in Makueni region and its environs was found more sensitive to fungal infections than was maize line GAF4. This

sensitivity is one possible reason why aflatoxicosis outbreaks are more frequent in Makueni than in Nandi, Kisumu and Homa Bay. The use of atoxigenic *A. flavus* strain during co-infection showed possible maximum inhibition of aflatoxin production by their aflatoxigenic counterparts. Moreover, the method developed as a biocontrol for mycotoxin mitigation using locally adopted atoxigenic strains of *A. flavus* exhibited a reduction in toxin profiles on the tested samples at co-infection ratios 50:50 for both atoxigenic and aflatoxigenic strains.

CHAPTER SEVEN

General Conclusion

7.0 Summary

The objective of the current study was to isolate and characterise *A. flavus* strains from maize kernels sampled from four different Kenyan counties which had different climatic conditions. The characterisation of the *A. flavus* isolates covered phenotypic, morphological and phylogenetic analyses together with examining their VCG diversity, gene expression of aflatoxin biosynthetic genes and metabolite and aflatoxin profile. The study was also extended into determining whether specific maize lines that are grown in the four counties influence aflatoxin production by aflatoxigenic strains and whether atoxigenic *A. flavus* isolates could be used as a biocontrol to limit aflatoxin production by aflatoxigenic strains.

In chapter two, the study revealed that maize kernels collected from Makueni, Homa Bay and Kisumu regions had high fungal contamination. The genus *Aspergillus* was the dominant strain isolated across the four regions. In terms of sclerotia presence and production, Makueni was the only county whose *A. flavus* isolates exhibited both S and L-morphotypes, whereas isolates from Nandi, Kisumu and Homa Bay regions lacked the S-type morphology. This could be a reason why Makueni frequently experiences chronic aflatoxicosis incidences over the last few decades as compared to other regions. Determining the primary causal agents of aflatoxin contamination is critical for predicting risk of contamination events and designing and implementing management strategies. Thus, aflatoxin management strategies that reduce frequencies of the S-morphotype strain may be particularly effective at reducing subsequent contamination.

VCG genetic diversity, aflatoxin-producing ability, fluorescence emissions, macro- and micro-morphological characters were the dominant differences between and within the *A. flavus* isolates sampled from the four climatic regions surveyed in chapter three. Out of thirty-seven isolates identified using culturable methods and molecular tools, the *nit* mutants' complementation test revealed twenty VCGs. Isolates from each region were self-compatible within the locality but incompatible between the regions and locality with a few exceptions. Heterokaryon incompatibility was stronger between

Nandi and Makueni isolates. Same strains from Nandi, Kisumu, and Homa Bay were vegetatively compatible and formed dense hyaline mycelia at the point of intersection. Kisumu and Makueni isolates were widely distributed. Additionally, Kisumu isolates were diversified compared to other regions with Nandi being the least diverse. ITS1 and ITS2 primers discriminated *A. flavus* isolates with Kisumu isolates more closely related to isolates from Nandi compared to isolates from Homa Bay region.

In chapter four, the deletion patterns of aflatoxin biosynthetic pathway gene cluster were dominant for *afID* and *afIR* whereas *afIO*, *afIS* and *afIP* had no deletion patterns. Yu *et al.*, (2004a), postulated that aflatoxin pathway genes are expressed concurrently and organized in cluster in the genome of *A. flavus* and *A. parasiticus*. One-way analysis of variance (1-way ANOVA) and Post-test for linear trend revealed aflatoxin biosynthetic cluster genes exhibiting significant difference between atoxigenic and aflatoxigenic isolates ($P < 0.05$). Tukey's Multiple Comparison Test (TMCT) revealed significant variation for different biosynthetic genes. Interestingly, *afIP* gene did not exhibit significant variation in expression for the isolates between induced and uninduced isolates except for isolate KSM012. This observation suggests that *afIP* might not be a good marker in discriminating between aflatoxigenic and atoxigenic isolates. However, *afID*, *afIR* and *afIS* revealed significant variation for aflatoxigenic isolate KSM014 in transcript abundance between induced and uninduced states. False detection of *afID* gene transcript was evident in both induced and uninduced states for isolate KSM012 (atoxigenic strain). This result was similar to Bhatnagar *et al.*, 2003 who also observed false positive and negative transcription signals for this gene. One-way ANOVA and Bartlett's test for equal variances revealed significant increase in transcript abundance, ($P < 0.05$) of *afIP*, *afIS* and *afIO* compared to *afID* and *afIR*. The *afIP*, *afIS*, *afIR* and *afIO* transcripts were the most upregulated genes across the tested isolates. *afIP* was highly expressed followed by *afIS* and *afIO* whereas *afIR* and *afID* were expressed the least. *afIS* and *afIO* were always found to be expressed in both induced and uninduced isolates.

In chapter five, a qPCR based fungal biomass assay revealed no significant variation between uninfected and infected maize plant tissues for the GAF4 maize line. However, the KDV1 maize line had significant ($P < 0.05$) *A. flavus* biomass in infected shoots and roots compared to the uninfected control. This suggests that the GAF4

maize line may have possible resistance to *A. flavus* infection compared to KDV1, a maize line cultivated in Makueni region. The observation of KDV1 maize line susceptibility to fungal infection could possibly be another reason why Makueni and the neighbouring regions experiences more frequent aflatoxicosis outbreaks and high levels of aflatoxin contamination of maize. The β -Tubulin and *Ef1a* markers were precise and able to detect and quantify the fungal biomass load with infected tissues only giving positive results. The MEP primer was observed to be plant specific by the absence of cross-reaction with fungal gDNA. As far as we are aware, this is the first report of a qPCR fungal biomass assay for the quantification of *A. flavus* using a β -*Tubulin* gene.

Chapter six focussed on the optimisation of bioanalytical methods such as TLC, HPLC and a pilot LC-MS/MS to detect and quantify aflatoxin presence. Though, LC-MS/MS was not adequately exhausted due to time constraints and financial wherewithal the TLC and HPLC were effective methods for detecting aflatoxins. Atoxicogenic isolate KSM012 was used as a biocontrol to reduce aflatoxin production by the toxigenic isolate KSM014. The sensitive KDV1 maize line showed a significant reduction of both aflatoxins at co-infection ratio 50:50 and 75:25 for atoxicogenic/aflatoxicogenic where there was no detection of aflatoxins using HPLC. This observation suggests that upon colonisation of kernels by an aflatoxicogenic isolate the atoxicogenic strain limits aflatoxin production by possibly outcompeting the aflatoxicogenic strain.

7.1 Significance and limitations of the study

This study is believed to be the first to compare *A. flavus* isolates from four different climatic regions in Kenya. Previous studies (Ngindu *et al.*, 1982; Gieseke 2004a; Cotty & Jaime-Garcia, 2007; Probst *et al.*, 2012) focussed on Makueni and neighbouring regions due to the endemic aflatoxicoses in this area, but there has been no study that characterised *A. flavus* isolates from all four climatic regions. The findings show that there are clear differences in the genetic diversity and distribution of the *A. flavus* isolates when comparing the other regions with Makueni. that the Toxigenic isolate, KSM015, which had the S-morphotype but produced both aflatoxins B and G was unique to the region. In addition, the maize line, KDV1, grown in this region was susceptible to infection by aflatoxicogenic isolate KSM015 and KSM014.

These factors could be among the reasons for the relatively frequent aflatoxicosis outbreaks in Makueni.

Molecular techniques such as qPCR and chromatography based bio-analytical techniques were useful in identifying aflatoxigenic isolates and associated aflatoxin production. As far as we are aware, this is the first report where a qPCR assay was used to determine the *A. flavus* biomass in an infected maize plant. Endemic atoxigenic *A. flavus* isolates from one of these Kenyan counties could be used for the development of a biocontrol strategy against aflatoxigenic isolates.

This study had also certain challenges and weaknesses that hampered the study and limited its scope. An initial problem was obtaining substantial quantity of maize kernels from certain regions such Makueni, Kisumu and Homa Bay regions due to poor harvests and maize necrosis disease. There was insufficient research funding to expand fieldwork and sampling across a greater area and across more regions of the administrative counties. Due to experimental design constraints, it was only possible to examine one aflatoxigenic and atoxigenic isolate respectively for the *in vitro* biocontrol study. Similarly, only two Kenyan maize lines: KDV1 and GAF4 was selected for testing the infectivity of an aflatoxigenic isolate. One should therefore be cautious in drawing too many conclusions from these results without testing more atoxigenic *A. flavus* isolates and including a greater variety of maize lines to validate these findings.

The bio-analytical techniques were largely successful in detecting and/or quantifying aflatoxins and other metabolites, that the nature of the experimental design and associated costs prevented more *A. flavus* isolates from being analysed. The use of aflatoxin standards in HPLC may be problematic in developing and third world countries due to logistical issues and lack of bioanalytical infrastructure. This also applies to the whole metabolomics approaches with LC-MS/MS, which though powerful, is not a practical approach to screen aflatoxin contaminated samples in countries like Kenya. Subsequent data analyses were complicated by the limited availability of specific *A. flavus* and aflatoxin databases which could give exhaustive information the metabolites produced and detected.

