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IDENTIFICATION OF SUITABLE GENES FOR RNAi SILENCING OF AFLATOXIGENIC *ASPERGILLUS FLAVUS* ISOLATED FROM GROUNDNUTS IN UGANDA

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Declaration

I, **Ssekandi Joseph**, declare that this dissertation is my original work and has never been submitted to any University for the award of any degree.

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List of abbreviations

DNA	Deoxyribonucleic acid
dsRNA	double stranded Ribonucleic Acid
EDTA	Ethylene Diamine Tetra-acetic Acid
FAO	Food Agricultural Organization
GPS	Global Positioning System
HDPE	High Density Polyethylene
IARC	International Agency for Research in Carcinogenesis
IRT	Inverted Repeat Transgenes
miRNA	Micro Ribonucleic Acid
NARL	National Agricultural Research Laboratories
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
piRNA	PIWI-interacting RNA
RISC	RNA-Induced Silencing Complex
RNAi	Ribonucleic Acid interference
siRNA	short interfering RNA
UBOS	Uganda Bureau of Statistics
UNBS	Uganda National Bureau of Standards
UTR	Un-Translated Region
UV	Ultra Violet
WHO	World Health Organization
ppb	Parts per billion

PRO	Principle research officer
DMSO	Dimethyl sulfoxide
KCl	Potassium chloride
USA	United States of America

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Abstract

Aflatoxins are lethal and carcinogenic secondary metabolites produced mainly by fungi of the Aspergillus genus, key among which is *Aspergillus flavus*. Previous research has shown that Ugandans are chronically exposed to these carcinogens and yet breeding *A. flavus* resistant varieties can be a sustainable solution to this problem. This study aimed at providing novel knowledge to groundnut breeders on which genes are suitable to silence in *A. flavus* so as to inhibit its growth using the RNAi technique triggered by dsRNA. Ten *A. flavus* isolates were isolated on Modified Rose Bengal Agar from 43 groundnut seed samples collected from Tororo, Soroti and Kamuli districts in Eastern Uganda and an Agraquant total aflatoxin ELISA kit was used to determine the amount of aflatoxins they produce. Only one isolate was non-toxigenic while others produced total aflatoxin ranging from five to over 2000 parts per billion. Thus there is diversity in *A. flavus* based on aflatoxin production ability. To determine the suitable genes for RNAi silencing in *A. flavus*, dsRNAs specific to 13 essential genes were transcribed *in vitro* using a T7 transcription kit. *A. flavus* spores were then exposed to these dsRNAs or sterile water for the controls and plated on ¼ strength PDA. Percentage spore growth inhibition was calculated and this spore growth inhibition data was subjected to ANOVA. Spore growth inhibition was significantly higher in all spores treated with dsRNAs targeting *A. flavus* essential genes in comparison to the controls treated with sterile water ($df = 13$, $p < 0.001$). Amongst the dsRNA treatments, silencing UBI, COH, 26s, ATP, PPK, IMP and ABC genes resulted in significantly higher spore growth inhibition than silencing GTT, HEL, PTRs, EXP, DRF and MOT genes ($df = 13$, $p < 0.001$). This study thus recommends UBI, COH, 26s, ATP, PPK, IMP and ABC as the suitable genes for RNAi silencing of *A. flavus in vivo* and also recommends a follow up study to establish the molecular basis for *A. flavus* diversity based on aflatoxin production ability.

CHAPTER 1: INTRODUCTION

1.1 Background

Filamentous fungi produce many secondary metabolites, some of which have tremendous impact on society for example some are exploited for their antibiotic and pharmaceutical activities while others are involved in disease interactions with plants or animals (Fox *et al.*, 2008). The secondary metabolites involved in such disease interactions are known as mycotoxins. These are small molecules that to date have not been proven to be essential for normal growth or development of the fungi. Thus the importance of these secondary metabolites to the fungi that produce them remains a mystery (Fox *et al.*, 2008). These toxins are introduced into human and animal food through direct infestation of edible plant tissue by toxigenic fungi, which produce toxic secondary metabolites. This contamination is especially worrying because many mycotoxins can survive food processing procedures like very high temperatures above 100^0C (Scott, 1991).

There are currently more than 400 mycotoxins known, amongst which 6 major classes frequently occur; Aflatoxins, Trichothecenes, Fumonisins, Zearalenone, Ochratoxins and Ergot alkaloids (Richard *et al.*, 2003). This study focused on Aflatoxins which are very dangerous if ingested by humans and animals. Aflatoxins for example if consumed in large quantities can cause acute aflatoxicosis which results in to quick human death. For example in 2004, in Kenya, 125 people died after ingesting aflatoxin contaminated maize (Lewis *et al.*, 2005). They could also cause massive animal death as was the case that led to their discovery in the 1960s when about 100,000 turkeys in England died due to consumption of a mold infested groundnut meal. The mold was found to be *Aspergillus flavus* and the toxic compounds were termed as aflatoxins (Richard, 2008).

If ingested in lower amounts than can cause acute aflatoxicosis, they may instead cause chronic aflatoxicosis effects like mutagenesis of the liver cells. This is because aflatoxins, especially aflatoxin B_1 are capable of binding to protein, DNA and RNA, thereby interfering with the normal cellular functions resulting in initiation of mutagenesis (Okello *et al.*, 2013a). Mutagenesis of liver cells can result into liver cancer and thus these toxins are also considered carcinogenic (David *et al*, 2013). Aflatoxin contamination of maize and groundnuts was found to be one of the causative factors of liver cancer in sub-Saharan Africa (Jemal *et al.*, 2012).

Apart from ingestion of aflatoxin contaminated plant derived food, human exposure to aflatoxins can also result from the carryover of aflatoxin metabolites in animal products such as milk, meat and eggs (Richard *et al.*, 2003) or inhalation of air and dust containing those aflatoxins (Jarvis, 2002). However, ingestion of contaminated plant derived food is the commonest means of human exposure to aflatoxins.

In Uganda, groundnut (*Arachis hypogaea* L) is the second most consumed legume after beans (Okello *et al*, 2010). Unfortunately, groundnuts are highly contaminated with aflatoxins, and accordingly aflatoxin research in Uganda has been focused mainly on groundnuts and maize (Kaaya *et al.*, 2005). Kaaya *et al.* (2006a), reported that the concentration of aflatoxins in groundnuts sold in the biggest markets in Kampala (St. Balikuddembe, Nakawa and Kalerwe), were found to exceed the 20 parts per billion (ppb) limit for human consumption set by the World Health Organization (WHO). These high aflatoxin concentrations expose Ugandans to many adverse effects key among which is liver cancer (Jemal *et al.*, 2012).

Apart from the health impacts, Uganda could face many negative economic impacts due to *A. flavus* infestation of groundnuts and subsequent aflatoxin production, for example; yield loss due to diseases induced by toxigenic fungi, low crop value as a result of aflatoxin contamination, and losses in animal productivity due to aflatoxin ingestion. Aflatoxin management, which includes aflatoxin prevention, sampling, mitigation, litigation, and research, is also very expensive. These expenses on aflatoxin management affect the whole food and feed supply chains, beginning with the crop and animal producers, grain handlers, distributors, processors, consumers, and the community as a whole (Schmale *et al.*, 2009).

There are many methods that have been proposed to control aflatoxin contamination of groundnuts. These include pre-harvest measures like irrigation (Payne *et al.*, 1986) and use of bio-control agents like non-toxigenic *A. flavus* isolates (Cole *et al.*, 1990). Post-harvest methods include proper handling of kernels during drying for example avoiding of kernel damage and drying the groundnuts on clean polythene bags or clean cemented floor, instead of bare ground (Okello *et al.*, 2010). Other control measures are control of insect and mite damage (Hell *et al.*, 2000), and adding mycotoxin binding agents such as Mont-morillonite or bentonite clay which can effectively adsorb some of the aflatoxins (Ramos *et al.*, 1996). Aflatoxin deactivation by means of enzymes like esterase, epoxidase and other yeast enzymes has also been improvised especially in animal feeds (Raju *et al.*, 2007).

Other aflatoxin control methods include physical sorting, washing, heat-treatment, radiation, and extraction with solvents (Grenier *et al.*, 2014). All the above do not exhaustively remove the aflatoxins from the groundnuts and most are quite expensive. Breeding of groundnut varieties which are resistant to colonization by toxigenic *A. flavus* and subsequent aflatoxin production, thus remains as the most sustainable strategy to control aflatoxin contamination (Williams, 2006). This is because each kernel can prevent fungal attack and this resistance is inherited. Genetic resistance can be achieved through conventional or molecular breeding, but Okello *et al.* (2010) noted, that conventionally bred fungi resistant varieties are less preferred by the local farmers. This could be attributed to the process of conventional breeding, which involves mixing and independent assortment of very many genes. This can result in undesired traits being inherited together with the desired traits (Manshardt, 2004).

Molecular breeding provides a better option because it preserves the parental genotype except for the insertion of a small either coding or non-coding DNA segment which is responsible for a specific function (Manshardt, 2004). The coding DNA fragments code for a protein, while the non-coding DNA fragments can be transcribed into functional non-coding RNA for example ribosomal RNA, transfer RNA and regulatory RNA which control the translation of different transcripts (Morris, 2012).

This study was geared at determining which regulatory RNA sequences are most ideal in inhibiting *A. flavus* growth by causing the degradation of a particular essential gene transcript to which they have sequence homology in a process known as RNA interference (RNAi). The regulatory RNA that were used in this study were double stranded RNAs (dsRNAs) which were transcribed from the PCR amplicons of the genes that the dsRNAs targeted. The 13 target genes were selected by a collaborating company (Venganza Plant Biotechnology and Research) in the United States of America (USA), basing on their unpublished RNAi research work on *Aspergillus flavus* isolated from maize. Transcription of dsRNA from the 13 target genes and determination of dsRNA toxicity on fungal growth in this study was done *in vitro* like in the bioassay of gene silencing constructs by Bailey *et al.* (2010), and this was geared at narrowing down the number of essential target genes to be silenced in later *in vivo* studies. This research thus provided new knowledge on essential genes for RNAi silencing to effectively inhibit *A. flavus* growth.

1.2 Problem statement

Aflatoxins are lethal to both humans and animals when consumed in high doses (Richard, 2008; Lewis *et al.*, 2005) and are classified as Group 1 chemical carcinogens by the International Agency for Research on Carcinogenesis (IARC). Their concentration in groundnuts sold for human consumption in the biggest markets in Kampala; St. Balikuddembe, Nakawa and Kalerwe, were found to exceed the 20 parts per billion (ppb) limit for human consumption set by the World Health Organization (WHO) (Kaaya *et al.*, 2006a). This high aflatoxin contamination of Uganda's second most consumed legume (Okello *et al.*, 2010), is one of the factors exposing Ugandans to liver cancer (Jemal *et al.*, 2012) cirrhosis, immune suppression and kwashiorkor among children (Prensner *et al.*, 2011).

Breeding of *A. flavus* resistant varieties is the most sustainable method of controlling aflatoxin contamination of crops (Williams, 2006). While conventional breeding for *A. flavus* resistance in groundnuts has been done, it is mainly for pre-harvest *A. flavus* control and results in production of new groundnut varieties which may have other undesirable attributes (Williams, 2006). The RNAi technique provides an alternative for both pre and post-harvest resistance against *A. flavus* and subsequent aflatoxin contamination of groundnut and this resistance can be introduced into any of the farmers' popular groundnut varieties. Previously RNAi has been used to silence aflatoxin biosynthesis genes and effectively reduced aflatoxin production in groundnuts (Arias *et al.*, 2015) but since infection of groundnut seeds with *A. flavus* also causes aflaroot (Ade *et al.*, 2010), RNAi silencing of *A. flavus* essential genes presents a better option of totally preventing *A. flavus* growth on groundnut seeds to control all adverse effects associated with it, key among which are aflatoxins. However, the essential genes which if silenced, could effectively inhibit *A. flavus* growth had not yet been established, and that is what prompted this study.

General objective

To identify essential genes in toxigenic *A. flavus* isolated from groundnuts in Uganda which if silenced using RNAi result in *A. flavus* growth inhibition.

1.3.1 Specific objectives

i. To isolate high aflatoxin producing *A. flavus* from groundnut seed samples collected from Soroti, Tororo and Kamuli districts.
ii. To synthesize 13 different dsRNAs targeting 13 selected essential genes of *A. flavus* isolated from Uganda through *in vitro* transcription.
iii. To determine the inhibition effect of the 13 different dsRNAs on *A. flavus* spore germination.

1.4 Research questions

i. Do groundnuts grown in eastern Uganda contain *A. flavus* isolates that produce high concentrations of aflatoxins?
ii. Can dsRNAs designed basing on *A. flavus* sequences of USA isolates be successfully transcribed from Ugandan *A. flavus* isolates?
iii. Do the different dsRNAs transcribed from the selected 13 essential genes have different inhibition effects on *A. flavus* spore germination?

1.5 Significance

This study was geared at providing new knowledge to breeders of groundnuts, on the suitable *A. flavus* essential genes to target with RNAi silencing so as to maximally inhibit *A. flavus* growth on groundnuts and subsequent aflatoxin production. Those genes have been established and thus this study has provided new knowledge for the development of *A. flavus* resistance in different groundnut varieties in Uganda using the RNAi technique.

Justification

Despite Uganda having the expertise to check for aflatoxins in groundnut food products (Okello *et al.*, 2010), enforcement of the maximum tolerable aflatoxin concentration of 10 parts per billion (ppb) in groundnuts and groundnut products sold within the Uganda has not been achieved. This could be attributed to the government having limited resources and most trade in groundnut products being done informally (Okello *et al.*, 2010). Therefore, there is need to develop cheap and sustainable means to control these aflatoxins in groundnuts and groundnut products sold in Uganda.

Breeding of *A. flavus* resistant groundnut varieties, based on the RNAi silencing of *A. flavus* essential genes could inhibit *A. flavus* colonization of groundnuts and thus control contamination of groundnut with aflatoxins. But there has been no study done on *A. flavus* isolated from groundnuts in Uganda to determine which particular *A. flavus* essential gene(s) conferred effective growth inhibition effect if targeted with RNAi silencing. Thus this study, in which dsRNAs were transcribed *in vitro*, which is quicker and cheaper than the *in vivo* methods when studying many genes, was required to screen through the proposed 13 *A. flavus* essential genes so as to establish which one(s) effectively inhibit *A. flavus* spore germination. Such genes if established would then be used by groundnut molecular breeders to modify popularly grown groundnut varieties for resistance to *A. flavus* based on the RNAi technique *in vivo*. These groundnuts would be both safe and consumer friendly as their color, taste, yield, amongst other characteristics would be the same as those of the varieties from which they were bred, except for resistance to *A. flavus* attack and subsequent accumulation of aflatoxins.

1.7 Scope of the study

In this study, *A. flavus* was isolated from groundnut seed samples collected from Tororo, Soroti and Kamuli districts which were purposively chosen as major groundnut growing areas in Uganda. Transcription of dsRNA was done *in vitro* and 13 *A. flavus* essential genes were investigated. These were proposed as plausible effective genes for RNAi silencing by a collaborating company that had done research on RNAi silencing of essential genes of *A. flavus* isolated from maize (Venganza Plant Biotechnology and Research, Florida – unpublished work).