7.2 Proposed future work

Sampling and isolation of *A. flavus* strains should be done in other Kenyan counties, followed by similar characterisation as in this study, so that a more comprehensive picture can be obtained. This will allow possible problem areas to be flagged and focussed agricultural programs to be developed to target these areas to limit *A. flavus* infection. This should be done in conjunction with identifying the maize lines grown in these regions so as to identify susceptible maize lines and allow maize germplasm to be selected and bred for resistance to fungal infection. As mentioned previously, more atoxigenic *A. flavus* isolates need to be included in the *in vitro* biocontrol assay with the long-term goal of testing this on maize lines under field-grown conditions. For example, a study on the potential of the atoxigenic fungi (KSM012) to produce other fungal metabolites, such as cyclopiazonic acid, which are also known to be toxic. There is a need for more molecular markers that can differentiate *A. flavus* strains within the species due to the contradicting findings reported by many researchers in this field. Ideally, a standard marker which can resolve the phylogeny within the species level would allow molecular systematic studies from different researchers to be compared. The use qPCR assay for *A. flavus* biomass quantification needs to be expanded into other areas such as screening of maize lines resistant to *A. flavus* infection, monitoring disease progression, determining asymptomatic infection and plant-host studies.

The LC-MS/MS work in this study was very preliminary and this needs to be expanded and repeated to validate the data obtained. In addition, this should be linked with NMR spectroscopy to clarify the ambiguous structures, molecular masses and chromatograms for some overlapping metabolites and compounds. The advantage of this approach is that it will allow for finer discrimination between the different strains of *A. flavus* and metabolites produced by some specific isolates.

In conclusion, we have performed a comprehensive and holistic study on *A. flavus* isolates from four climatic regions of Kenya and this work will serve as a basis for future studies.

CHAPTER EIGHT

Publications and Conferences



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Vegetative compatibility and phenotypic characterization as a means of determining genetic diversity of *Aspergillus flavus* isolates

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Heterokaryon incompatibility/compatibility and phenotypic characterisation of *Aspergillus flavus* isolates in low and high risk zones in Kenya

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332T Heterokaryon incompatibility and phenotypic characterisation of *Aspergillus flavus* isolates in low and high risk zones in Kenya. Alfred Mitema^{1,2}, Sheila Okoth², Revel Iyer², Amelia Hilgart², Suhail Rafudeen² 1) Molecular and Cell Biology, University of Cape Town, Cape Town, Western Cape, ZA; 2) University of Nairobi, Kenya.

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Supplementary Data

S1: Questionnaire

We hired people (1-2) from the local community to aid in native language translation during the interview for ease of communication. Proposed questions were:

1. Do you cultivate maize or what types of crops do you cultivate?
2. How do you store maize kernels after harvest?
 - (i). In granaries []
 - (ii). In sacs []
 - (iii). On the floor []
 - (iv). Other.....
3. Do you know, or have you heard of any maize pathogens such as *Aspergillus* ear rot in your location/region?
4. Have you heard of any incidence(s) related to consumption of contaminated kernels with *Aspergillus* ear rot? If yes, which year?
5. How long have you been trading in maize as a cereal?
 - 0-12 months []
 - 1-2 years []
 - Over 2 years []
6. In which other markets do you also trade or sell your products?
 - (i).
 - (ii).
7. From where do you obtain the maize?

	Yes	No
(i). Directly from the farm	<input type="checkbox"/> []	<input type="checkbox"/> []
(ii). From other traders	<input type="checkbox"/> []	<input type="checkbox"/> []
(iii). Own farm	<input type="checkbox"/> []	<input type="checkbox"/> []
(iv) Other.....		

S2. Additional macro- and micro-morphological characteristics of *A. flavus* strains isolated from four climatic regions of Kenya. (NC: Nandi county; KSM: Kisumu; HB: Homa Bay; MC: Makueni county; CYA: Czapek yeast agar; CZ: Czapek dox agar; SEM: Scanning electron microscope) (Figs. S2.1-S2.43).

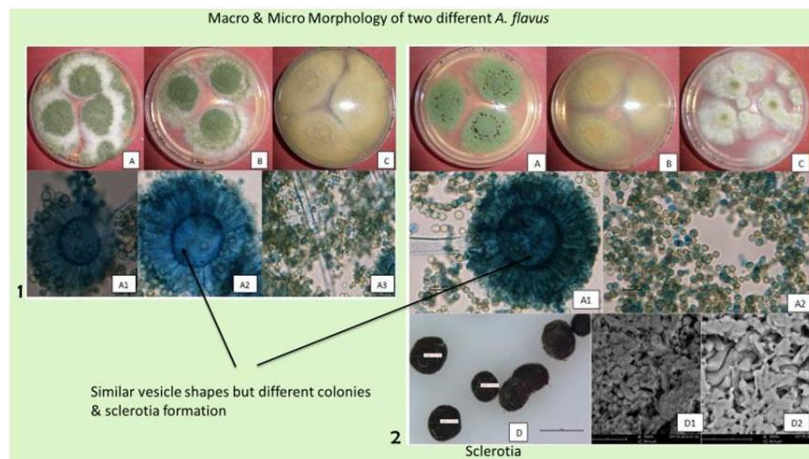


Figure S2.1 *Aspergillus flavus* isolate-HB-BOK 0021-1 (1) from left to right, colonies incubated at 25 ° C for 7 days on **A.** CYA; **B.** CZ showing front and reverse (**A, C**) colony appearance respectively. Conidiophores (**A1, A2**) and conidia (**A3**) and (2) *A. flavus* isolate-HB-CBR 0025. from left to right, **A.** CYA; **C.** CZ showing front and reverse (**A, B**) colony appearance respectively. Conidiophores (**A1**), conidia (**A2**) and SEM of sclerotia, surface and cross section (**D, D1, and D2**). (Scale bars 10 μ m and SEM X3000).

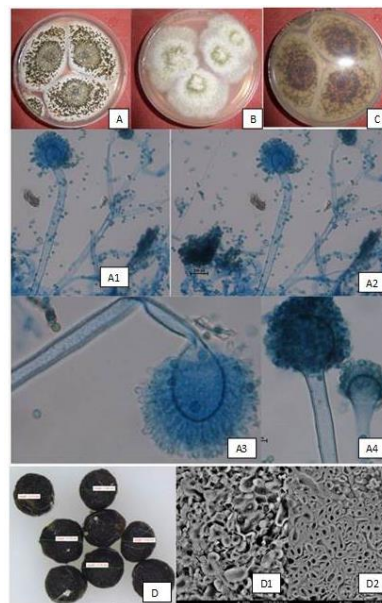


Figure S2.2 *Aspergillus parvisclerotigenus*-HB- 0029-1. from left to right, colonies incubated at 25 ° C for 7 days on **A.** CYA; **B.** CZ showing front and reverse colony (**A, C**) appearance, respectively. Branching conidiophores (**A1, A2, A3, A4**), conidia and SEM of sclerotia, surface and cross section (**D, D1, D2**). (Scale bars 10 μ m and SEM X3000).

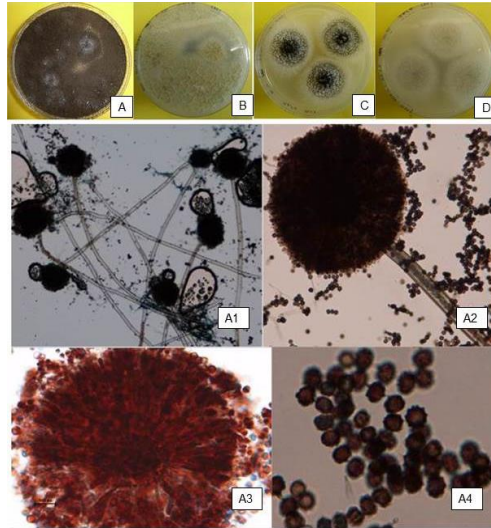


Figure S2.3 *Aspergillus niger* isolate-MC NZU (1) 0034-BK. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse (**A**, **B**, **C**, **D**) colony appearance, respectively. Conidiophores (**A1**, **A2** and **A3**) and conidia (**A4**), respectively. (Scale bars 10 μ m).

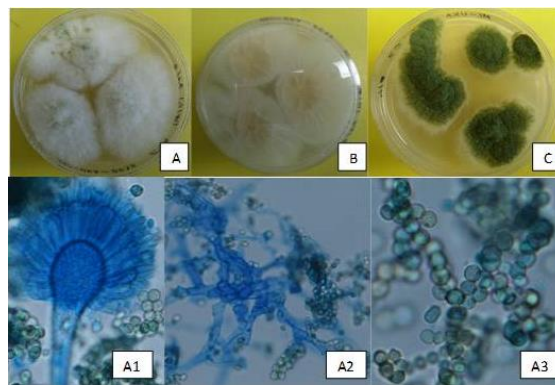


Figure S2.4 *Aspergillus flavus* isolate-MC UKA 0035-G. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse (**A**, **B**) colony appearance, respectively. Conidiophores (**A1**, **A2**) and conidia (**A3**), respectively. (Scale bars 10 μ m).

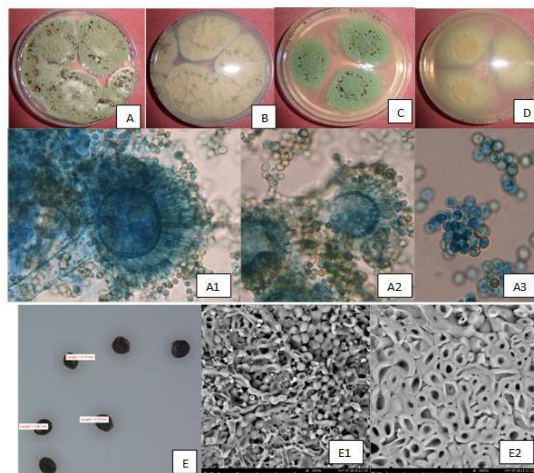


Figure S2.5 *Aspergillus flavus* isolate- NC-CHB 002-1. From left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse colony (**A**, **B**; **C**, **D**) appearance respectively. Conidiophores (**A1**, **A2**) and conidia (**A3**). SEM of sclerotia, surface and cross section (**E**, **E1**, **E2**). (Scale bars 10 µm and SEM X 1000).

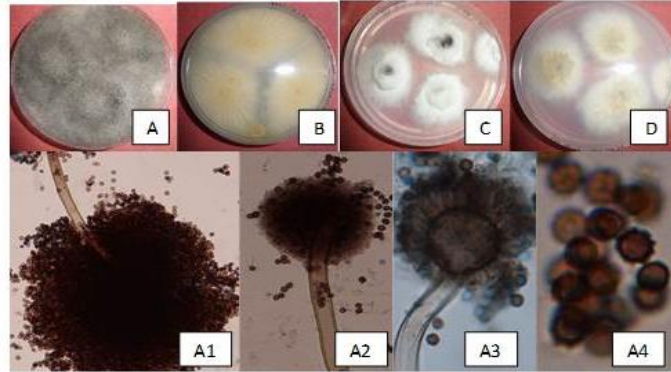


Figure S2.6 *Aspergillus carbonarius* isolate-NC-STW 004-1. From left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse colony (**A**, **B**; **C**, **D**) appearance respectively, conidiophores (**A1**, **A2**, **A3**) and conidia (**A4**). (Scale bars=10 µm).

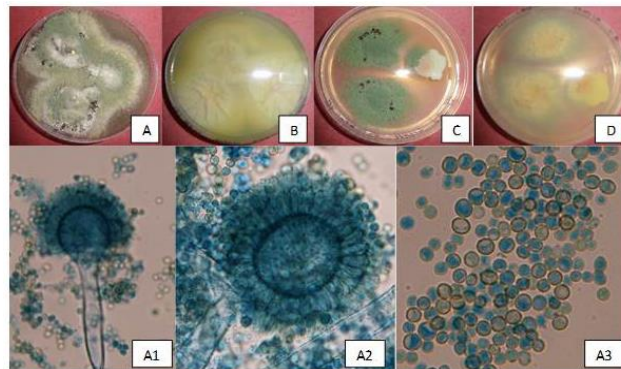


Figure S2.7 *Aspergillus flavus* isolate-NC-TBA 005-2. **A**. CYA; **C**. CZ showing front and reverse colony (**A**, **B**; **C**, **D**) appearance respectively. Conidiophores (**A1**, **A2**) and conidia (**A3**).

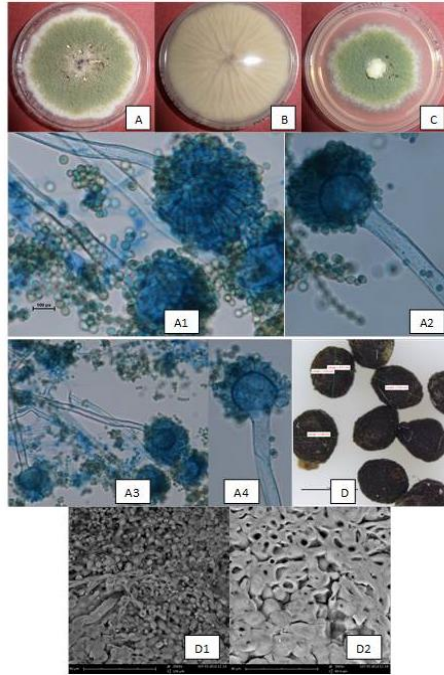


Figure S2.8 *Aspergillus minisclerotium* isolate-NC-TBA 005-3. From left to right colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse (**A**, **B**) colony appearance respectively. Conidiophores (**A1**, **A2**, **A3**, **A4**); SEM of sclerotia, surface and cross section (**D1**, **D2**, **D3**). (Scale bars 10 µm and SEM X3000).

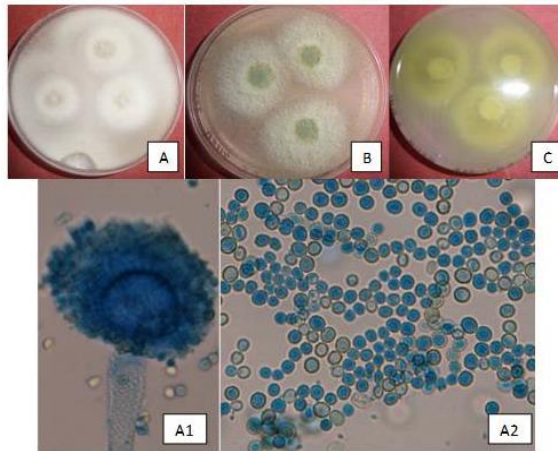


Figure S2.9 *Aspergillus aculeatus* isolate-NC-KBY 006-1 from left to right colonies incubated at 25 ° C for 7 days on. **A**. CYA; **B**. CZ showing front and reverse colony (**B**, **C**) appearance respectively.

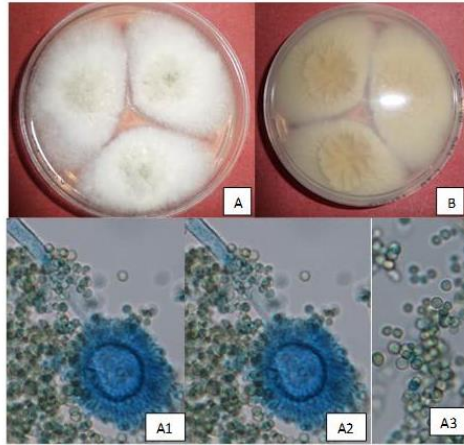


Figure S2.10 *Aspergillus flavus* strains isolate-NC-MRB 007 colonies incubated at 25 ° C for 7 days on **A**. CYA showing front and reverse (**A**, **B**) colony appearance respectively, conidiophores (**A1**, **A2**) and conidia (**A3**). (Scale bars 10 µm).

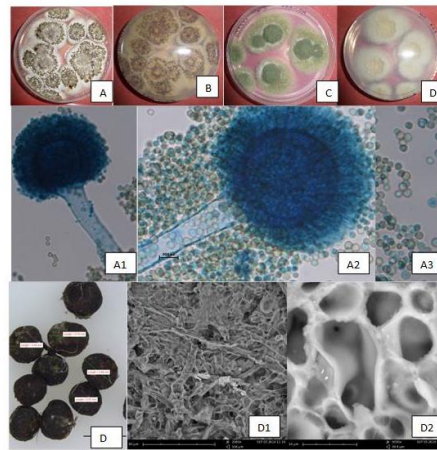


Figure S2.11 *Aspergillus flavus* strains isolates-NC- 009-2. colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse (**A**, **B**, **C**, **D**) colony appearance respectively. Conidiophores (**A1**, **A2**), conidia (**A3**), SEM of sclerotia, surface and cross section (**D**, **D1**, **D2**) respectively. (Scale bars 10 µm and SEM X3000).

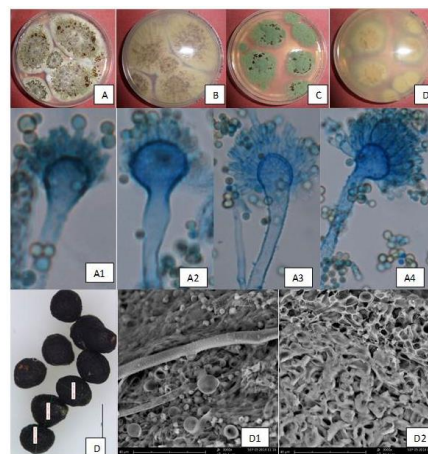


Figure S2.12 *Aspergillus flavus* strains isolate-NC- MTT 0010-1 colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse (**A**, **B**, **C**, **D**) colony appearance

respectively. Conidiophores (**A1, A2, A3, A3**), SEM of sclerotia, surface and cross section (**D, D1, D2**). (Scale bars 10 μ m and SEM X3000).