1.8 Conceptual framework

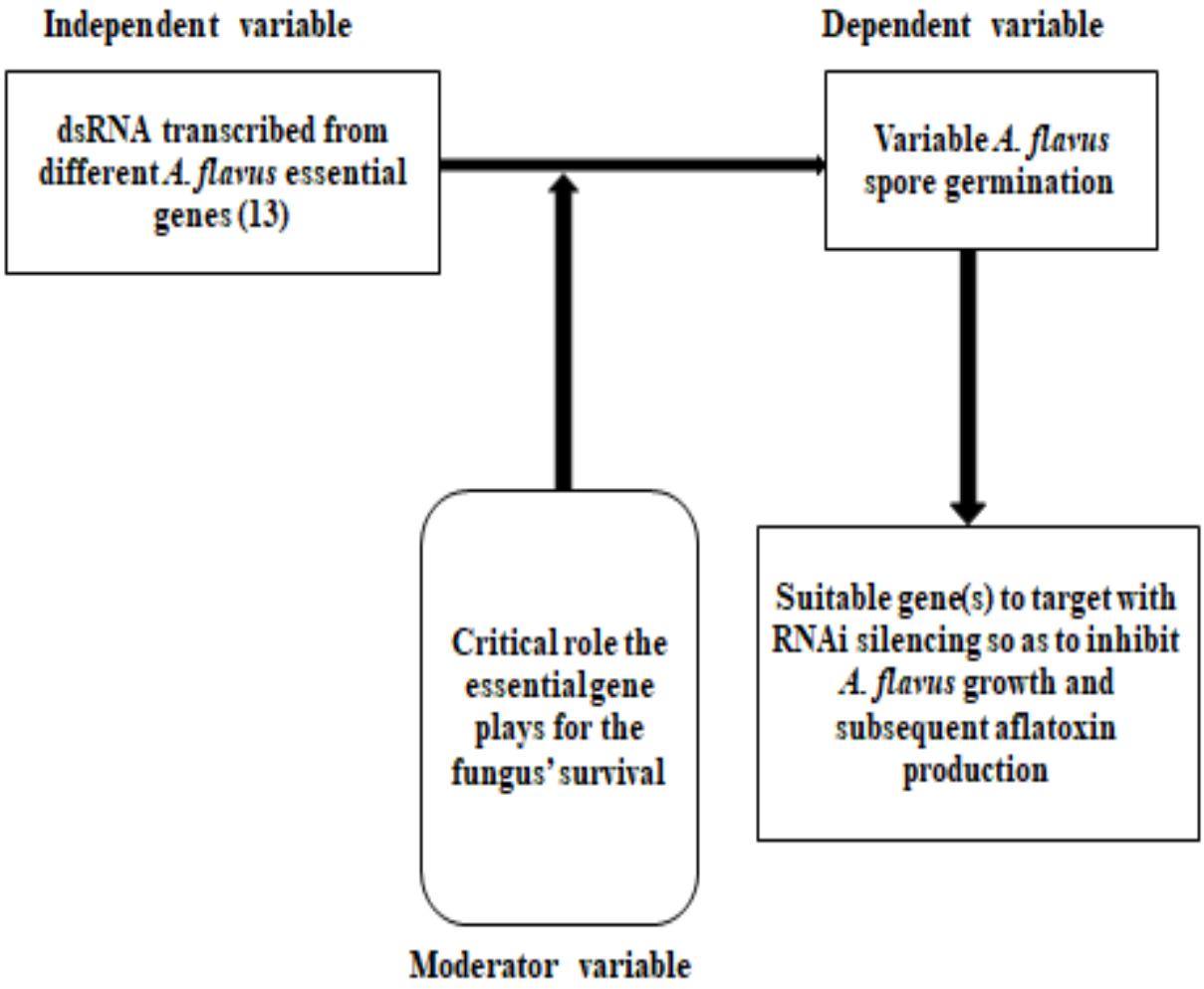


Figure 1: Conceptual framework

Double stranded RNA transcribed from different *A. flavus* essential genes could have different inhibitory effects on *A. flavus* spore germination, mainly based on how critical the silenced essential gene is for the fungus' survival. This has provided new information on the effective genes to target with RNAi silencing in *A. flavus* so as to inhibit its growth and subsequent aflatoxin production in groundnuts. Those genes which when silenced with RNAi, resulted in highest *A. flavus* spore growth inhibition were considered as the suitable targets.

CHAPTER 2: LITERATURE REVIEW

2. 1 Groundnuts and their importance

Groundnuts are the second most important legumes after beans in Uganda (Okello *et al.*, 2013a) and they are mostly grown in the eastern and northern parts of Uganda (Kaaya *et al.*, 2006b). Groundnuts constitute an important part of Ugandans' diet, since they contain 40 - 50% fat, 20 - 50% protein and 10 - 20% carbohydrate depending on the variety. Thus with the costs of animal protein becoming increasingly high, groundnuts have become an even more important source of protein. Groundnut seeds also contain vitamin E, niacin, magnesium, zinc, iron, riboflavin, thiamine, calcium, phosphorus, and potassium (Savage *et al.*, 1994) which are all very important in the human diet.

Groundnuts are grown mainly in the northern, eastern and southern parts of the country, with the eastern region being the highest producer. In Northern and Eastern Uganda, groundnuts are produced mainly on light, loose and sandy loams, but in Southern Uganda they are also grown in clay loams. In Uganda, groundnut growing primarily depends on rainfall. Apart from the Northern region which experiences one long rainy season (April - October), groundnuts are grown during two rainy seasons in the southern, eastern and central regions, with highest production in the first season. The first harvest is during the dry periods in July and the second is in the November - December dry season. The first rainy season, which lasts from March to June, has more reliable rain than the second rainy season from August to September (Nalyongo *et al.*, 1987). Groundnuts grown in Uganda are either bunchy or spreading. While both types are grown, farmers tend to prefer growing bunchy types because they are usually early maturing and easier to harvest (Nalyongo *et al.*, 1987).

2. 2 Groundnut pests and diseases

There are many groundnut pests and diseases, some attacking the crop during pre-harvest, drying or during storage. The diseases are caused by bacteria, viruses, fungi and nematodes. The commonest bacterial disease is bacterial wilt caused by *Pseudomonas solanacearum*. Viral groundnut diseases include peanut clump caused by furovirus, groundnut yellow mosaic caused by Geminivirus, peanut chlorotic leaf streak caused by caulimovirus while cucumovirus causes peanut stunt (Sreenivasulu *et al.*, 1991). On the other hand many nematodes parasitize groundnuts for example root-knot nematodes (*Meloidogyne arenaria, Meloidogyne hapla, Meloidogyne javanica*), root lesion nematode (*Pratylenchus*

brachyurus), ring nematode (*Macroposthonia ornata*), sting nematode *(Belonolaimus longicaudatus)*, and testa nematode (*Aphelenchoides arachidis*) (Singh *et al.*, 1992).

There are also many fungal groundnut diseases which can be broadly grouped as either, seed rots and seedling diseases, or foliar diseases. Foliar fungal diseases include rust *(Puccinia arachidia* Speg.), late leaf spot (*Phaeoisariopsis peraonata*), and early leaf spot (*Cercospora arachidicola*). *Fusarium solani* and *Fusarium oxysporum* also cause wilt in groundnuts (Singh *et al.*, 1992). Seed rots and seedling diseases are mainly caused by *Rhizopus arrhizus* (gray spores), *Aspergillus niger* (black spores), *Penicillium* sp (green or blue spores) and *A. flavus* (yellow-green spores) which produces aflatoxin and causes aflaroot of groundnuts.

Aflaroot symptoms include appearance of yellow-green spore heads of the *A. flavus* on the groundnut cotyledons after the emergence of seedlings. The *A. flavus* is translocated throughout the seedling in the transpiration stream. Infected plants generally become stunted with symptoms of vein clearing chlorosis on the leaflets. Such seedlings lack a secondary root system, a condition known as "aflaroot" (Singh *et al.*, 1992).

2. 3 *Aspergillus flavus* mycotoxin production and accrued economic burden

The Food and Agriculture Organization (FAO) estimates that 25% of the world's crops are affected by mycotoxins each year, with annual losses of around 1 billion metric tons of foods and food products (Schmale *et al.*, 2009). Plant infection with *A. flavus* and subsequent aflatoxin production lead to economic losses in four main ways; yield loss due to diseases induced by *A. flavus* for example aflaroot, reduced crop value resulting from aflatoxin contamination, losses in animal productivity from health problems related to aflatoxins and human health costs. Therefore it is very important to manage these mycotoxins, but mycotoxin management is also very costly for example the estimated cost of mycotoxin management in the USA and Canada in 2008 was $5 billion (Schmale *et al.*, 2009). In west Africa region for example groundnut export has decreased since 1960 while so many losses are made due to aflatoxin contaminated groundnut products in South Africa where aflatoxins in Food are regulated (Anthony *et al.*, 2012).

In Uganda however, aflatoxin management costs have not yet been incurred by most groundnut farmers and traders because they mainly sell their groundnut produce locally , and in addition, Uganda National Bureau of Standards (UNBS) has not yet been able to enforce the 1oppb maximum aflatoxin concentration limit in food that it set (Okello & Kaaya, 2010). Therefore the main aflatoxin burden in Uganda is felt in terms of the health implications that

the aflatoxins pose on the Ugandan population, key among which is liver cancer which in 2008 was the leading cause of cancer deaths among men in Africa and third in women (Jemal *et al.*, 2012). Worldwide the highest number of hepatocellular carcinoma cases which were attributed to aflatoxin consumption were recorded in Nigeria (Liu *et al.*, 2010).

2.4.0 Types of Mycotoxins

There are currently more than 400 mycotoxins known, amongst which six major classes frequently occur; Aflatoxins, Trichothecenes, Fumonisins, Zearalenone, Ochratoxins and Ergot alkaloids (Richard *et al.*, 2003). Different fungi produce different types of toxins for example; *Aspergillus flavus, Aspergillus parasiticus, Aspergillus nominus* and *Aspergillus psuedotamarii* produce aflatoxins (B_1, B_2, G_1 and G_2). *Aspergillus ochraceus* produces Ochratoxins, *Fusarium proliferatum* and *Fusarium verticilliodes* produce fumonisins, while *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium sporotrichioides* produce Zearalenones (Weidenbörner, 2001). *Penicillium* species produce ochratoxin A, citrinin, patulin, Pr toxin, poguefortine and pentirem A. *Claviceps species* produce penitrem A, clavines, lysegic acid and ergopeptines. The *Neotyphodium species* produce toxins known as ergot alkaloids, lolines and peramine while the *Pithomyces species* produce sporidesmin (Weidenbörner, 2001). Focus in this study was placed on *A. flavus* whose most dangerous toxin is aflatoxin B_1 (AFB_1) and B2 (AFB2).

2.4.1 Aflatoxins' history, structure and toxicity

Aflatoxins in general were discovered in the early 1960s. They were isolated and characterized after the death of about 100,000 turkeys in England, whose death was associated to the consumption of a mold-contaminated peanut feed (turkey X disease). The mold in the feed was found to be *A. flavus* and the toxic components in the groundnut meal came to be known as aflatoxins (Richard, 2008). Aflatoxins are secondary metabolites of mostly two fungi; *A. flavus* and *A. parasiticus* but other *Aspergillus species* like *A. nomius, A. pseudotamarii, A. bombycis, A. ochraceoroseus,* and *A. australis* also produce aflatoxins (IARC, 2002). The major aflatoxins are B_1, B_2, G_1, and G_2 (based on their blue or green fluorescence under Ultra Violet (UV) light). Others are M_1 and M_2 which were isolated from milk and dairy products (D'mello *et al.*, 1997).

Aflatoxin B_1 is not only the commonest, but the most genotoxic amongst aflatoxins and the most potent natural carcinogen known (Zain, 2011). It is mostly produced by both *A. flavus* and *A. parasiticus*, and is most prevalent in groundnuts and maize. The chemical structure of

Aflatoxin B_2 is the dihydroxy derivative of Aflatoxin B_1 as shown in Figure 1, but both aflatoxins B_1 and B_2 are detected by blue fluorescence under UV-light. Also both Aflatoxin B_1 and Aflatoxin B_2 have metabolites isolated from milk namely; Aflatoxin M_1 and Aflatoxin M_2 respectively. Aflatoxin M_1 is a 4-hydroxy aflatoxin B_1, while Aflatoxin M_2 is 4-dihydroxy aflatoxin B_2. Aflatoxin G_1 and aflatoxin G_2 are exclusively produced by *A. parasiticus* and they emit a yellow-green fluorescence under UV-light. Like in the Aflatoxin B series, Aflatoxins G_2 is the dihydroxy derivative of Aflatoxin G_1 as shown in Figure 1 (Zain, 2011).

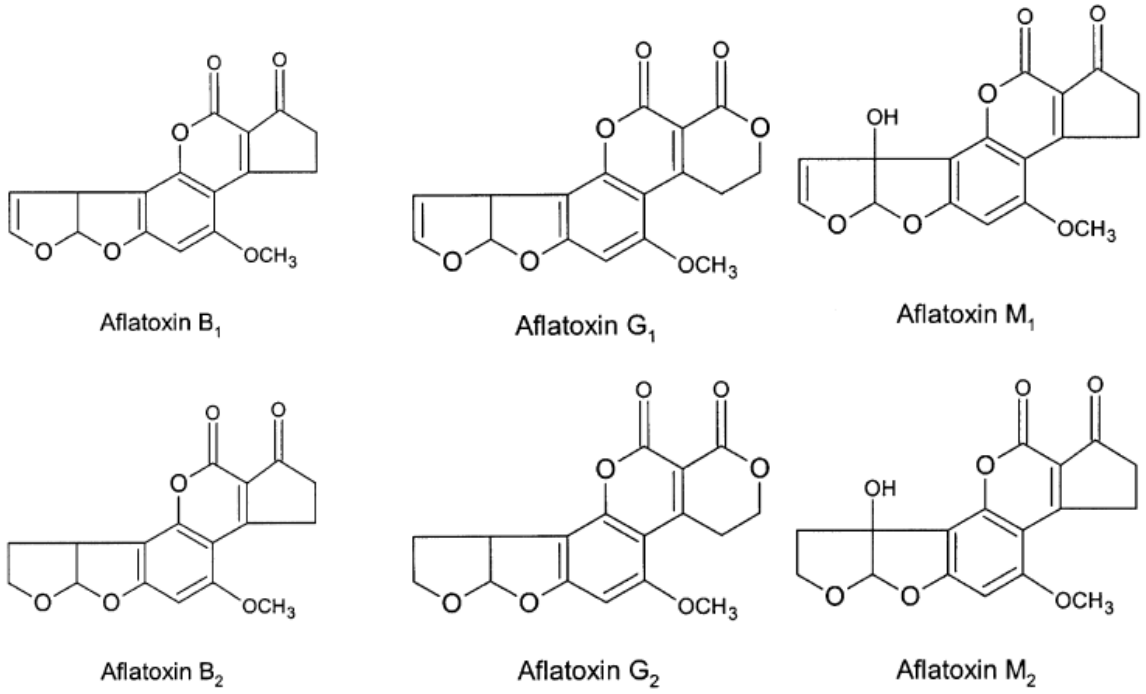


Figure 2: Chemical structure of aflatoxin B_1, B_2, G_1, G_2, M_1 and M_2 (Zain, 2011)

The different mycotoxins are differentiated in terms of their chemical structure (Fig.1) and accordingly their adverse effects differ. Aflatoxins in general are associated to the following deleterious effects; carcinogenicity, genotoxicity, nephrotoxicity, hepatotoxicity, oestrogenicity, immune suppression, reproductive disorders or even dermal disorders (Bryden, 2012). Given that each Aflatoxin has a different molecular structure, each causes different adverse effects or the level of severity of the same adverse effect may differ. Despite the influence of many factors for example species, sex, breed, age, health, immune status and possible synergism between mycotoxins simultaneously present in feed or food, on the type and magnitude of the mycotoxin effects (Richard, 2007), Aflatoxin B_1 remains the most potent genotoxic and carcinogenic of all the known aflatoxins.