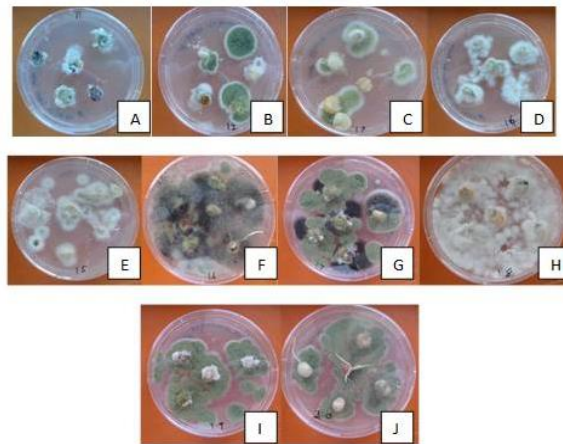


Figure S2.13 Growth of *Aspergillus* species on maize kernels collected from different locations/sites in Kisumu county. Maize kernels were incubated on PDA at 30 ° C for 4 days (**A**: KSM-KRU-0011; **B**: KSM-KOW-0012; **C**: KSM-SAT-0013; **D**: KSM-KAB-0014; **E**: KSM-OFN-0015; **F**: KSM-AHR-0016; **G**: KSM-CHRA-0017; **H**: KSM-OJR-0018; **I**: KSM-MHN-0019 and **J**: KSM-KPR-0020).

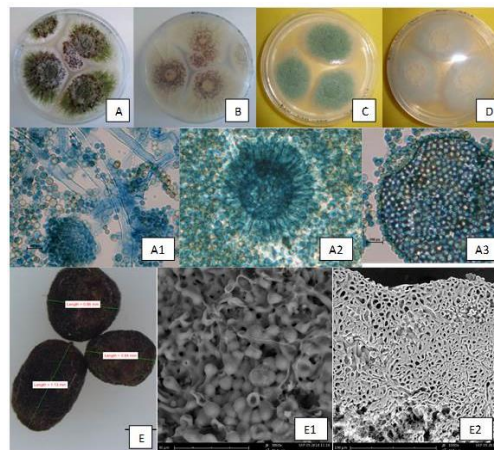


Figure S2.14 *Aspergillus flavus* isolate-KSM KOW 0012. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse (**A, B, C, D**) colony appearance respectively. Conidiophores (**A1, A2,**), conidia (**A3**) and SEM of sclerotia, surface and cross section (**E, E1, E2**), respectively. (Scale bars 10 μ m and SEM X3000).

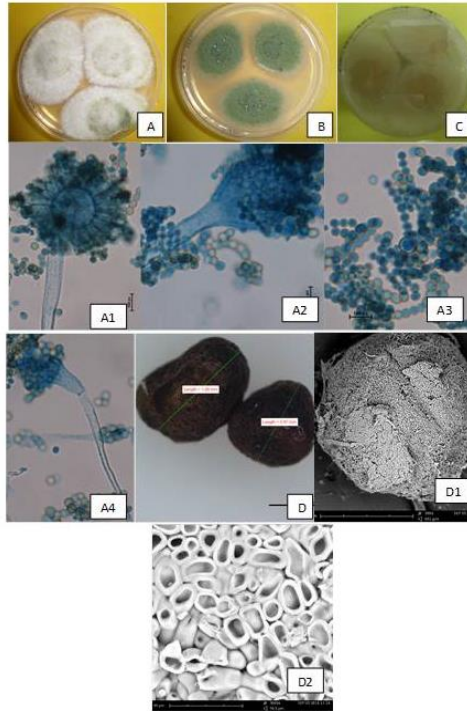


Figure S2.15 *Aspergillus nomius*-KSM SAT 0013. From left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **B**. CZ showing front and reverse (**A**, **C**) colony appearance respectively. Conidiophores (**A1**, **A2**, **A4**) conidia (**A3**) and SEM of sclerotia, surface and cross section (**D**, **D1**, **D2**). (Scale bars 10 µm and SEM X3000).

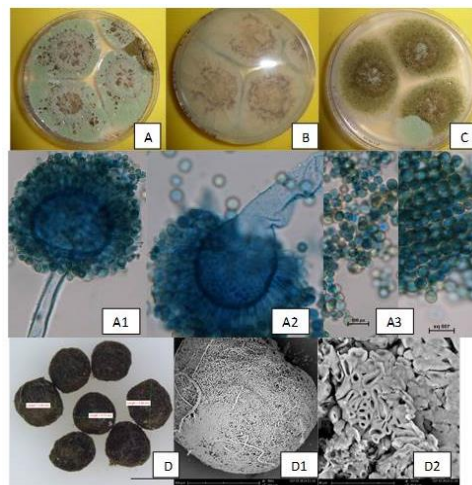


Figure S2.16 *Aspergillus oryzae*-KSM KAB 0014. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse (**A**, **B**) colony appearance respectively. Conidiophores (**A1**, **A2**), conidia (**A3**) and SEM of sclerotia, surface and cross section (**D**, **D1**, **D2**). (Scale bars 10 µm and SEM X3000).

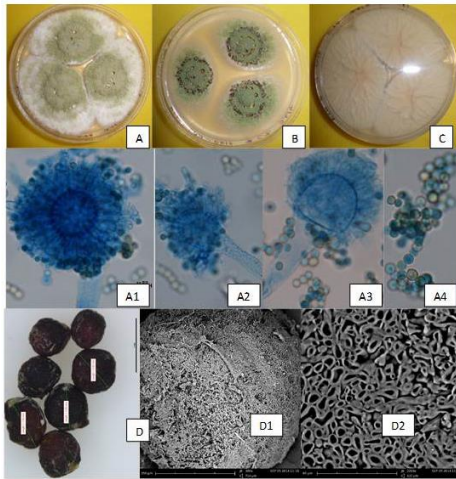


Figure S2.17 *Aspergillus arachidicola*-KSM 0FN 0015. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **B**. CZ showing front and reverse (**A**, **C**) colony appearance respectively. Conidiophores (**A1**, **A2**, **A3**), conidia (**A4**) and SEM of sclerotia, surface and cross section (**D**, **D1**, **D2**). (Scale bars 10 μ m and SEM X3000).

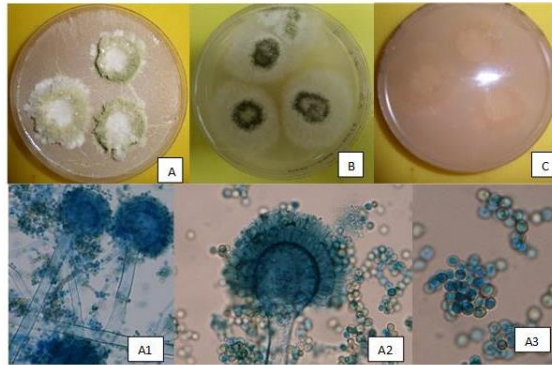


Figure S2.18 *Aspergillus togoensis*-KSM AHR 0016-G. From left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **B**. CZ showing front and reverse (**A**, **C**) colony appearances respectively. Conidiophores (**A1**, **A2**) and conidia (**A3**). (Scale bars 10 μ m).

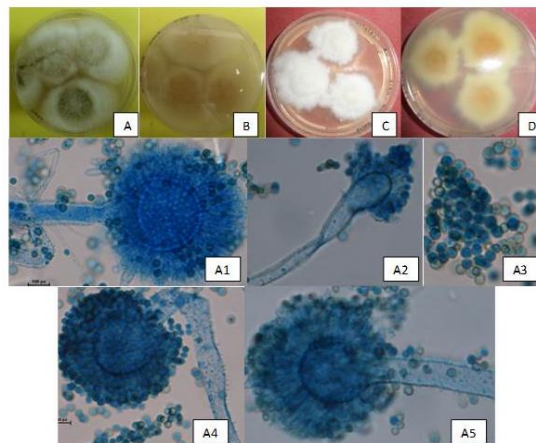


Figure S2.19 *Aspergillus togoensis*-KSM AHR 0016-Y. From left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse (**A**, **B**, **C**, **D**) colony

appearance respectively. Conidiophores (**A1, A2, A4, A5**) and conidia (**A3**). (Scale bars 10 μ m).

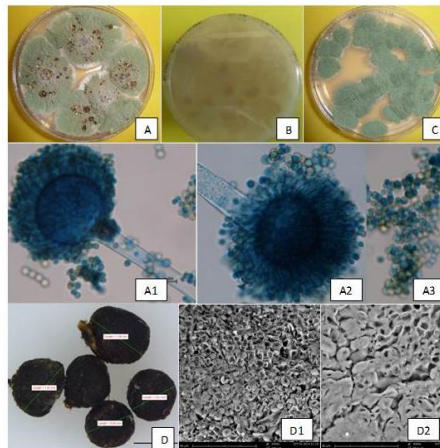


Figure S2.20 *Aspergillus flavus* isolate-KSM CHRA 0017. From left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse colony appearance respectively. Conidiophores (**A1, A2**), conidia (**A3**) and SEM of sclerotia, surface and cross section (**D, D1, D2**). (Scale bars 10 μ m and SEM X3000).