2.5.0 Measures for aflatoxin control in groundnuts

Different researchers have proposed various solutions to control *A. flavus* groundnut infection and subsequent aflatoxin production. These can be applied pre-harvest or post-harvest although some can offer *A. flavus* resistance both pre and post-harvest for example breeding resistant groundnut varieties. The pre-harvest measures include irrigation and use of bio-control agents while post-harvest measures include proper handling and drying of kernels, control of insect and mite infestation as well as enterosorption and chemoprotection as further explained below.

2.5.1 Pre harvest control measures

2.5.1.1 Irrigation

Drought stress favors *A. flavus* attack on crops *(Okello et al., 2010)*. This is because drought causes strains on pods and testa, and this provides entry points for fungal infection (Okello *et al.*, 2010). For example in a four year experiment by Payne *et al,* (1986) on the effect of irrigation on aflatoxin crop contamination, aflatoxin levels in the non-irrigated treatment were significantly higher than in all irrigated treatments (Payne *et al.*, 1986). Thus irrigation or timely planting of groundnut varieties whose maturity period fits in the rainfall cycle could reduce groundnut aflatoxin contamination that would have resulted from drought.

2.5.1.2 Bio control agents

As bio-control agents, non-toxigenic strains of *A. parasiticus* or *A. flavus*, which ideally occupy the same ecological niche as the toxigenic strains, are the best option. Using other bio-competitive agents for example bacteria could have been effective, however, they become inactive under the extreme conditions of high temperature and drought, associated with pre-harvest aflatoxin contamination, implying that the bacteria do not share a similar ecological niche. Thus they would not be good bio competitive agents. A three year research by Cole *et al* (1990), at the United States of America's National Peanut Research Laboratories (USA-NPRL) using a non-toxigenic *A. parasiticus* strain realized a significant difference in aflatoxin contamination in groundnuts from the bio agent (BA) inoculated plots than in the non BA inoculated soils (Cole *et al.*, 1990).

2.5.2 Post-harvest control practices

2.5.2.1 Moisture content, relative humidity and temperature control

Drying of seeds to very low moisture content helps in controlling mold growth on stored seeds. This is because excessive moisture weakens the pods and testa, thereby providing entry points for fungal infection (Okello *et al.*, 2010). Temperature is also important in determining groundnut contamination but its impact is synergistic with moisture content and relative humidity of the stored seeds. Different fungi have different optimum temperatures for growth and mycotoxin production. *Aspergillus flavus* for example, grows best between 10°C and 45°C at a relative humidity of 75% or more although the optimum conditions for aflatoxin production are between 25°C and 30°C, at 85% relative humidity (FAO, 1998). For fungi in general, far lower moisture content, temperature and relative humidity can control groundnut fungal infestation and subsequent mycotoxin production therein (Okello *et al.*, 2010).

2.5.2.2 Handling and drying

Mechanical damage to kernels, mainly incurred during harvesting and shelling, increases their vulnerability to invasion by storage fungi (Sauer *et al.*, 1987). Groundnut drying should better be done in pods. The groundnuts can be dried on a raised platform for example on papyrus mats supported at about 1.5-meter height from the ground. Alternatively drying would be done on a clean cemented floor or on clean polythene bags in or out doors. The outdoor dried groundnuts should be occasionally carried in door in case of postharvest rains, so that optimum low kernel moisture content can be achieved (Kaaya *et al.*, 2006b; Okello *et al.*, 2010).

2.5.2.3 Control of insect and mite infestation or damage

Not only can insects and mites damage stored grain, but they can also aid fungi in colonizing grain for example by carrying and spreading fungal spores in the stored grain. Due to insect damage, toxigenic fungi can infect growing crops and may consequently produce toxins prior to harvest, when harvesting or during storage (Okello *et al.*, 2013a). The metabolic heat and water of insects can increase the water activity and temperature of stored grain, thus making it suitable for fungal growth. It is thus important to control insect and mite attacks both pre and post-harvest (Hell *et al.*, 2000).

2.5.2.4 Enterosorption and chemoprotection

These two processes can be adopted in case groundnuts are already contaminated with aflatoxins. Enterosorption involves mechanisms of detoxifying mycotoxins if already consumed by animals. Chemoprotection against aflatoxin implies the use of compounds that either improve an animal's detoxification process or prevent the production of damage causing compounds which would otherwise target various body parts. Development of enterosorption and chemoprotection stemmed from the discovery of some zeolytic minerals which could selectively adsorb aflatoxins tightly enough and consequently prevent their absorption through the intestinal walls (Ramos *et al.*, 1996). The above methods of aflatoxin control are rather expensive and are not easy to sustain especially in developing countries like Uganda. The chemicals used in detoxification could also have detrimental effects on humans and thus development of *A. flavus* resistant groundnuts offers a better solution.

2.5.2.5 Conventional and Molecular breeding approaches

Breeding to develop resistance against *A. flavus* and its mycotoxin production are preferable to the above aflatoxin control strategies. This is because the resistance introduced into groundnuts due to breeding is passed on from one generation to another in the crops which makes it a cheaper and more sustainable than all the agronomical, enterosorption and chemoprotection methods. This does not mean that developing resistant varieties to *A. flavus* is entirely adequate to erase aflatoxin groundnut contamination caused by *A. flavus*, but rather that it should be the backbone of all the other control strategies.

Conventional breeding for resistant varieties against fungal infection has been done, but with a major weakness of the farmers disliking most of the resistant varieties on basis of their color and taste (Okello *et al.*, 2013a). This is because a lot of genetic material (so many genes) is exchanged in conventional breeding, unlike in molecular breeding which is more specific. Molecular breeding advanced quickly in groundnuts since the successful establishment of groundnut regeneration and transformation protocols. Some transformations have been made with whole resistance genes against fungi in groundnuts, for example a rice chitinase gene was transformed into groundnuts to help prevent invasion by fungal pathogens. And in the *in vitro* seed inoculations, the transformants showed resistance to *A. flavus* infection (< 10% infection) (Prasad *et al*, 2013). Another very promising molecular breeding approach is by transforming with genetically engineered dsRNA constructs which can initiate RNAi process.

The RNAi approach is especially desirable because it does not involve protein production given that the otherwise produced proteins may not only be toxic to the fungi but also to humans and animals that end up consuming the groundnuts. Secondly RNAi is sequence specific and thus off target silencing should not be expected. The RNAi discovery and mechanism is explained further below.

2.5.3.0 Use of RNAi as a biotechnological approach to control aflatoxin contamination in groundnuts

In fungi, RNAi was initially known as quelling, a phenomenon that was discovered by Romano and Machino in 1992, when they observed that the expression of a carotenoid biosynthesis endogenous gene, albino-1 (al-1), was attenuated by a transformation with homologous, al-1, sequences in the fungus *Neurospora crassa* (Romano *et al.*, 1992). A similar phenomenon in plants had been reported by (Van der Krol *et al.* (1990); Matzke *et al.*, 1989) in which ectopic transgenes were found to suppress their own expression and that of endogenous homologous genes. They referred to it as "co-suppression". Later in 1993, RNAi was discovered in the form of a single microRNA (miRNA) in the *Caenorhabditis elegans* genome, where short transcripts from *lin-4* (22-61) were found to contain complementary sequences to a 3' un-translated region (UTR) of *lin-14*. This led to the suggestion that *lin-4* which regulates *lin-14* effects the regulation through an antisense RNA-RNA interaction (Lee *et al.*, 1993). Which means that *lin-4* and *lin-14* share sequence homology. Since sequence homology between the DNA sequences associated to silencing a particular gene was common to both quelling and co-suppression, all these phenomena were later clearly explained as RNA interference (RNAi).

Ribo-nucleic acid interference can thus be defined as a post-transcriptional regulation of genes through sequence specific interception and degradation of messenger RNA (mRNA) (Mumbanza *et al.*, 2013). The mRNA degradation is triggered by a double stranded RNA (dsRNA) homologous to it (Nakayashiki *et al.*, 2008). The short transcript of *lin-4* was termed as micro RNA (miRNA). Over time very many miRNAs were discovered; for example as much as 5% of the human genome is dedicated to encoding and producing the >1,000 miRNAs that regulate at least 30% of human genes (Jinek *et al.*, 2009; MacFarlane *et al.*, 2010).

Currently there are three known pathways of RNAi, based on; microRNA (miRNA), short interfering RNA (siRNA) and, PIWI-interacting RNA (piRNA). The piRNAs and miRNAs are derived from the genome, whereas siRNAs may be endogenous or exogenous including viral infection sources (Carthew *et al.*, 2009). All the three share a common mode of action. In all, a ribonucleo-protein complex comprising an Argonaute family protein is bound to a single-stranded ~20- to 30-nt RNA which grants specificity via base-pairing interactions with the target gene transcript (mRNA or viral RNA). Degradation of target RNA is then effected by argonaute protein at the point of sequence homology. In miRNA and siRNA pathways, the ribonucleo-protein is known as the RNA-induced silencing complex (RISC) (Wilson *et al.*, 2013).

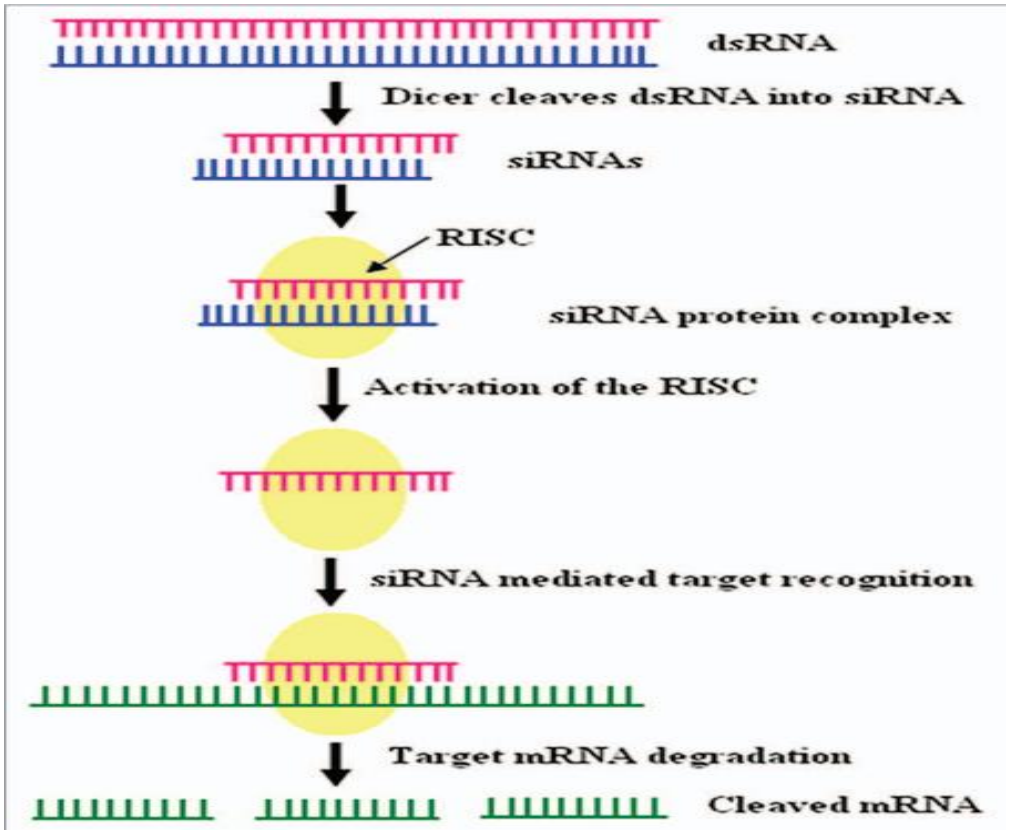


Figure 3: RNAi pathway triggered by dsRNA (Ali *et al.*, 2010)

Ribo nucleic acid interference is very important in many vital cellular processes for example cell growth, tissue differentiation, heterochromatin formation, and cell proliferation. Accordingly, RNAi dysfunction is linked to cardiovascular disease, neurological disorders, and many types of cancers (Lu *et al.*, 2008).

In addition, organisms could use RNAi as a natural defense mechanism whereby they recognize as foreign any double-stranded RNA (dsRNA) molecule and hydrolyze it with a ribonuclease named dicer. Such hydrolysis can degrade double stranded viruses in cells too (Niblett *et al.*, 2012). The RNAi technique is worthwhile investigating for resistance against *A. flavus* in groundnut varieties because of; the minimal side effects that would result from eating groundnuts transformed with RNAi inducing constructs since they code for no protein. It is also specific, and this controls for harming of off target, and probably useful microorganisms which a protein could indiscriminately target.

2.5.3.1 Rational for targeting essential genes of *A. flavus*

Essential genes are ones which are critical for the survival of an organism. In other words those are the genes that are critical to maintain life, given that all the organism's nutrients are available in its environment (Zhang *et al.*, 2009). Most essential genes have been found to encode proteins that are involved in central metabolism, DNA replication, mRNA translation, cellular structure maintenance and mediation of transport processes into and out of the cells (Zhang *et al.*, 2009). Many *in vitro* studies have succeeded in silencing aflatoxin pathway genes and observed significant reduction in aflatoxin production. For example McDonald *et al.* (2005), transformed *A. flavus* and *A. parasiticus* with inverted repeat transgenes (IRT) which contained sequences homologous to aflatoxin-specific regulatory genes and thus could initiate RNAi process in the fungi, and they successfully suppressed aflatoxin production in both fungi. But since infection of groundnut seed with *A. flavus* also causes aflaroot (Ade *et al.*, 2010), RNAi silencing of *A. flavus* essential genes presents a better option of totally preventing *A. flavus* growth on groundnut seeds to control all adverse effects associated with it, key among which are aflatoxins. The essential genes silenced in this research are shown in Table 1.

Table 1: Target genes and their predicted biological functions and references.

Target gene	Predicted biological function
Exportin (*EXP)*	It mediates nuclear export of pre-microRNAs and short hairpin RNAs (Yi *et al.*, 2003)
Cohesin (*COH*)	Affects embryonic viability and separation of sister chromatids during cell division (Pasierbek *et al.*, 2001).
Inosine monophosphate (*IMP*)	Important for purine metabolism (Davies *et al.*, 2012)
Modifier of Transcription (*MOT1*)	Activates and represses transcription by direct ATPase-dependent mechanism (Auble *et al.*, 1994)
Polyphosphate kinase (*PPK*)	Catalyzes synthesis polyphosphate (Poly P) (Lee *et al.*, 2003)
Glucose 1 Phosphate Thymidylyl Transferase (*GTT*)	Important in glycosylation (Madduri *et al.*, 2001)
Polyubiquitin (*UBI*)	For germ cell differentiation (Ozkaynak *et al.*, 1987)
Dehydration Responsive Factor (*DRF7*)	Transcription factor activity, sequence-specific DNA binding (Xue *et al.*, 2004)
Hevein-like preproprotein (*HEL*)	Chitin binding and ribonuclease activity (Potter *et al.*, 1993)
ATP-binding cassette transporters (*ABC*)	For making transmembrane transporter proteins which bind and hydrolyse adenosine triphosphate (ATP) so as to effect certain biological processes including translocation, translation of RNA and DNA repair (Allikmets *et al.*, 1996).
*26s r*RNA	Encodes one of the ATPase subunits (Otsuka *et al.*, 1983)
Peptide transporters (*Ptrs*)	Cellular transport proteins (Tsay *et al.*, 2007)
ATP gene family (*ATP*)	Provide instructions for making transporter proteins called ATPases (Corradi *et al.*, 2006).

CHAPTER 3: MATERIALS AND METHODS

3. 1 Study site

The study was carried out in three districts of Eastern Uganda, namely Soroti, Tororo and Kamuli (sampling sites are shown in Figure 2). These purposively constituted the sampling area because they are major groundnut growing areas in Uganda (Mahmoud *et al.*, 1991). Soroti district for example, was the greatest groundnut producer (19,599 tonnes) in 2014 (UBOS, 2015). The soils in this region are light, loose and sandy which favor groundnut growing (Okello *et al.*, 2013a). Rainfall is bimodal and the average annual rainfall ranges between 1374mm to 2058mm (Kansiime *et al.*, 2013). This annual rainfall amount is adequate for groundnut growing since annual rainfall between 450 mm and 1250 mm is adequate for good groundnut growth (Okello *et al.*, 2013b). Groundnut seed samples were picked in August 2015, while *A. flavus* isolation from the groundnut samples and RNAi experiments were carried out from June 2016 at the National Agricultural Research Laboratories (NARL) located at in Wakiso central Uganda.

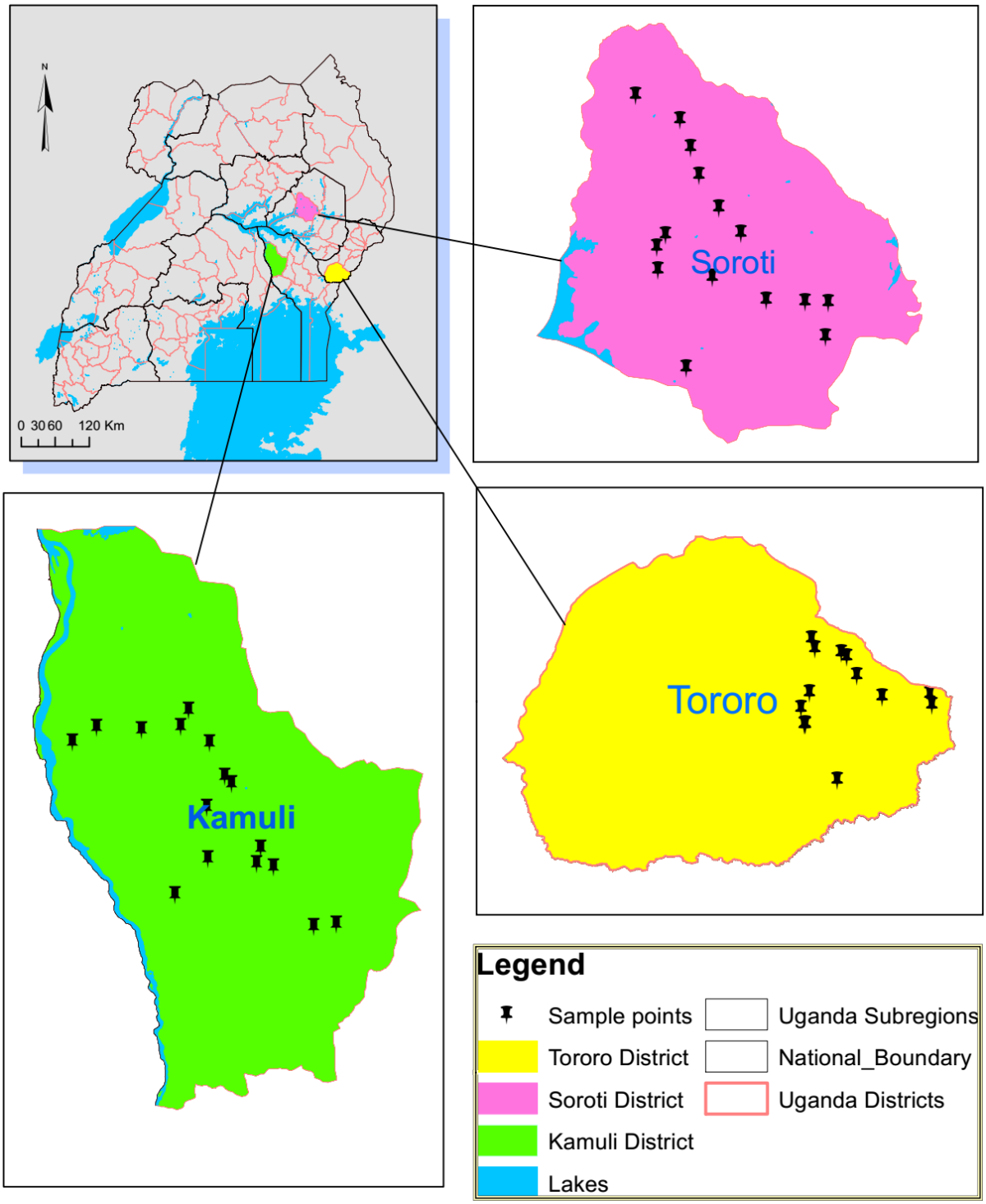


Figure 4: Map of Uganda showing the study sites

3. 2 Study design

A cross-sectional design was used to obtain aflatoxin producing *A. flavus* isolates from the study area, while an experimental design was used to determine the effect of dsRNA on *A. flavus* spore germination.

3.3.0 Sampling strategy

An agricultural extension worker in each district was contacted in advance for guidance to the different groundnut growers. A total of 14, 15 and 16 samples were systematically picked from Tororo, Soroti and Kamuli districts respectively. About 0.5 kg of unshelled groundnuts from each garden or home granary constituted a sample. The first groundnut garden or home in each district was randomly chosen near the district boundary, subsequent ones were chosen after every five kilometers, and Global Positioning System (GPS) coordinates were taken for each place where a sample was picked using a GPS machine (Etrex-Garmin). Farmers were first requested for oral consent after explaining the study aims before picking samples from their fields or granaries and three thousand shillings (3000shs) compensation was offered to them for provision of samples. Additional information about farm location and whether the sample was got from farmer's field or granary was recorded as indicated in the sample collection data sheets in Appendix VI. Samples were placed in paper bags and transported at room temperature to the National Agricultural Research Laboratories (NARL) at Kawanda. At NARL, they were first sun dried on clean high density polyethylene (HDPE) sheets for four days, then hand shelled and stored at 4^0C until fungal isolation was done.

3.3.1 Sample size determination

Sample size was estimated using the following formula (Metcalfe, 2001)

$n= Z^2 P (1-P)/d^2$

Where, n = sample size,

Z = Z statistic for a level of confidence,

P = expected prevalence or proportion,

d = precision.

Using aflatoxin prevalence (P) of 50% and d of 15% for a preliminary study (Naing *et al.*, 2006) and at 95% confidence, the sample size according to the above formula by Metcalfe (2001), was 43 groundnut seed samples.

3.4 *Aspergillus flavus* isolation from groundnut samples

Isolation of *A. flavus* involved surface sterilization of groundnuts according to Olwari *et al.* (2013) but with a few modifications. Briefly, the kernels were rinsed in sterile water, soaked in 70% commercial sodium hypochlorite for two minutes, and then rinsed three times with sterile water. Sterilized seeds were aseptically initiated on modified Rose Bengal Agar (prepared according to Appendix III) and incubated at 28°C for 48 hours. *Aspergillus flavus* was morphologically identified as white mycelia with yellow-green spores (Arias *et al.*, 2015). Using a compound microscope, *A. flavus* was identified as having radiate conidial heads which split to form bi-seriate columns, and with phialides borne directly on the vesicle which rests on a long hyaline conidiophore, with globose and finely echinulate conidia (Rodrigues *et al.*, 2007). All *A. flavus* isolated from the same groundnut sample was considered as one isolate.

Confirmation of aflatoxin producing *A. flavus* isolates

A competitive ELISA, using Agraquant total Aflatoxin kit (1 to 20 ppb, Romer Labs) was used to confirm aflatoxin producing isolates and even quantify the amount of aflatoxins they produce. All procedures were according to manufacturer's instructions as elaborated in Appendix II. Briefly, to extract the aflatoxins: 600mg of fresh *A. flavus* mycelia was ground using a clean motor and pestle in 3ml of 70/30 (v/v) methanol/water extraction solution, for 3 minutes. The solution was filtered through Whatman #1 filter paper, filtrate collected and diluted 100 times in 70/30 methanol/water solution (1 sample volume: 99 volumes of methanol/water solution). Note: the following biosafety measures were taken while extracting aflatoxins from *A.flavus*. All extraction was done from a Fume hood, Nitrile gloves were worn while handling the samples, a 0.45nm pore nose mask was worn to inhibit inhalation of *A. flavus* spores and laboratory eye glasses were worn to protect the eyes. Quantification of the extracted toxins proceeded as follows: to 200µl of conjugate was added 100µl of sample extract or standard (i.e. 0, 4, 10, and 20 ppb) in dilution microwell strips placed in a microwell strip holder and mixed by pipetting up and down three times. Then 100µl of that mixture of sample extract and conjugate or standard and conjugate was pipetted into antibody coated microwells and chilled at room temperature for 15 minutes. The microwell contents were then emptied in a waste container and the microwells washed by filling with sterile distilled water and then dumping the water from the microwell strips. This step was repeated five times and the microwell strips were tapped on paper towels to expel as much residual water as possible. Thereafter, 100 µl of substrate was added into each microwell and incubated at room temperature for five minutes.

A blue color was observed. Then 100µl of stop solution was added into each microwell, and the color changed from blue to yellow. The wells were read in a microwell reader using a 450nm filter and the obtained ODs were used to construct a dose response curve using the 5 standards as reference points. Since the amount of aflatoxin in each standard was known, the unknown sample aflatoxin concentrations were deduced by interpolation from the standard curve. In addition, the deduced concentrations from the standard curve for each sample were multiplied by the dilution factor which was 100 since the original samples had been diluted 100 times. Thus for a sample whose OD lies at 1ppb on the standard curve, it's actual concentration was 100ppb.

3.6.0 *In vitro* dsRNA transcription from different target genes of *A. flavus*

3.6.1 DNA extraction

DNA was extracted from seven day old cultures of aflatoxigenic *A. flavus* isolate KAf 5 which was chosen for downstream assays because it produced the highest quantified total aflatoxins. DNA extraction was done according to a modified protocol of Mahuku (2004), shown in Appendix III. Briefly, 150mg of fresh mycelia was homogenized by macerating for two minutes with a clean motor and pestle containing sterilized sea sand and 500µl of TES buffer (0.2M Tris-HCL [pH8], 10mM EDTA [pH8], 0.5M NaCl, 1% SDS and proteinase K 50µg/µl). The homogenate was then transferred to a 1.5ml micro-centrifuge tube, vortexed and incubated at 65°C for 30 minutes. Thereafter one half volume (250µl) of 7.5M ammonium acetate was added, and the sample was vortexed and incubated on ice for 10 minutes followed by centrifuging for 15 minutes at 13000rpm. 500µl of the supernatant was transferred to a new tube and an equal volume of isopropanol was added, and incubated at -20°C overnight. This was followed by centrifuging for ten minutes at 13000rpm to pellet the DNA. The supernatant was decanted and pellet was washed by adding 800µl of 70% ethanol and centrifuging at 13000rpm for five minutes. The supernatant was discarded and the pellet was then left to air dry on a sterile paper towel for six hours. The dry DNA pellet was resuspended in nuclease free water, quantified using a Nanodrop and was then diluted to 200ng/µl and stored at -20°C for later amplification of target essential genes.

3.6.2 Amplification of target essential genes

The PCR was performed using primers specific to the 13 essential genes of *A. flavus*. These primers (Table 2) were designed by a collaborating private company called Venganza Plant Biotechnology Consulting and Research (Florida, USA), using sequences of *A. flavus* isolated from maize grain samples but they did not disclose the primer designing details for patenting purposes. To each of these primers was attached a T7 RNA polymerase promoter. By running gradient PCRs an optimum annealing temperature of 62°C was obtained for all the different primers. The PCR reaction mixture contained 10µM each of forward and reverse primers (0.5µl), 5u/µl *Taq* DNA Polymerase - Bioneer (0.2µl;), 5X GoTaq® Reaction Buffer - Promega (4µl), 10µM deoxyribonucleotides (dNTP; 0.5µl), Dimethyl sulfoxide (DMSO) 5% of total reaction, 500mg/µl Potassium chloride (KCl; 6µl), 200ng/µl genomic DNA of *A. flavus* (1µl) and then adjusted with sterile water to 20µl final volume. Sterile water was added (1µl) instead of genomic DNA for the negative control.

The PCR conditions were modified from Mumbanza *et al.* (2013) that is; five minutes initial denaturation at 95°C, 35 cycles of 30 seconds of denaturation at 94°C, one minute primer annealing at 62°C, 40 seconds extension at 72°C and 10 minute final extension at 72°C. The PCR products were separated by agarose gel electrophoresis using a 1.5% agarose gel in 1X TAE buffer (40mM Tris, 20mM acetate and 1mM EDTA) at 100 volts for 60 minutes.

The gel was stained with ethidium bromide at 0.5µg/ml final concentration and then the bands were visualized using ultra violet light in a Gel documentation machine. The amplicons were then excised from the gel and purified using the Gene Elute ™ Gel Extraction kit according to the manufacturer's instructions elaborated in Appendix IV. Briefly, the DNA fragment of interest was excised from an agarose gel using a clean, sharp scalpel, placed in a 1.5ml Eppendorf tube and weighed. Three gel volumes of the gel solubilization solution was added to the slice (for example for 100mg of gel was added 300ml of gel solubilization solution). The gel mixture was then incubated at 60°C until the gel was completely dissolved. Binding columns were prepared by placing them in 2ml collection tubes, adding 500µl of column preparation solution to each of them and centrifuging at 12000rpm for one minute. One gel volume of isopropanol was added in to the solubilized gel mixture and this mixture was pipetted into a prepared binding column and centrifuged at 12000rpm for one minute. The binding column was removed from the 2ml collection tube and the flow through liquid was discarded. Then it was returned into the collection tube and 700µl of wash solution was added, centrifuged at 12000rpm for one minute and the flow through liquid was discarded too. Lastly the binding column was transferred to a fresh collection tube and 50 µl of elution solution was added at the center of the membrane, incubated for one minute at room temperature followed by centrifuging at 12000rpm for one minute. The DNA contained in the flow through was then quantified using a Nano-drop and then stored at -20°C for use in dsRNA transcription.

3.6.3 Transcription and Quantification of dsRNA

The purified PCR amplicons were used to synthesize dsRNAs using a MEGAscript® T7 high yield transcription kit according to the manufacturer's instructions (Manual, 2009), as explained in Appendix V. Briefly, a ribonucleotide mixture was made by mixing 3µl of each of the 4 ribonucleotides (ATP, CTP, GTP and UTP). Then 8 µl of this mixture was added to the other reaction components which included; 2µl of 10X reaction buffer, 1µl of linear template, 2µl of reverse transcriptase enzyme and 7µl of sterile water for a 20µl reaction. The negative control constituted of 8µl of ribonucleotide mixture, 2µl of 10X reaction buffer, 1µl of linear template, 9µl of sterile water and no reverse transcriptase enzyme was added. The mixture was vortexed thoroughly and then incubated at 37°C for 4 hours. To this mixture was then added 1µl of Turbo DNase, vortexed and incubated again at 37°C for 15 minutes so as to degrade the template DNA. This was followed by incubating at 65°C for 30 minutes to in-activate the TURBO DNase enzyme. Quantitation of dsRNA was done using a Nano drop and 1µl of the dsRNA was run in 1.5% agarose gel at 80 volts for 45 minutes. Thereafter the gel was stained in 0.5µg/ml ethidium bromide and dsRNA bands were visualized under U.V light in a gel documentation system to ascertain presence of the dsRNA. The remaining transcribed dsRNA was then stored at -20°C for use in the dsRNA assays.