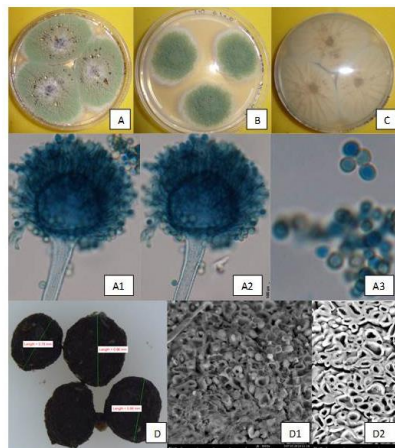


Figure S2.21 *Aspergillus caelatus* KSM-CHRA 0017-Y. From left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **B**. CZ showing front and reverse (**A, C**) colony appearance, respectively. Conidiophores (**A1, A2**), conidia (**A3**) and SEM of sclerotia, surface and cross section (**D1, D2, D3**). (Scale bars 10 μ m and SEM X3000).

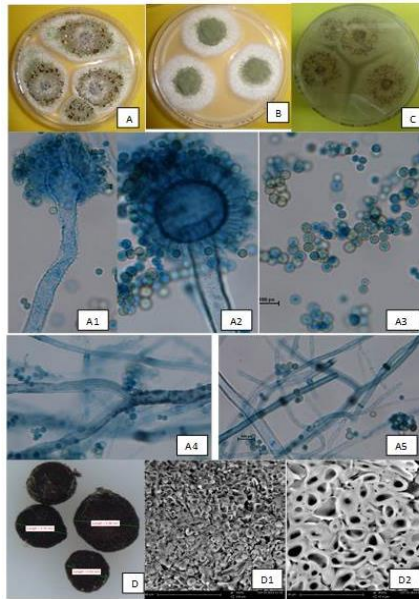


Figure S2.22 *Aspergillus minisclerotium*-KSM OJR 0018. From left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **B**. CZ showing front and reverse (**A**, **C**) colony appearance respectively. Conidiophores (**A1**, **A2**), conidia (**A3**), septate mycelia network (**A4**, **A5**), and SEM of sclerotia, surface and cross section (**D**, **D1**, **D2**). (Scale bars 10 μ m and SEM X3000).

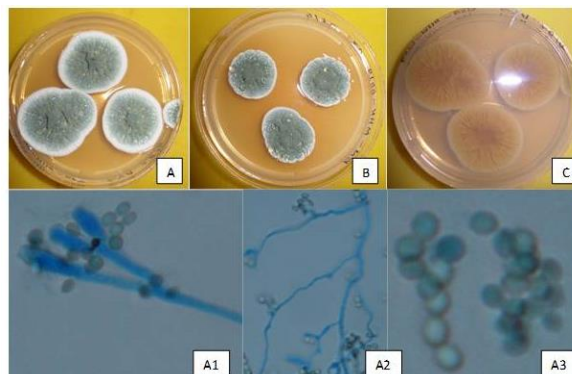


Figure S2.23 *Aspergillus* species isolate-KSM MHN 0019. From left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **B**. CZ showing front and reverse colony (**A**, **C**) appearance respectively. Conidiophores (**A1**, **A2**) and conidia (**A3**). (Scale bars 10 μ m).

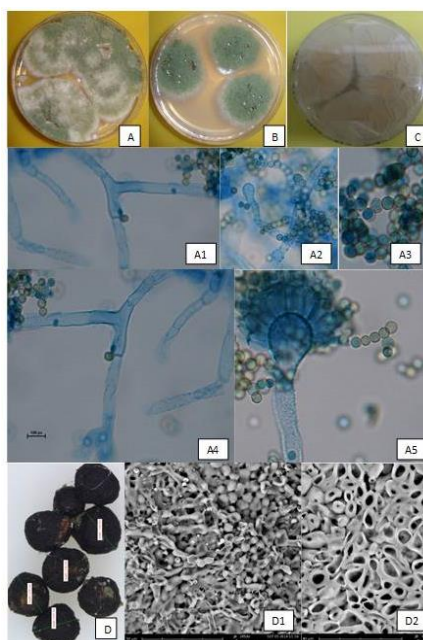


Figure S2.24 *Aspergillus flavus* isolate-KSM KPR 0020. From left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **B**. CZ showing front and reverse (**A**, **C**) colony appearance respectively. Septate mycelia (**A1**, **A4**), young conidiophore (**A2**), conidia (**A3**), conidiophore (**A5**) and SEM of sclerotia, surface and cross section (**D**, **D1**, **D2**). (Scale bars 10 μ m and SEM X3000).

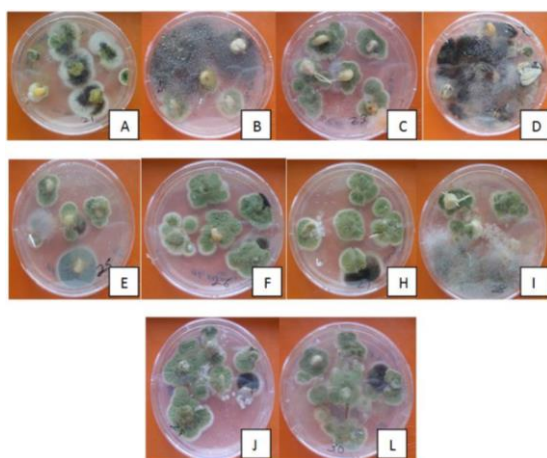


Figure S2.25 Fungal growth (*Aspergillus* species) on maize kernels from different locations/sites in Homa Bay county. Maize kernels were incubated on PDA at 30 ° C for four days. **A**: HB-BOK-0021; **B**: HB-RNG-2-0022; **C**: HB-MIK-0023; **D**: HB-RNG-1-0024; **E**: HB-CBR-0025; **F**: HB-KDG-2-0026; **G**: HB-KDG-1-0027; **H**: HB-OYG-0028; **I**: HB- 0029 and **J**: HB-KOL-0030 respectively.

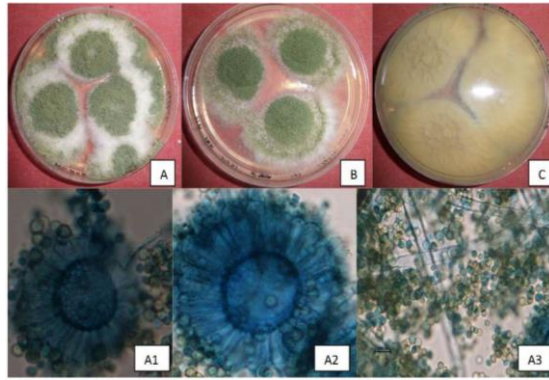


Figure S2.26 *Aspergillus flavus* isolate-HB-BOK 0021-1. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **B**. CZ showing front and reverse (**A**, **C**) colony appearance respectively. Conidiophores (**A1**, **A2**) and conidia (**A3**). (Scale bars 10 μm).

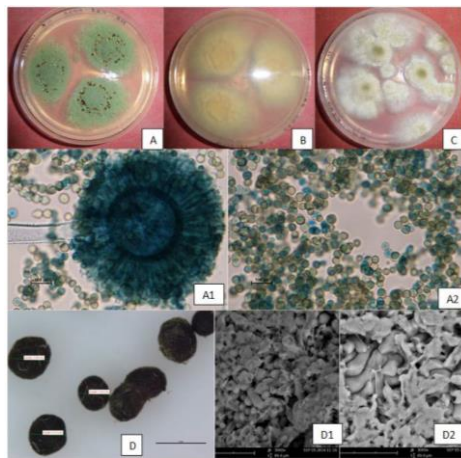


Figure S2.27 *Aspergillus flavus* isolate-HB-CBR 0025. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse (**A**, **B**) colony appearance respectively. Conidiophores (**A1**), conidia (**A2**) and SEM of sclerotia, surface and cross section (**D**, **D1**, and **D2**). (Scale bars 10 μm and SEM X3000).

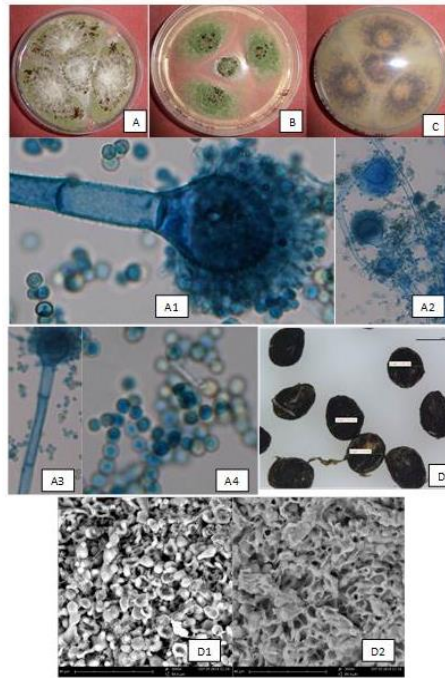


Figure S2.28 *Aspergillus flavus* isolate-HB-KDG 0026-Y. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **B**. CZ showing front and reverse (**A**, **C**) colony appearance respectively. Septate stipe (**A3**), conidiophores (**A1**, **A2**, **A3**), conidia (**A4**) and SEM of sclerotia, surface and cross section (**D**, **D1** and **D2**). (Scale bars 10 μ m and SEM X3000).

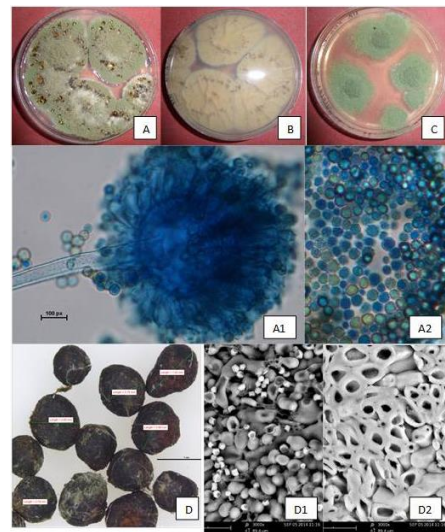


Figure S2.29 *Aspergillus flavus* isolate-HB-KDG 0027. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse colony (**A**, **B**) appearance respectively. Conidiophore (**A1**), conidia (**A2**) and SEM of sclerotia, surface and cross section (**D**, **D1** and **D2**). (Scale bars 10 μ m and SEM X3000).

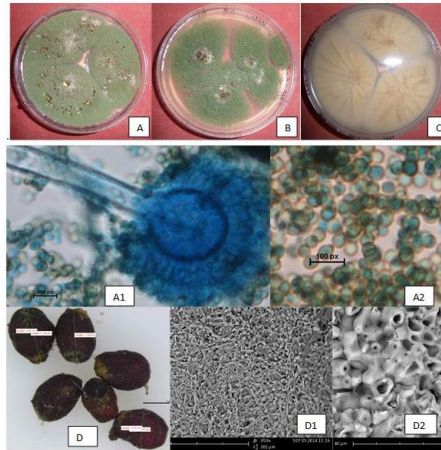


Figure S2.30 *Aspergillus flavus* isolate-HB-OYG 0028-1. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **B**. CZ showing front and reverse colony (**A**, **C**) appearance, respectively. Conidiophore (**A1**), conidia (**A2**) and SEM of sclerotia, surface and cross section (**D**, **D1**, **D2**). (Scale bars 10 μ m and SEM X3000).

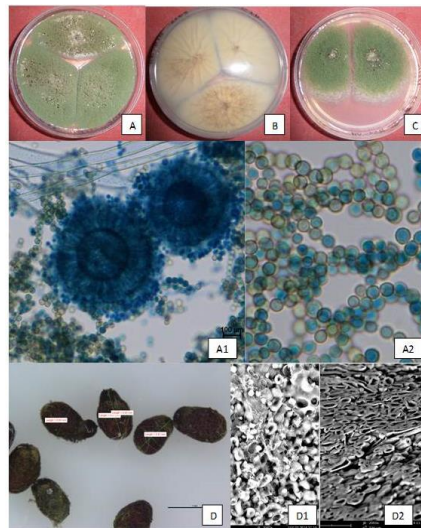


Figure S2.31 *Aspergillus flavus* isolate-HB-OYG 0028-2. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse colony (**A**, **B**) appearance, respectively. Conidiophores, conidia and SEM of sclerotia, surface and cross section (**D**, **D1**, and **D2**). (Scale bars 10 μ m and SEM X3000).

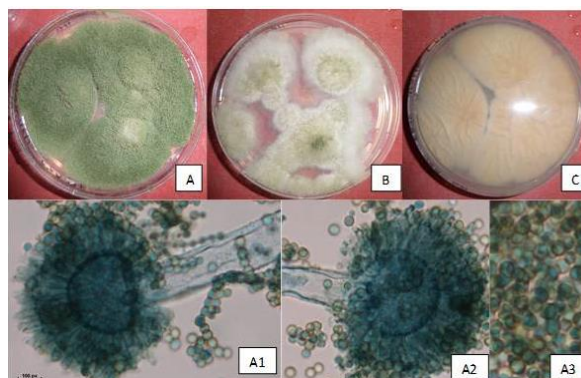


Figure S2.32 *Aspergillus flavus* isolate-HB- KOL 0030-1. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **B**. CZ showing front and reverse colony (**A**, **C**) appearance, respectively. Conidiophores (**A1**, **A2**) and conidia (**A3**). (Scale bars 10 µm).

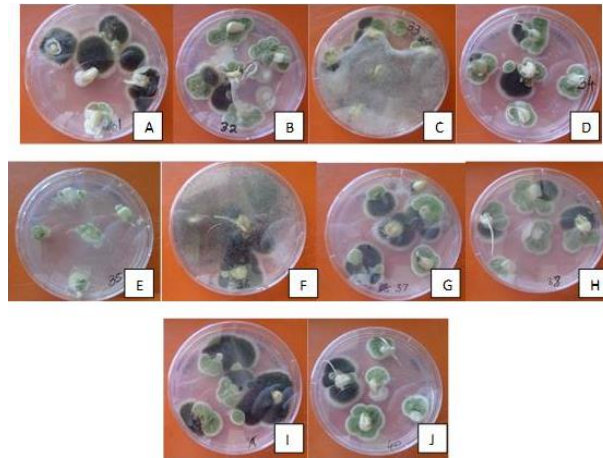


Figure S2.33 Growth of *Aspergillus* species on maize kernels collected from different locations/sites. Incubated kernels on PDA at 30 ° C for 4 days (**A**: MC-ST. LAW-0031; **B**: MC-MKY-0032; **B**: MC-NZU-2-0033; **C**: MC-NZU-1-0034; **D**: MC-UKA-0035; **E**: MC-KVN-0036; **F**: MC-MAL'09-0037; **G**: MC-MAL'11-0038; **H**: MC-MAL'12-0039 and **J**: MC-MAL'13-0040), respectively.

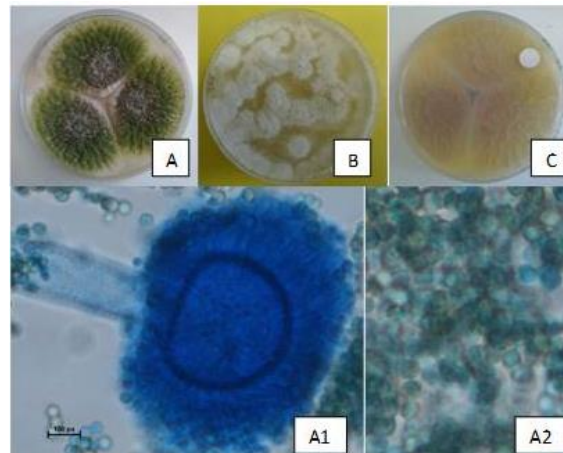


Figure S2.34 *Aspergillus flavus* isolate-MC St. LAW 0031. from left to right upper row, colonies incubated at 25 ° C for 7 days on **A**.CYA; **B**.CZ showing front (**A**, **B**) and reverse (**C**) colony appearance, respectively. Conidiophore (**A1**) and conidia (**A2**). (Scale bars 10 µm).

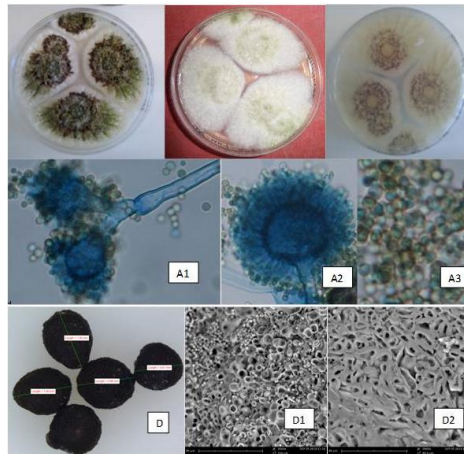


Figure S2.35 *Aspergillus sojae* isolate-MC MKY 0032 G. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **B**. CZ showing front (**A**, **B**) and reverse (**C**) colony appearance, respectively. Conidiophores (**A1**, **A2**), conidia (**A3**) and SEM of sclerotia, surface and cross section (**D**, **D1**, and **D2**) respectively. (Scale bars 10 μ m and SEM X3000).

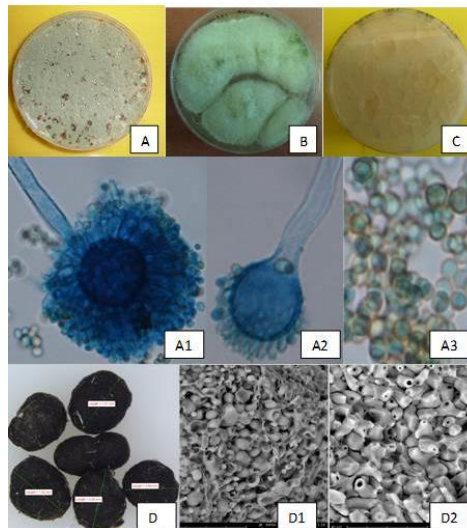


Figure S2.36 *Aspergillus sojae* isolate-MKY 0032-B. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA, **B**. CZ showing front (**A**, **B**) and reverse(**C**) colony appearance, respectively. Conidiophores (**A1**, **A2**) conidia (**A3**) and SEM of sclerotia, surface and cross section (**D**, **D1** and **D2**), respectively. (Scale bars 10 μ m and SEM X3000).