Table 2 : Target genes and their forward and reverse primer sequences

The underlined sequence on each primer is for the T7 RNA polymerase promoter which was added to each essential gene primer.

Gene	Primer sequences	Product size (bp)
Exportin (*EXP*)	AfExpFPT7 GCGTAATACGACTCACTATAGGGAGAGCATTGTTTGCCCTCGCTTCG	450
	AfExpRPT7 GCGTAATACGACTCACTATAGGGAGAAGAGTGCCGAGAAGAGGAAG	
Cohesin (*COH*)	AfCohFPT7 GCGTAATACGACTCACTATAGGGAGAGACGATCGATTACGAGCCAGG	500
	AfCohRPT7 GCGTAATACGACTCACTATAGGGAGAGCGTGCTCCCTCTTTGCATCC	
Inosine monophosphate (*IMP*)	AfImpFPT7 GCGTAATACGACTCACTATAGGGAGATCACTTCCTGATCTTGCCTGG	500
	AfImpRPT7 GCGTAATACGACTCACTATAGGGAGAGTTTCCACTTTCAGTTACAGG	
Modifier of Transcription (*MOT1*)	AfMotFPT7 GCGTAATACGACTCACTATAGGGAGAACTGAGTCTGCTCGCTCTGCT	450
	AfMotRPT7 GCGTAATACGACTCACTATAGGGAGACCCCTTGACCGCAGCAGTACC	
Polyphosphate kinase (*PPK*)	AfPpkFPT7 GCGTAATACGACTCACTATAGGGAGAGGGTCATTATGTCTCCTTCGC	400
	AfPpkRPT7 GCGTAATACGACTCACTATAGGGAGAGTGTCCCAACGGACCATCCGG	
Glucose1PhosphateThymidylyl Transferase (*GTT*)	AfGttFPT7 GCGTAATACGACTCACTATAGGGAGACCAGATCATTCATTCCAGTCG	500
	AfGttRPT7 GCGTAATACGACTCACTATAGGGAGAGTCCTGCACCGCTTCAAGCAG	
Polyubiquitin (*UBI*)	AfUbiFPT7 GCGTAATACGACTCACTATAGGGAGACCGTACTCTTTCAATTTGAGG	350

	AfUbiRPT7 GCGTAATACGACTCACTATAGGGAGATCCCATTTGGTAAACCTTGCG	
Dehydration Responsive Factor (*DRF7*)	AfDrfFPT7 GCGTAATACGACTCACTATAGGGAGAGTCTTGTCATATGGAGAAGAC	450
	AfDrfRPT7 GCGTAATACGACTCACTATAGGGAGACTCGGAGGCAAGTTGGTCTTG	
Hevein-like preproprotein (*HEL*)	AfHelFPT7 GCGTAATACGACTCACTATAGGGAGAGACATCTCGACATCATGGCCC	500
	AfHelRPT7 GCGTAATACGACTCACTATAGGGAGAGCCAGCCGACTTGCAAATGGG	
ATP-binding cassette transporters (*ABC*)	AfABCFPT7 GCGTAATACGACTCACTATAGGGAGACATGACTCAGCCTTCTTGGAG	450
	AfABCRPT7 GCGTAATACGACTCACTATAGGGAGACTGCTTTATTCGTCGTTGTCC	
*26s r*RNA	Af26sFPT7 GCGTAATACGACTCACTATAGGGAGACTCGGCCGCTGTTGGCCGTAC	450
	Af26sRPT7 GCGTAATACGACTCACTATAGGGAGACACTTGCTCAATGTCTTCCTC	
ATP gene family (*ATP*)	AfAtpFPT7 GCGTAATACGACTCACTATAGGGAGACTTATCACTCCGAGAACTGCC	450
	AfAtpRPT7 GCGTAATACGACTCACTATAGGGAGAATGTCCGAAGAATCTTCCCAG	
PTRs gene (*Ptrs*)	AFPTRsFPT7 GCGTAATACGACTCACTATAGGGAGAACTTCAGCCAGACGAGAA	500
	AFPTRsRPT7 GCGTAATACGACTCACTATAGGGAGATATCATGGCGTCCAATCAAG	

3.7 Double stranded RNA inhibition Assays on *A. flavus* spores

The dsRNAs were administered to the *A. flavus* spores by imbibition, and their fungal growth inhibition effect were tested based on reduction in colony number on the plates where spores treated with the different dsRNAs were plated compared to the control plates on which spores treated with sterile water were plated (Bailey *et al.*, 2010). This was a step by step assay which involved collecting and quantifying *A. flavus* spores in sterile conditions, exposing the spores to dsRNA or sterile water and lastly plating the spores of the different treatments on fresh PDA to observe the effect of the different treatments as detailed in the subsection 3.7.1 and 3.7.2.

3.7.1 Collection and quantification *A. flavus* spores

The *A. flavus* isolate from which dsRNAs were synthesized (KAf 5), was cultured on ¼ strength PDA (10g of PDA powder and 10g of agar per liter of distilled water, supplemented with 1ml of cefotaxime at 300mg/ml) at 28°C for 15days. Collection of spores was done as in Mumbanza *et al.* (2013) with a few modifications. Briefly, 10ml of sterile distilled water were pipetted onto each culture plate and the cultures were gently rubbed with a sterile bent glass rod to release the spores into the water. The formed spore suspension was filtered through a sterile double layer of cheese cloth positioned in a funnel, into a sterile falcon tube, so as to separate the spores (filtrate) from the mycelia (residue). The conidia were then centrifuged at 1000rpm for 30 minutes, the supernatant poured off while the conidia pellet was resuspended in 3ml of sterile distilled water. Then the conidia concentration was determined by counting them using a compound microscope (Olympus CH-2) and a heamocytometer (Weber Scientific International Ltd). Briefly 10µl of the spore suspension was slowly pipetted between a cover slip and the heamocytometer. The spores in 5 squares of the central cube of the heamocytometer were then counted using a compound microscope at 100X magnification. The average of spores in the 5 squares was the number of *A. flavus* spores per 0.1µl of solution (because the dimensions of each small cube in the central heamocytometer cube is 1 x 1 x 0.1 µm). The concentration of spores per 0.1 µl was then used to calculate the approximate number of spores in a microliter of the spore suspension and the spore suspension was then diluted to 200 spores per µl.

3.7.2 Exposure of *A. flavus* spores to the different dsRNA

Using a micropipette, 10µl of the 200 spores/µl suspension was pipetted into a 1.5ml microfuge tube, and to this was added 15µl of a 0.04µg/µl dsRNAs solution, so that the spores are exposed to a total of 0.6µg of dsRNA in each resultant 25µl suspension. On the other hand, 15µl of distilled sterile water was added to the 10µl of the 200 spores/µl suspension for the controls. The assay tubes were then incubated at 28°C for 1 week (Mumbanza *et al.*, 2013). After incubation, the 25ml suspension was topped up to 1000µl with distilled sterile water. The resultant spore suspension concentration was 2spores/µl. Then 250µl of this solution was plated on ¼ strength PDA (10g of PDA powder and 10g agar per liter of water supplemented with cefotaxime at 300mg/l) in petri dishes, and incubated for 24 hours at 28°C (Mumbanza *et al.*, 2013). The established individual colonies were counted by marking them out in day light. This experiment was conducted twice and each experimental treatment and control had three replicates.

3.8 Data analysis and interpretation

Percentage inhibition was calculated using the formula;

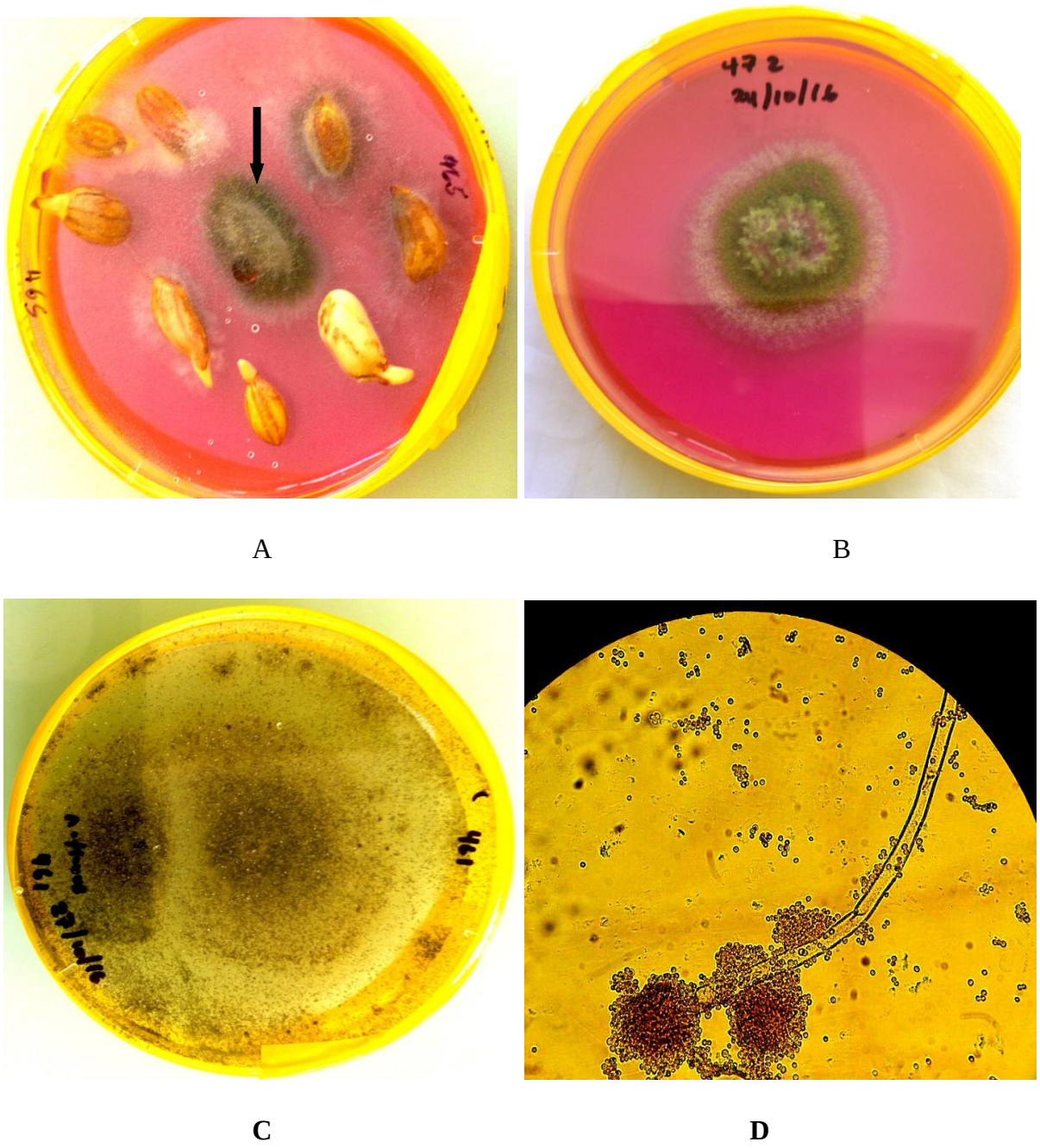
$$\%\ \text{inhibition} = 100 - \left(\frac{(\text{No.colonies in treated})}{(\text{No.colonies in control})} \times 100\right)$$ (Mumbanza *et al.* (2013)).

Percentage inhibition data which was collected from the inhibition assays targeting different essential genes in *A. flavus* was subjected to One-way ANOVA to establish if there was any significant difference in *A. flavus* spore growth inhibition due to silencing the 13 different essential genes. Data analysis was performed using Gen-Start software.

CHAPTER 4: RESULTS

4.1 *Aspergillus flavus* isolated from groundnut seed samples of Kamuli, Soroti and Tororo districts

In total, 43 groundnut seed samples were collected, 14 from Tororo, 15 from Soroti and 16 from Kamuli district (Figure 2). *A. flavus* isolation from groundnut seeds was done on Modified Rose Bengal media according to Appendix I. and *A. flavus* was observed as white mycelia with yellow-green spores (Figure 3-B). The morphologically identified *A. flavus* isolates were then confirmed microscopically and they were observed to have conidia in seriate columns borne directly on the vesicle which rests on long hyaline conidiophores as shown in Figure 3-D. Out of the 43 samples, 10 *A. flavus* isolates were obtained, six were isolated from Tororo district groundnut seed samples, three from Soroti and only one from Kamuli district groundnut seed samples as shown in Table 3.



A B

C D

Figure 5: *Aspergillus flavus* isolated from groundnut samples

A – Arrow pointing at *A. flavus* isolated from a groundnut seed sample on Modified Rose Bengal Media (MRBM), B - *A. flavus* isolate transferred to fresh MRBM for Morphological identification , C - *A. flavus* isolate transferred to 1/4 strength PDA for aflatoxin production quantification and D - Microscopic appearance of *A. flavus* isolate at magnification 400X.

***Aspergillus flavus* isolates produced varying total aflatoxin quantities on ¼ strength PDA**

A competitive ELISA to determine the quantity of total aflatoxin produced by each isolate when cultured on ¼ strength PDA for 2 weeks was performed and nine out of the ten isolates were found to be toxigenic while only one was non-toxigenic. The non-toxigenic *A. flavus* isolate KAf10 was obtained from Kamuli district and this was the only isolate from the groundnut samples of Kamuli district. Seven isolates produced from 5 parts per billion (ppb) to 990 ppb of total aflatoxin, while two produced over 2000 ppb of total aflatoxin. The two most (KAf4) and Soroti (KAf9) as shown in Table 3.

Table 3: Varying total aflatoxin quantities produced by different *A. flavus* isolates

Isolate	Sample source (district)	Approximate total Aflatoxin concentration (ppb)
KAf1	Tororo	510
KAf2	Tororo	570
KAf3	Tororo	175
KAf4	Tororo	>2000
KAf5	Tororo	990
KAf6	Tororo	5
KAf7	Soroti	55
KAf8	Soroti	390
KAf9	Soroti	>2000
KAf10	Kamuli	0

***Aspergillus flavus* essential target genes amplified from isolate KAf5**

DNA was extracted from an aflatoxigenic isolate KAf5 according to the DNA extraction protocol in Appendix III, and PCR was performed using primers targeting the selected 13 essential genes. The obtained PCR amplicons were separated on a 1.5% agarose gel and visualized using Ultra Violet (U.V) light. All 13 target genes were amplified and the expected amplicon sizes were obtained (350 to 500 bp – Table 2) as shown in Figure 4.

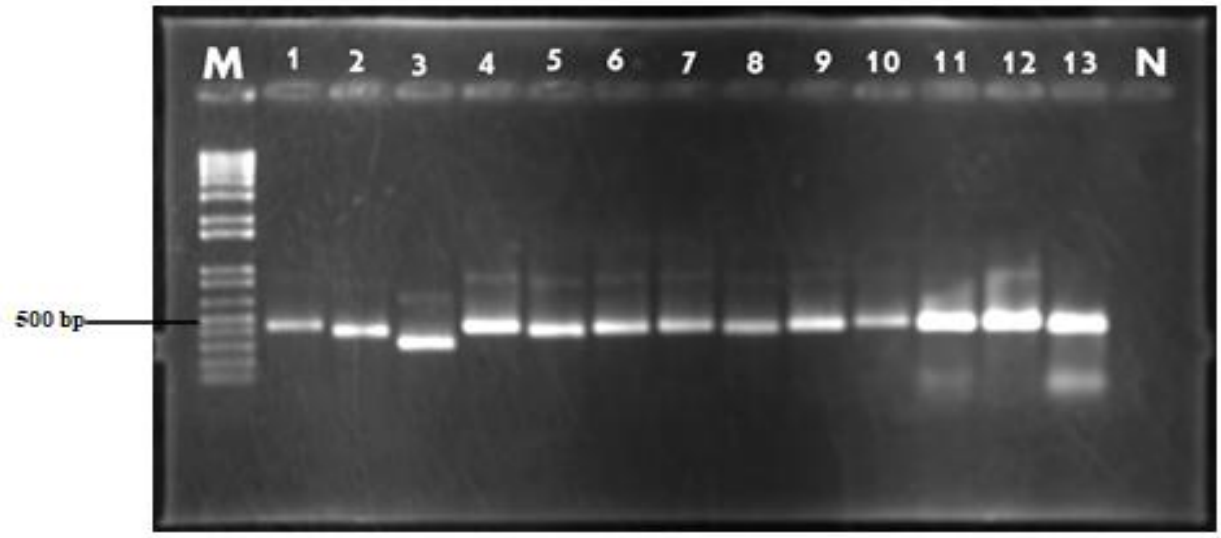


Figure 6: A 1.5% agarose gel showing PCR amplicons of the 13 target essential genes of *A. flavus*

M=Invitrogen 1kb plus DNA Ladder (Thermo Fisher scientific), 1=*Mot1*, 2=*PPK*, 3=*UBI*, 4=*HEL*, 5=*ABC*, 6=*ATP*, 7=*26s*, 8=*Ptrs*, 9=*DRF*, 10=*EXP*, 11=*COH*, 12=*IMP*, 13=*GTT* and N=Negative control (sterile water added instead of genomic DNA), annealing temperature for all 13gene primers was optimized to 62°C.

Double stranded RNAs transcribed from the 13 gene specific amplicons.

The PCR amplicons were used as templates in transcribing corresponding dsRNA using the MEGA script transcription kit according to the manufacturer's instructions. Obtained dsRNA were quantified using a Nano Drop (2000), their quality was checked by running them on 1.5% agarose gel after DNase treatment, and all target gene dsRNA were visualized under U.V after staining with ethidium bromide at 0.5µg/ml (Figure 5). Double stranded RNA was absent in the negative control in which sterile water was added to GTT amplicon in the reverse transcription reaction instead of the reverse transcriptase enzyme as shown in Figure 5.

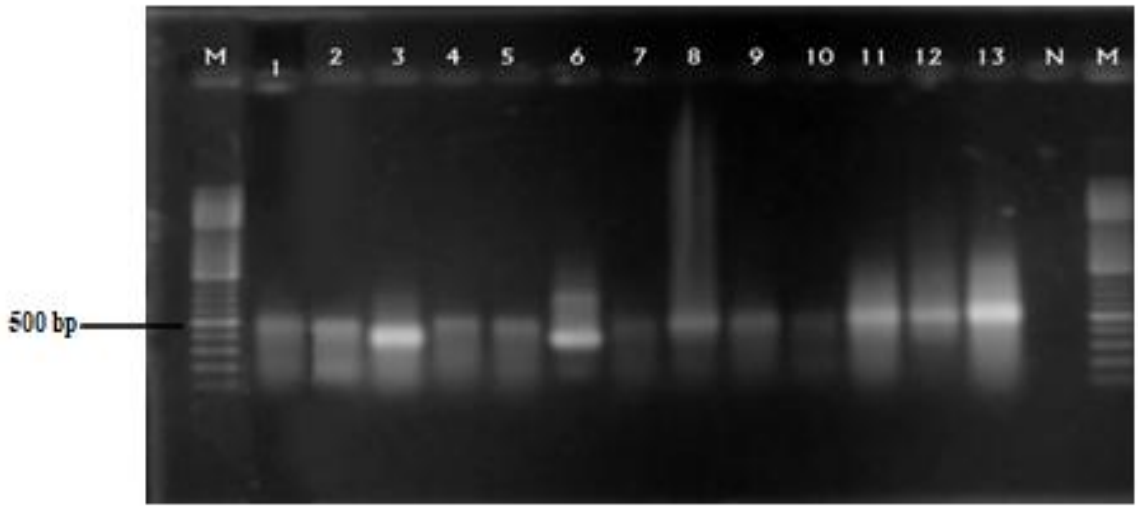


Figure 7: A 1.5% agarose gel showing double stranded RNA transcripts of 13 target essential genes of *A. flavus*

M=100bp DNA Ladder (Bioneer), 1=*Mot1*, 2=*PPK*, 3=*UBI*, 4=*HE*L, 5=*ABC*, 6=*ATP*, 7=*26s*, 8=*Ptrs*, 9=*DRF*, 10=*EXP*, 11=*COH*, 12=*IMP* and 13=*GTT* and N=Negative control (sterile water added instead of reverse transcriptase enzyme)

4.5 *Aspergillus flavus* spore germination inhibition due to exposure to dsRNAs

In this study, exposure of KAf5 *A. flavus* spores to different dsRNAs or sterile water for the controls, resulted in a marked difference in the number of spores that germinated. The controls had the highest average *A. flavus* spore germination of 552 spores per plate. Plates whose spores were exposed to the different dsRNAs showed variable spore germination, ranging from 17 to 228 spores per plate. The plates whose spores were exposed to dsRNA targeting *UBI, COH, 26s* and *PPK* genes recorded the lowest average spore growth per plate which was 17, 23, 37 and 41 respectively. Those treated with dsRNAs of *IMP, ATP,* and *ABC* genes followed with average spore growths of 60, 65, and 70 respectively. Figures 7 and 8, show a comparison in spore germination between plates whose spores were treated with the six most effective dsRNAs at inhibiting *A. flavus* growth (*UBI, COH, 26s, PPK, IMP,* and *ATP* dsRNAs) and the controls whose spores were treated with sterile water. Amongst the dsRNA treated spores, highest average spore growth was recorded on plates with spores treated with dsRNAs targeting *GTT, HEL, EXP, PTRs, DRF* and *MOT* genes, on which an average 117, 125,156, 181, 188 and 228 spores germinated respectively as shown in Figure 8.

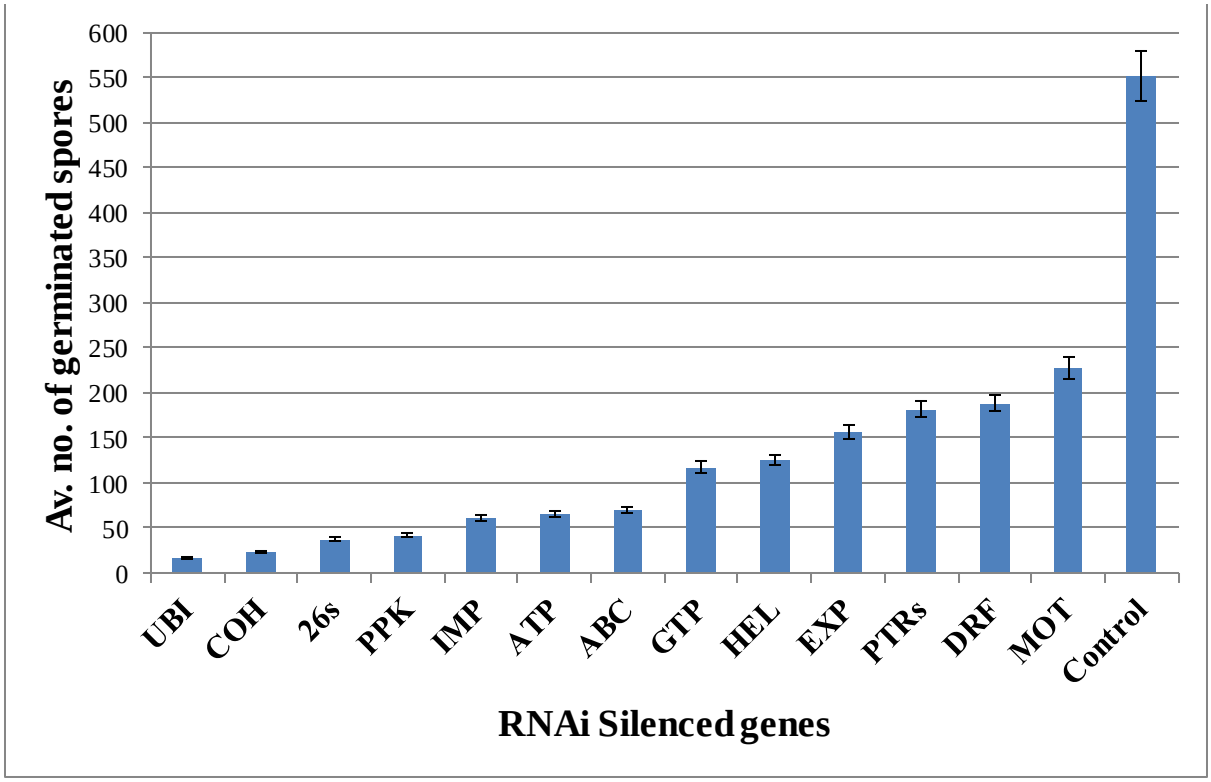


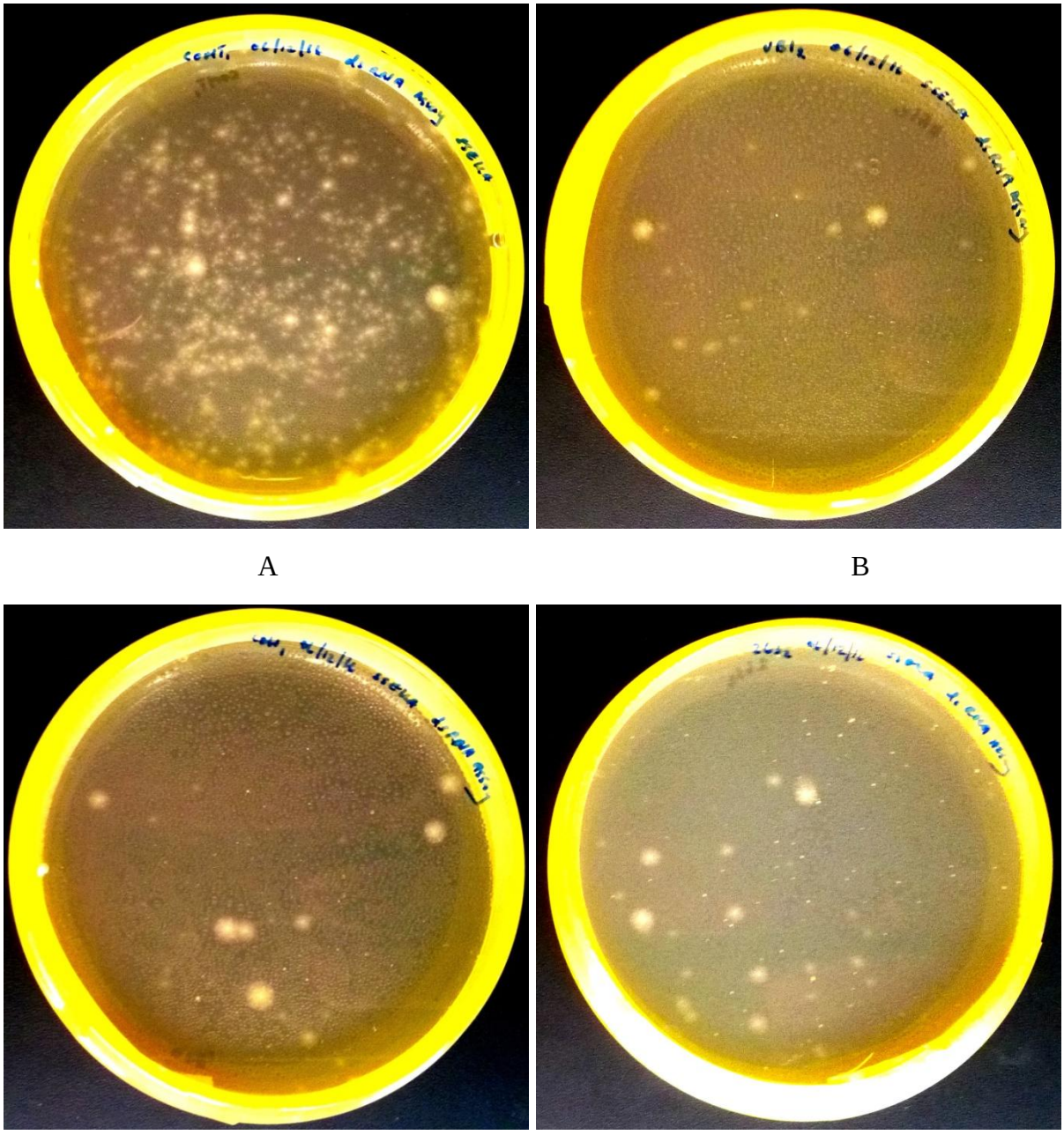
Figure 8: Average number of spores that germinated after treatment with different dsRNAs ($p<0.05$), Control (sterile water was added instead of dsRNA).

Percentage spore germination inhibition due to exposure of *A. flavus* spores to the different dsRNAs was subsequently calculated according to the formula: $\%\ \text{inhibition} = 100 - \left(\frac{(\text{No.colonies in treated})}{(\text{No.colonies in control})} \times 100\right)$. Six percentage inhibitions per each different dsRNA were calculated from the 6 replicates of each dsRNA treatment and all these were subjected to ANOVA. Results from ANOVA showed that spore germination inhibition was significantly higher in all treatments with dsRNAs compared to the controls ($F = 21.66$, $df = 13$, $p < 0.001$). Amongst dsRNA treatments, exposure of *A. flavus* spores to *UBI, COH, 26s, ATP, PPK, IMP* and *ABC* dsRNAs resulted in the highest mean spore growth inhibitions of 96.8%, 95.0%, 91.6, 90.2%, 89.0%, 88.4% and 82.1% respectively. Mean spore growth inhibition by dsRNAs targeting the above seven genes was significantly higher than the inhibition by the rest of the remaining six genes. Amongst these six, mean spore growth inhibition by dsRNAs targeting *GTP, HEL* and *PTRs* genes which was 79.5%, 73.5% and 66.1% respectively, was significantly higher than inhibition by dsRNAs targeting *EXP, DRF* and *MOT* genes which was 63.0%, 54.8% and 54.3% respectively. ANOVA results thus cluster these genes into 3 significantly different groups based on the least significant difference (l.s.d) of 15.54, at $p < 0.001$. The estimated standard error for each of the mean spore growth inhibitions was 5.48 while the coefficient of variation was 18.4 as shown in Table 4.