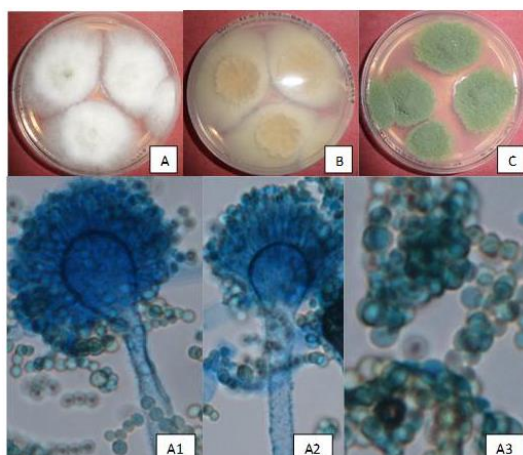


Figure S2.37 *Aspergillus flavus* isolate-MC NZU (2) 0033. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front (**A**, **C**) and reverse (**B**) colony appearance, respectively. Conidiophores and conidia (**A1**, **A2** and **A3**) respectively. (Scale bars 10 μ m).

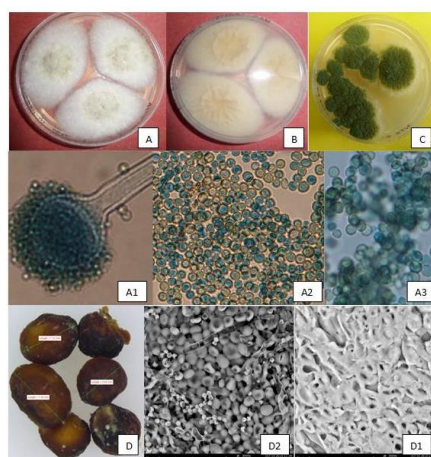


Figure S2.38 *Aspergillus flavus* isolate-MC NZU (1) 0034 G/Y. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front (**A**, **C**) and reverse (**B**) colony appearance, respectively. Conidiophore (**A1**), conidia (**A2**, **A3**) and SEM of sclerotia, surface and cross section (**D**, **D1** and **D2**) respectively. (Scale bars 10 μ m and SEM X3000).

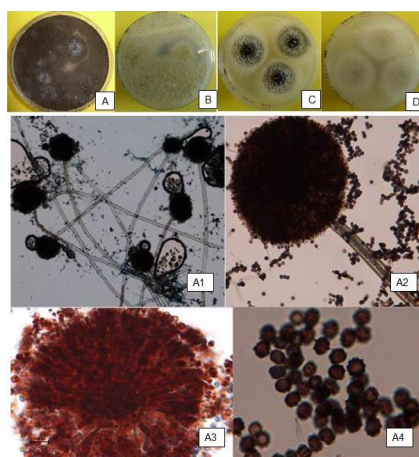


Figure S2.39 *Aspergillus niger* isolate-MC NZU (1) 0034-BK. from left to right, colonies incubated at 2525 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse (**A**, **B**, **C**, **D**)

colony appearance, respectively. Conidiophores (**A1**, **A2** and **A3**) and conidia (**A4**), respectively. (Scale bars 10 μ m).

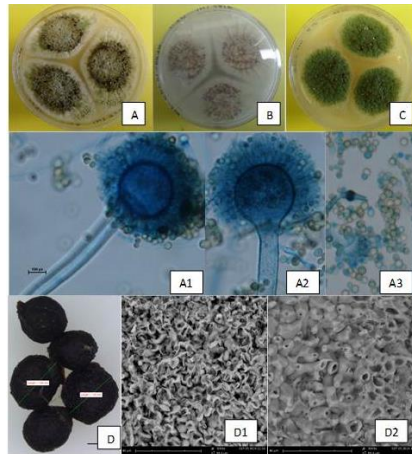


Figure S2.40 *Aspergillus minisclerotium*-MC NZU (1) 0034-W. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse (**A**, **B**) colony appearance, respectively. Conidiophores (**A1**, **A2**), conidia (**A3**) and SEM of sclerotia, surface and cross section (**D**, **D1**, **D2**), respectively. (Scale bars 10 μ m and SEM X3000).

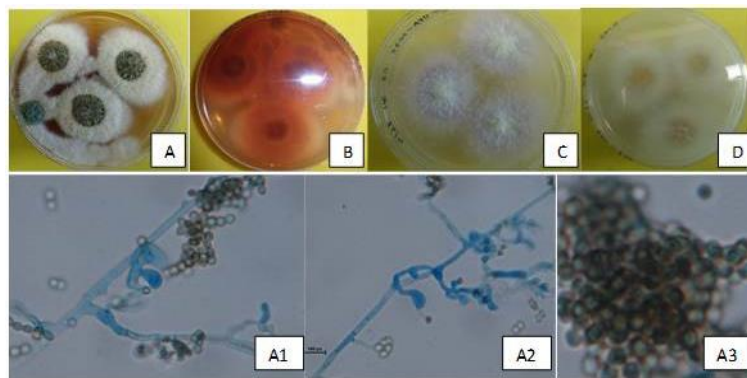


Figure S2.41 *Aspergillus carbonarius*-MC UKA 0035-W. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse colony appearance, respectively. Conidiophores (**A1**, **A2**) and conidia (**A3**). (Scale bars 10 μ m).

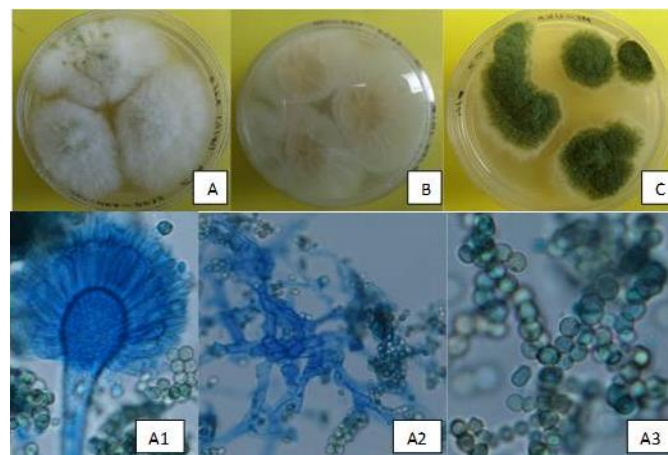


Figure S2.42 *Aspergillus flavus* isolate-MC UKA 0035-G. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse (**A**, **B**) colony appearance, respectively. Conidiophores (**A1**, **A2**) and conidia (**A3**), respectively. (Scale bars 10 µm).

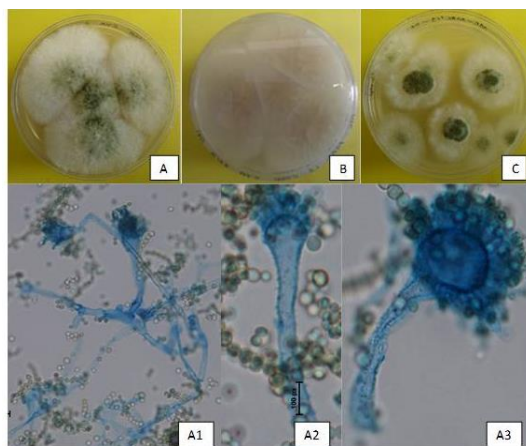


Figure S2.43 *Aspergillus nomius* isolate-MC MAL'13 0040-G. from left to right, colonies incubated at 25 ° C for 7 days on **A**. CYA; **C**. CZ showing front and reverse (**A**, **B**) colony appearance, respectively. Conidiophores (**A1**, **A2**, **A3**) and conidia. (Scale bars 10 µm).

Table S3.1 NCBI accession numbers of ITS1 and ITS2 domains of *Aspergillus flavus* strains isolated from maize kernels collected from four different climatic regions of Kenya. The sequences were generated using Sanger sequencing. The names assigned generated from NCBI website with Basic Local Alignment Search Tool nucleotide were identified as *Aspergillus flavus* strains with ≥ 99 % identity from NCBI GenBank.

<i>Aspergillus flavus</i> strain	NCBI accession no.	<i>Aspergillus flavus</i> strain	NCBI accession no.
NC01_1	MG430299	HB021_1	MG430317
NC02_1	MG430300	HB025	MG430318
NC04_1	MG430301	HB028_1	MG430319
NC05_2	MG430302	HB028_2	MG430320
NC05_3	MG430303	HB029_1	MG430321
NC06_1	MG430304	HB027_	MG430322
NC007	MG430305	HB030_1	MG430323
NC09_2	MG430306	MC032_G	MG430324
NC010_1	MG430307	MC031	MG430325
KSM013	MG430308	MC032_B	MG430326
KSM015	MG430309	MC033	MG430327
KSM016_G	MG430310	MC034_GY	MG430328
KSM016_Y	MG430311	MC034_W	MG430329
KSM017	MG430312	MC035_G	MG430330
KSM017_Y	MG430313	MC035_W	MG430331
KSM018	MG430314	MC040	MG430332
KSM019	MG430315	KSM012	MG385137
KSM020	MG430316	KSM014	MG385138

Figure S3.1 Aligned *Aspergillus flavus* sequences (gDNA isolated from maize kernels collected from four climatic regions of Kenya) generated from NCBI BLASTn.