Table 4: Analysis of variance, of the percentage inhibitions to *A. flavus* spore germination

Target gene	% inhibition	Significantly Different groups	l.s.d	s.e	% CV	p value
UBI	96.8					
COH	95.0					
26s	91.6					
ATP	90.2	A				
PPK	89.0					
IMP	88.4					
ABC	82.1		15.54	5.48	18.4	p < 0.001
GTT	79.5					
HEL	73.6	B				
Ptrs	66.1					
EXP	63.0	C				
DRF	54.8					
MOT	54.3					

l.s.d = least significant difference between these means, s.e = Standard Error and % C.V = percentage Coefficient of Variance.

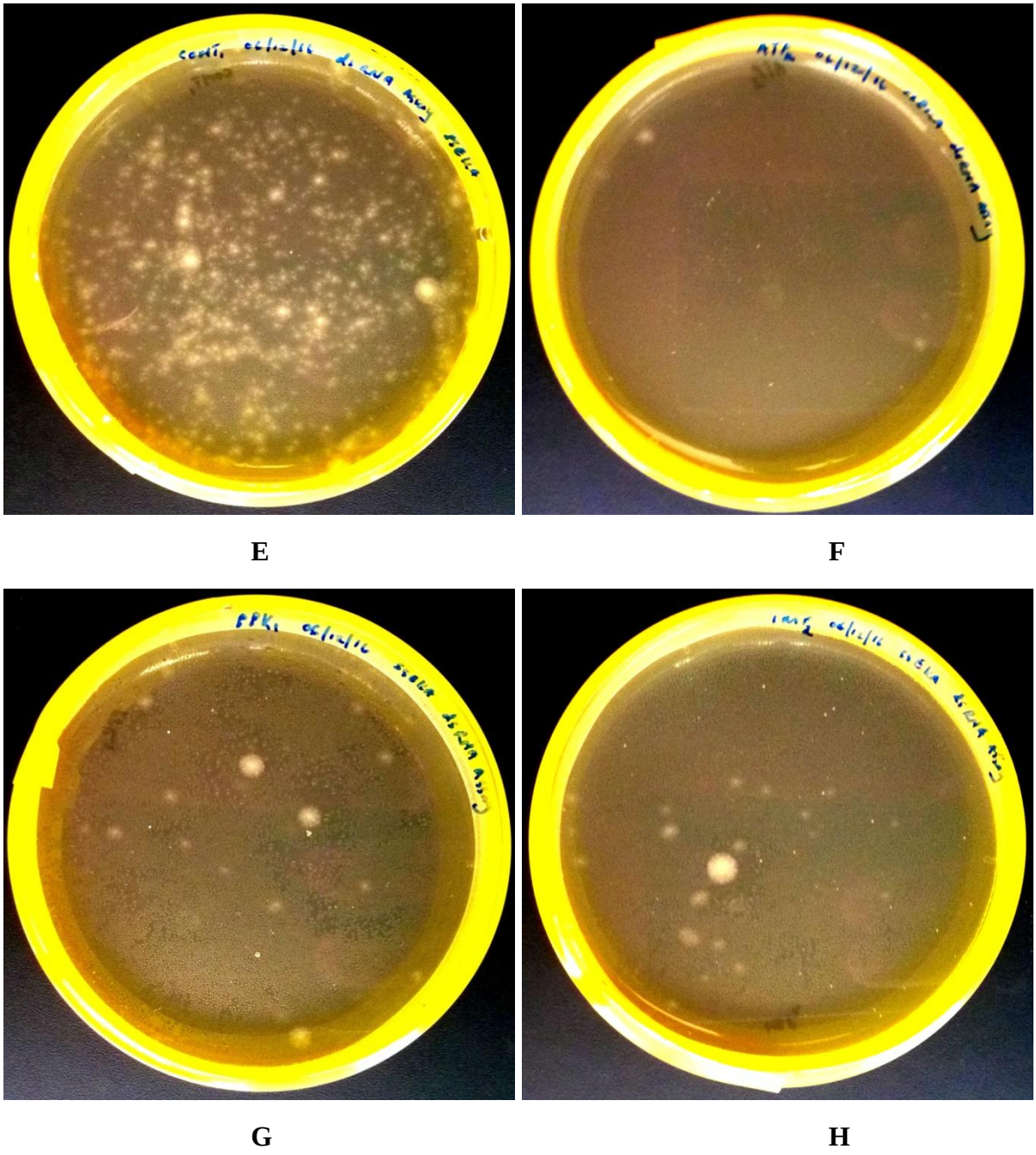


A B

C D

Figure 9: *Aspergillus flavus* spore germination inhibition by dsRNAs targeting UBI, COH and 26s genes

Double stranded RNAs targeting *UBI* (B), *COH*(C), *26s* (D), effectively inhibited *A. flavus* spore germination compared to the control (A) where spores were treated with sterile water.



E **F**

G **H**

Figure 10: *Aspergillus flavus* spore germination inhibition by dsRNAs targeting ATP, PPK and IMP genes

Double stranded RNAs targeting *ATP* (F), *PPK* (G), and *IMP* (H) genes effectively inhibited *A. flavus* spore germination compared to the control (E) where spores were treated with sterile water.

CHAPTER 5: DISCUSSION

This is the first study geared at identifying the most critical essential genes which if silenced in toxigenic *A. flavus,* isolated from groundnuts grown in Uganda, can impart resistance to the fungus' growth. Since no Ugandan *A. flavus* isolates were available in the National Agricultural Research Organization (NARO) institutes, the isolates used in this study had to be obtained from groundnut samples picked from Ugandan farmers' gardens or granaries. From the *A. flavus* isolations made, Tororo and Sororti districts had the highest number and the most toxigenic *A. flavus* isolates compared to Kamuli district which had one non-toxigenic *A. flavus* isolate. Some of the isolates were producing from 600ppb to >2000ppb of aflatoxin and such concentrations if consumed in food would constitute acute doses (Krishnamachari *et al.*, 1975), while many others were producing >20ppb to 990ppb and these lower aflatoxin doses could result in chronic effects like liver cancer, stunting of children and animals among others (Turner, 2013).

It is important to note that such high aflatoxin contamination can occur even before harvest and can be carried on through the food chain to the final human consumer. This is because, *A. flavus* being a soil pathogen, can contaminate the tender freshly fertilized groundnut ovary with aflatoxins as it penetrates the soil. Since the aflatoxins have a bio-accumulating effect (Castillo-Urueta *et al.*, 2004), they would remain in the groundnuts till consumption by animals and consequently consumed by humans in animal products like eggs or milk or people can directly consume these contaminated groundnuts and get exposed to their deleterious effects. This presents a big aflatoxin contamination problem which needs to be addressed well before harvest of the groundnuts, and thus the need to breed groundnuts that can resist colonization by *A. flavus* even when still in the soil.

This study, investigated the ability to resist *A. flavus* spore germination by silencing its essential genes using the RNAi technique. The precursors of RNAi used in this study were dsRNAs which were synthesized *in-vitro* using PCR amplicons of target genes as templates and the MEGAscript® T7 high yield transcription kit. Synthesized dsRNA was detected by agarose gel electrophoresis instead of serological methods like ELISA because it was cheaper and the transcription kit used can synthesize over 100µg of dsRNA per reaction which was easily detectable by gel electrophoresis. These dsRNAs were then applied to the spores by soaking the spores in a concentrated solution of dsRNA for 2 weeks, prior to plating the spores on PDA media. Many different scholars have reported that dsRNA can be introduced

into cells by imbibition, if cells are placed into a solution containing the dsRNA (Bailey *et al.*, 2010; Mumbanza *et al.*, 2013).

Thus the imbibed dsRNA should have started the silencing effect of the different essential genes, through the RNAi process, and depending on how critical the gene was for the survival of *A. flavus*, different numbers of spores were able to survive and germinate in the different treatments.

On average, close to the estimated number of untreated spores germinated on the control plates, that is to say, 552, which was close to the estimated 500 that were plated on each petri dish. This shows that the conditions under which the spores were kept were conducive for their survival and germination, except if a limiting factor were to be introduced in the treatments and in this study dsRNA were used as the limiting factor. On plates treated with the different dsRNAs, only 17 to 228 spores germinated on average. The lowest average spore germination of 17, 23, 37, 41, 60, 65 and 70 spores per petri dish was recorded on plates treated with dsRNAs targeting *UBI, COH, 26s, PPK, IMP, ATP* and *ABC* genes respectively. Percentage spore growth inhibition was calculated according to the formula $\%\,\text{inhibition} = 100 - \left(\frac{(\text{No.colonies in treated})}{(\text{No.colonies in control})} \times 100\right)$ and subjected to ANOVA to establish if there was any significant difference in *A. flavus* spore growth inhibition, when the spores are exposed to dsRNAs targeting particular essential genes.

The ANOVA results showed that imbibition of dsRNA by spores and consequent silencing of the target essential genes resulted in significant spore germination inhibition in all dsRNA treated spore solutions compared to their respective controls ($df = 13$, $p < 0.001$). These results show that all the silenced genes were essential for the survival of *A. flavus* spores and are in agreement with the findings of an *in vitro* study by Mumbanza *et al.* (2013) where the number of colonies that were recorded on plates treated with synthetic dsRNA targeting 12 essential genes of *Mycosphearella figiensis* and 14 essential genes of *Fusarium oxysporum f.s.p. cubense* varied significantly from those on untreated control plates.

In addition, amongst the treated plates, there was a significant difference between the percentage spore germination inhibition amongst dsRNA targeting different genes ($F= 21.66$, $df = 13$, $p < 0.001$). Basing on the least significant difference, there was a significant difference in spore growth inhibition due to RNAi silencing of different essential genes, thus placing the genes in to three groups labeled A, B and C in Table 4. The first group A consists of seven genes namely; *UBI, COH, 26s, ATP, PPK, IMP* and *ABC*. Silencing these seven

genes resulted in significantly higher spore growth inhibition than was realized when genes in group B and C were silenced. This implies that the above seven genes are better targets for RNAi silencing of *A. flavus* than the six genes in group B and C. Silencing genes in group B, (*GTT, HEL* and *PTRs*) resulted in significantly higher spore growth inhibition than silencing the genes in group C, (*EXP, DRF* and *MOT*) and are thus better target genes for RNAi silencing of *A. flavus* than the genes in group C which had the least spore growth inhibition effect on *A. flavus* spores. Since results in this study clearly show that the limiting factor for *A. flavus* spore germination was dsRNAs, which dsRNAs are known to be precursors of RNAi and consequent gene silencing (Prentice *et al.*, 2017), genes in group A could be better targets for RNAi silencing of *A. flavus* because they are more critical for the survival of *A. flavus* than the essential genes in group B and C.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

- There is a diversity in the types of *A. flavus,* found in eastern Uganda, some are non-toxigenic, some produce low concentrations of aflatoxins, while some produce very high concentrations of aflatoxins.
- Of the 13 essential genes targeted in this study, the suitable genes to silence in *A. flavus* so as to inhibit its growth are *UBI, COH, 26, ATP, PPK, IMP* and *ABC*.

6.2 Recommendations

- A detailed study should be carried out to establish the molecular diversity of *A. flavus* in Uganda so as to establish the molecular basis for some isolates producing high concentrations of aflatoxins; others produce very low concentrations while others do not produce any aflatoxins.
- Another study should be carried out to test percentage growth inhibition of *A. flavus* when U*BI, COH, 26s, ATP, PPK, IMP* and *ABC* genes are silenced by RNAi induced by dsRNA transcribed by groundnut hosts *in vivo*.

REFERENCES

Ade, A. B., & Gangawane, L. (2010). Biocontrol of aflaroot and dry root rot of groundnut by transgenic Rhizobium. *Indian Phytopathology, 63*(1), 42-44.

Ali, N., Datta, S. K., & Datta, K. (2010). RNA interference in designing transgenic crops. *GM crops, 1*(4), 207-213.

Allikmets, R., Gerrard, B., Hutchinson, A., & Dean, M. (1996). Characterization of the human ABC superfamily: isolation and mapping of 21 new genes using the expressed sequence tags database. *Human Molecular Genetics, 5*(10), 1649-1655.

Anthony, M. H., Francis, D. M., Berka, N. P., Ayinla, G. T., & Haruna, O. G. (2012). Aflatoxin contamination in foods and feeds: A special focus on Africa *Trends in Vital Food and Control Engineering*: InTech.

Arias, R., Dang, P., & Sobolev, V. (2015). RNAi-mediated Control of Aflatoxins in Peanut: Method to Analyze Mycotoxin Production and Transgene Expression in the Peanut/*Aspergillus* Pathosystem. *Journal of visualized experiments: JoVE*(106), 53398-53398.

Auble, D. T., Hansen, K. E., Mueller, C., Lane, W. S., Thorner, J., & Hahn, S. (1994). Mot1, a global repressor of RNA polymerase II transcription, inhibits TBP binding to DNA by an ATP-dependent mechanism. *Genes & development, 8*(16), 1920-1934.

Baggerman, W. (1981). A modified rose bengal medium for the enumeration of yeasts and moulds from foods. *Applied Microbiology and Biotechnology, 12*(4), 242-247.

Bailey, A. M., & Niblett, C. (2010). Bioassay for gene silencing constructs: Google Patents.

Bryden, W. L. (2012). Food and feed, mycotoxins and the perpetual pentagram in a changing animal production environment. *Animal production science, 52*(7), 383-397.

Carthew, R. W., & Sontheimer, E. J. (2009). Origins and mechanisms of miRNAs and siRNAs. *cell, 136*(4), 642-655.

Castillo-Urueta, P., García-Gómez, R. S., & Durán-de-Bazúa, C. (2004). Aflatoxinas en Maíz Amarillo Usado para Elaborar Jarabes de Fructosa:¿ Existen Riesgos para la Salud? *Industria alimentaria, 26*(4), 28-36.

Cole, R. J., & Cotty, P. J. (1990). Biocontrol of aflatoxin production by using biocompetitive agents. *A Perspective on Aflatoxin in Field Crops and Animal Food Products in the United States: A Symposium*, 62-66.

Corradi, N., & Sanders, I. R. (2006). Evolution of the P-type II ATPase gene family in the fungi and presence of structural genomic changes among isolates of Glomus intraradices. *BMC Evolutionary Biology, 6*(1), 21-36.

D'mello, J., & Macdonald, A. (1997). Mycotoxins. *Animal Feed Science and Technology, 69*(1), 155-166.

Davies, O., Mendes, P., Smallbone, K., & Malys, N. (2012). Characterisation of multiple substrate-specific (d) ITP/(d) XTPase and modelling of deaminated purine nucleotide metabolism. *BMB reports, 45*(4), 259-264.

Fox, E. M., & Howlett, B. J. (2008). Secondary metabolism: regulation and role in fungal biology. *Current opinion in microbiology, 11*(6), 481-487.

Grenier, B., Loureiro-Bracarense, A.-P., Leslie, J. F., & Oswald, I. P. (2014). Physical and chemical methods for mycotoxin decontamination in maize. *Mycotoxin Reduct. Grain Chains*, 116-129.

Hell, K., Cardwell, K., Setamou, M., & Poehling, H.-M. (2000). The influence of storage practices on aflatoxin contamination in maize in four agroecological zones of Benin, West Africa. *Journal of Stored Products Research, 36*(4), 365-382.

IARC. (2002). Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. *Iarc Monographs on the Evaluation of Carcinogenic Risks to Humans, 82*, 1-3.

Jarvis, B. B. (2002). Chemistry and toxicology of molds isolated from water-damaged buildings *Mycotoxins and Food Safety* (pp. 43-52): Springer.

Jemal, A., Bray, F., Forman, D., O'brien, M., Ferlay, J., Center, M., & Parkin, D. M. (2012). Cancer burden in Africa and opportunities for prevention. *Cancer, 118*(18), 4372-4384.