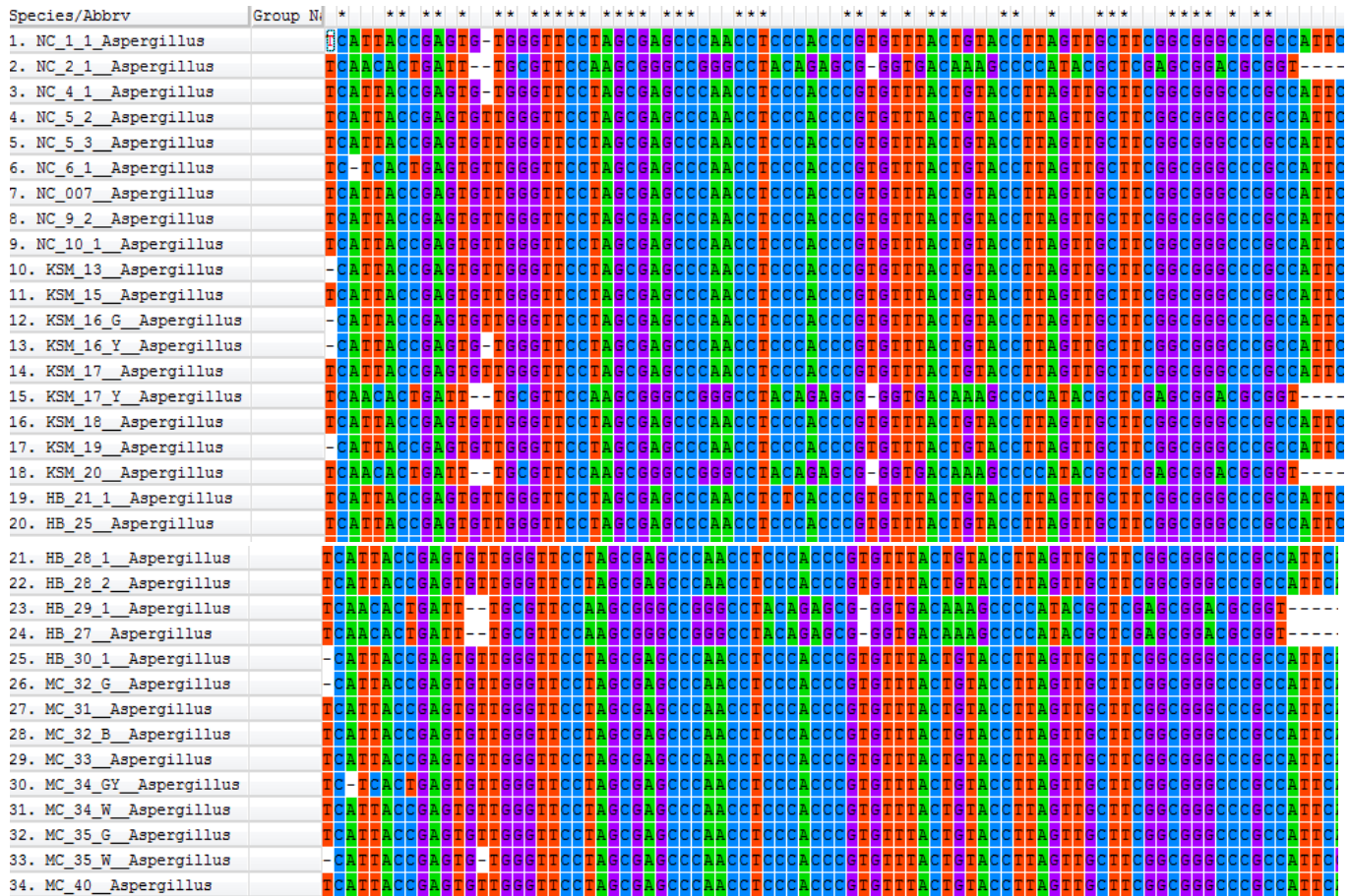


Table S4.1 Integrity and quality of RNA assessed on Nano Drop spectrophotometer used for downstream analysis.

<i>A. flavus</i> strains	State	ng/μl	260/280	260/230
KSM012	induced	1181.9	2.01	1.95
KSM012	uninduced	1539.3	2.03	2.0
KSM014	induced	1521.3	2.01	2.02
KSM014	uninduced	1201.0	1.91	2.13
HB021	induced	1634.6	1.96	2.11
HB021	uninduced	414.6	2.05	1.97
HB026	induced	2171.9	2.01	1.98
HB026	uninduced	1539.3	2.03	2.0
HB027	induced	1067.6	1.88	2.24
HB027	uninduced	1017.0	1.94	2.3

Table S5.1 Phenotypic characteristic measurements of control and infected (GAF and KDV1) maize lines with *Aspergillus flavus* isolate KSM014 ($n = 3$) taken after 14 days of growth. Massive variation was observed on roots and shoots of both maize lines, with KDV1 exhibiting worse symptoms of stunted growth.

Phenotypic characteristic																
Maize line	Control roots				Control shoots				Infected roots				Infected shoots			
	Exp 1	Exp 2	Exp 3	Average (mm)	Exp 1	Exp 2	Exp 3	Average (mm)	Exp 1	Exp 2	Exp 3	Average (mm)	Exp 1	Exp 2	Exp 3	Average (mm)
GAF4	285	260	278	274.33	352	322	312	328.67	134	113	98	115	142	185	111	146
KDV1	272	252	232	252	344	300	323	322.33	78	83	84	81.67	82	87	91	86.67

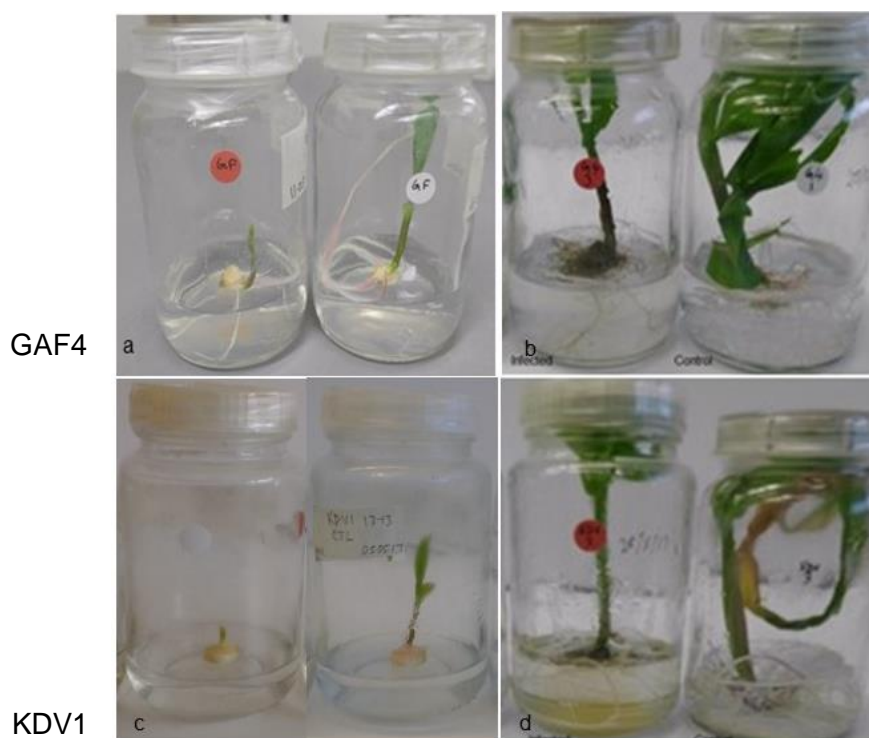


Figure S5.1 The GAF4 and KDV1 maize lines after 3 days (a, c) and 14 days (b, d) of growth with and without *A. flavus* KSM014 infection. The red sticker shows infected maize plants while the white stickers are the control, uninfected maize plants.

Table S6.1 Retention time and solvent gradients (ACN: Acetonitrile; H₂O: ultrapure water; TFA: Trifluoroacetic acid).

Time	Solvent C (100 % ACN with 0.005 % TFA)	Solvent D (H ₂ O with 0.005 % TFA)	Flow rate	Pressure (bars)
0	15	85	1	400
20	51	49	1	400
23	100	0	1	400
27	15	85	1	400

Table S6.2 The linear gradient used for running the samples and aflatoxin standards on the LC-MS/MS. (Solvent A: ultra-pure water; Solvent B: Acetonitrile).

Time [min]	Solvent A [%]	Solvent B [%]	Flow rate [ml/min]	Max. Pressure Limit [bar]
0.00	97.00	3.00	0.3	800
1.00	97.00	3.00	0.3	800
14.00	3.00	97.00	0.3	800
16.00	3.00	97.00	0.3	800
18.00	97.00	3.00	0.3	800

Figure S6.1 The standard curves for individual aflatoxin generated used for quantification of aflatoxins were revealed.