Jinek, M., & Doudna, J. A. (2009). A three-dimensional view of the molecular machinery of RNA interference. *Nature, 457*(7228), 405-412.

Kaaya, A., Harris, C., & Eigel, W. (2006a). Peanut aflatoxin levels on farms and in markets of Uganda. *Peanut Science, 33*(1), 68-75.

Kaaya, A., Kyamuhangire, W., & Kyamanywa, S. (2006b). Factors affecting aflatoxin contamination of harvested maize in the three agroecological zones of Uganda. *Journal of Applied Sciences, 6*, 2401-2407.

Kaaya, N., & Warren, H. (2005). Review of past and present research on Aflatoxin in Uganda. *African Journal of Food, Agriculture, Nutrition and Development, 5*(1).

Kansiime, M. K., Wambugu, S. K., & Shisanya, C. A. (2013). Perceived and actual rainfall trends and variability in Eastern Uganda: Implications for community preparedness and response. *Journal of Natural Sciences Research, 3*(8), 179-194.

Kolosova, A. Y., Shim, W.-B., Yang, Z.-Y., Eremin, S. A., & Chung, D.-H. (2006). Direct competitive ELISA based on a monoclonal antibody for detection of aflatoxin B1. Stabilization of ELISA kit components and application to grain samples. *Analytical and bioanalytical chemistry, 384*(1), 286-294.

Krishnamachari, K., Bhat, R., Nagarajan, V., & Tilak, T. (1975). Investigations into an outbreak of hepatitis in parts of western India. *The Indian journal of medical research, 63*(7), 1036-1049.

Lee, R. C., Feinbaum, R. L., & Ambros, V. (1993). The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *cell, 75*(5), 843-854.

Lee, S.-J., Song, O.-R., Lee, Y.-C., & Choi, Y.-L. (2003). Molecular characterization of polyphosphate kinase (ppk) gene from *Serratia marcescens*. *Biotechnology letters, 25*(3), 191-197.

Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., Kieszak, S., . . . Misore, A. (2005). Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. *Environmental health perspectives*, 1763-1767.

Liu, Y., & Wu, F. (2010). Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. *Environmental health perspectives, 118*(6), 818.

Lu, M., Zhang, Q., Deng, M., Miao, J., & Guo, Y. (2008). An Analysis of Human MicroRNA and Disease Associations. *PLoS ONE, 3*(10), 3420-3424.

MacFarlane, L.-A., & Murphy, P. R. (2010). MicroRNA: biogenesis, function and role in cancer. *Current genomics, 11*(7), 537.

Madduri, K., Waldron, C., & Merlo, D. J. (2001). Rhamnose biosynthesis pathway supplies precursors for primary and secondary metabolism in Saccharopolyspora spinosa. *Journal of bacteriology, 183*(19), 5632-5638.

Mahmoud, M., Osman, A., Nalyongo, P., Wakjira, A., & David, C. (1991). Peanut in east Africa: 1981-1990. *Peanut, A Global Perspective: Proc. Intl. Workshop*, 22-29.

Mahuku, G. S. (2004). A simple extraction method suitable for PCR-based analysis of plant, fungal, and bacterial DNA. *Plant Molecular Biology Reporter, 22*(1), 71-81.

Manshardt, R. (2004). Crop improvement by conventional breeding or genetic engineering [electronic resource].

Manual, M. K. P. (2009). Ambion/Invitrogen website: http://tools. invitrogen. com/content/sfs/manuals/cms—072987. pdf. *Publication Date: Oct, 27*.

Matzke, M., Primig, M., Trnovsky, J., & Matzke, A. (1989). Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. *The EMBO Journal, 8*(3), 643-649.

McDonald, T., Brown, D., Keller, N. P., & Hammond, T. M. (2005). RNA silencing of mycotoxin production in *Aspergillus and Fusarium* species. *Molecular plant-microbe interactions, 18*(6), 539-545.

Metcalfe, C. (2001). Biostatistics: A Foundation for Analysis in the Health Sciences. 7th edn. Wayne W. Daniel, Wiley, 1999. No. of. pages: xiv+ 755+ appendices. Price:£ 28.95. ISBN 0- 471- 16386- 4. *Statistics in Medicine, 20*(2), 324-326.

Morris, K. V. (2012). *Non-coding RNAs and epigenetic regulation of gene expression: Drivers of natural selection*: Horizon Scientific Press.

Mumbanza, F. M., Kiggundu, A., Tusiime, G., Tushemereirwe, W. K., Niblett, C., & Bailey, A. (2013). *In vitro* antifungal activity of synthetic dsRNA molecules against two pathogens of banana, *Fusarium oxysporum f. sp. cubense* and *Mycosphaerella fijiensis*. *Pest management science, 69*(10), 1155-1162.

Naing, L., Winn, T., & Rusli, B. (2006). Practical issues in calculating the sample size for prevalence studies. *Archives of orofacial Sciences, 1*(1), 9-14.

Nakayashiki, H., & Nguyen, Q. B. (2008). RNA interference: roles in fungal biology. *Current opinion in microbiology, 11*(6), 494-502.

Nalyongo, P., & Emeetai-Areke, T. (1987). *Groundnut and pigeonpea production in Uganda*. Paper presented at the Consultative Group Meeting for Eastern and Central African Regional Research on Grain Legumes (Groundnut, Chickpea, and Pigeonpea), Addis Ababa (Ethiopia), 8-10 Dec 1986.

Niblett, C., & Bailey, A. (2012). Potential applications of gene silencing or RNA interference (RNAi) tocontrol disease and insect pests of date palm. *Emirates Journal of Food and Agriculture, 24*(5), 462-469.

Okello, D., Biruma, M., & Deom, C. (2013a). Overview of groundnuts research in Uganda: Past, present and future. *African Journal of Biotechnology, 9*(39), 6448-6459.

Okello, D., Kaaya, A., Bisikwa, J., Were, M., & Oloka, H. (2010). Management of Aflatoxins in Groundnuts: A Manual for Farmers, Processors, Traders, and Consumers in Uganda. *National Agricultural Research Organisation, Entebbe, Uganda, ISBN*, 978-9970.

Okello, D., Monyo, E., Deom, C., Ininda, J., & Oloka, H. (2013b). Groundnuts production guide for Uganda: Recommended practices for farmers. *National Agricultural Research Organisation, Entebbe. ISBN*, 978-9970.

Olwari, F., Bisikwa, J., Kaaya, A., & Okello, D. (2013). Tolerance levels of peanut varieties against *Aspergillus flavus* infection. *Journal of Plant Pathology & Microbiology, 4*(8), 1-4.

Otsuka, T., Nomiyama, H., Yoshida, H., Kukita, T., Kuhara, S., & Sakaki, Y. (1983). Complete nucleotide sequence of the 26S rRNA gene of *Physarum polycephalum*: its significance in gene evolution. *Proceedings of the National Academy of Sciences, 80*(11), 3163-3167.

Ozkaynak, E., Finley, D., Solomon, M., & Varshavsky, A. (1987). The yeast ubiquitin genes: a family of natural gene fusions. *The EMBO Journal, 6*(5), 1429-1439.

Pasierbek, P., Jantsch, M., Melcher, M., Schleiffer, A., Schweizer, D., & Loidl, J. (2001). A *Caenorhabditis elegans* cohesion protein with functions in meiotic chromosome pairing and disjunction. *Genes & development, 15*(11), 1349-1360.

Payne, G., Cassel, D., & Adkins, C. (1986). Reduction of aflatoxin contamination in corn by irrigation and tillage. *Phytopathology, 76*(7), 679-684.

Potter, S., Uknes, S., Lawton, K., Winter, A. M., Chandler, D., DiMaio, J., . . . Ryals, J. (1993). Regulation of a hevein-like gene in *Arabidopsis*. *MPMI-Molecular Plant Microbe Interactions, 6*(6), 680-685.

Prensner, J. R., Iyer, M. K., Balbin, O. A., Dhanasekaran, S. M., Cao, Q., Brenner, J. C., . . . Kominsky, H. D. (2011). Transcriptome sequencing across a prostate cancer cohort identifies PCAT-1, an unannotated lincRNA implicated in disease progression. *Nature biotechnology, 29*(8), 742.

Prentice, K., Christiaens, O., Pertry, I., Bailey, A., Niblett, C., Ghislain, M., . . . Smagghe, G. (2017). RNAi- based gene silencing through dsRNA injection or ingestion against the African sweet potato weevil Cylas puncticollis (Coleoptera: Brentidae). *Pest management science, 73*(1), 44-52.

Raju, M., Rama Rao, S., Radhika, K., Panda, A., Shyam Sunder, G., & Sharma, R. (2007). Efficacy of mycotoxin degrading enzymes in countering aflatoxicosis in broiler chicken1. *Indian Journal of Animal Nutrition, 24*(3), 136-141.

Ramos, A.-J., Fink-Gremmels, J., & Hernández, E. (1996). Prevention of toxic effects of mycotoxins by means of nonnutritive adsorbent compounds. *Journal of Food Protection®, 59*(6), 631-641.

Richard, J., Payne, G., eds, Desjardins, A., Maragos, C., Norred, W., & Pestka, J. (2003). Mycotoxins: risks in plant, animal and human systems. *CAST Task Force Report, 139*, 101-103.

Richard, J. L. (2007). Some major mycotoxins and their mycotoxicoses—An overview. *International journal of food microbiology, 119*(1), 3-10.

Richard, J. L. (2008). Discovery of aflatoxins and significant historical features. *Toxin Reviews, 27*(3-4), 171-201.

Richards, M. P., Poch, S. M., & McMurtry, J. P. (2005). Expression of insulin-like growth factor system genes in liver and brain tissue during embryonic and post-hatch development of the turkey. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 141*(1), 76-86.

Rodrigues, P., Soares, C., Kozakiewicz, Z., Paterson, R., Lima, N., & Venâncio, A. (2007). Identification and characterization of *Aspergillus flavus* and aflatoxins.

Romano, N., & Macino, G. (1992). Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. *Molecular microbiology, 6*(22), 3343-3353.

Sauer, D., & Tuite, J. (1987). *Conditions that affect growth of Aspergillus flavus and production of aflatoxin in stored maize.* Paper presented at the US Universities-CIMMYT Maize Aflatoxin Workshop, El Batan, Mexico (Mexico), 7-11 Apr 1987.

Savage, G., & Keenan, J. (1994). The composition and nutritive value of groundnut kernels *The Groundnut Crop* (pp. 173-213): Springer.

Schmale, D., & Munkvold, G. (2009). Mycotoxins in crops: A threat to human and domestic animal health. *The plant health instructor, 3*, 340-353.

Singh, F., & Oswalt, D. (1992). Major Diseases of Groundnut. *ICRISAT-skill development series*(6), 50-36.

Sreenivasulu, P., Kuhn, C., Naidu, R., Demski, J., Reddy, D., & Nayudu, M. (1991). Viruses infecting peanuts (*Arachis hypogaea*): Taxonomy, identification, and disease

management. *Research bulletin-University of Georgia, Agricultural Experiment Stations (USA)*.

Tsay, Y.-F., Chiu, C.-C., Tsai, C.-B., Ho, C.-H., & Hsu, P.-K. (2007). Nitrate transporters and peptide transporters. *FEBS letters, 581*(12), 2290-2300.

Turner, P. (2013). The molecular epidemiology of chronic aflatoxin driven impaired child growth. *Scientifica, 2013*, 152879-152879.

Van der Krol, A. R., Mur, L. A., Beld, M., Mol, J., & Stuitje, A. R. (1990). Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *The Plant Cell, 2*(4), 291-299.

Weidenbörner, M. (2001). *Encyclopedia of food mycotoxins* (Vol. 615): Springer Science & Business Media.

Williams, W. (2006). Breeding for resistance to aflatoxin accumulation in maize. *Mycotoxin research, 22*(1), 27-32.

Wilson, R. C., & Doudna, J. A. (2013). Molecular mechanisms of RNA interference. *Annual review of biophysics, 42*, 217-239.

Xue, G. P., & Loveridge, C. W. (2004). HvDRF1 is involved in abscisic acid- mediated gene regulation in barley and produces two forms of AP2 transcriptional activators, interacting preferably with a CT- rich element. *The Plant Journal, 37*(3), 326-339.

Yi, R., Qin, Y., Macara, I. G., & Cullen, B. R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes & development, 17*(24), 3011-3016.

Zain, M. E. (2011). Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society, 15*(2), 129-144.

Zhang, R., & Lin, Y. (2009). DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes. *Nucleic acids research, 37*(suppl 1), D455-D458.

APPENDICES

Appendix I: Modified Rose Bengal Agar Preparation Protocol (Baggerman, 1981)

- Weigh; Glucose (10g), Peptone (2.5g), Potassium phosphate (KH_2PO_4 – 1.0g), Magnesium sulphate ($MgSO_4.7H_2O$ – 0.5g), Yeast extracts (0.5g), Agar (18.0g) and add all to 1.0 liter of distilled water.
- Add 500µl of Rose Bengal stock solution prepared at (500mg of Rose Bengal in 30ml of ethanol in a falcon tube and topped up to 100ml with sterile water).
- Adjust the pH to 5.0 and autoclave at 121°C and 15 pa for 15 minutes.
- Allow to cool up to about 55°C in a laminar flow hood.
- Add 5ml of cefotaxime stock solution prepared at 60mg/ml.
- Add 100µl of Botran stock solution prepared at 0.04g of Botran powder dissolved in 5.0ml of acetone.
- Dispense in petri dishes and allow the plate to set in the laminar flow hood and store at 4°C.

Appendix II: Protocol for Competitive ELISA for Aflatoxin Detection and Quantification (Kolosova *et al.*, 2006)

Sample Preparation / Extraction

- Obtain 600mg of *A. flavus* mycelia (cultured on PDA 7days). Grind it using a clean motor and pestle in 3ml of 70/30 (v/v) methanol/water extraction solution for at least 3 minutes.
- Allow sample to settle, then filter the top layer of extract through a Whatman #1 filter paper and collect the filtrate.

Aflatoxin Quantification

- Place the required number of blue-bordered dilution Strips in a microwell strip holder, considering one dilution well for each standard, (i.e. 0, 4, 10, 20, & 40 ppb) or sample.
- Place an equal number of antibody coated microwell strips in a microwell strip holder as well.
- Measure 240µl/well of enzyme-conjugated aflatoxin (conjugate) in to a separate container.
- Pipette 200µl of that conjugate into each blue-bordered dilution well and add 100µL of each standard or sample into the appropriate dilution well containing 200µl of conjugate. (Note: a fresh pipette tip is used for each standard or sample and the pipette tip should be completely emptied)
- Mix each well's contents by carefully pipetting up and down 3 times
- Immediately transfer 100µL of the contents from each dilution well into a corresponding antibody coated microwell and incubate at room temperature for 15 minutes. (Note: the plate is not agitated to control well-to-well contamination)
- Empty the contents of the microwell strips into a waste container, and wash the microwell strips by filling each with sterile distilled, and then dumping the water from the microwell strips. This step is repeated 5 times

- After the fifth wash, lay several layers of absorbent paper towels on a flat surface and tap the microwell strips on the towels to expel as much residual water. Also dry the bottom of the microwells with a paper towel.
- Measure 120µl/well of Substrate and dispense into a separate container. Pipette 100µL of it into each microwell and incubate the mixture at room temperature for 5 minutes. A blue color should be observed.
- Measure 120µL/well of the stop solution and dispense into a separate container. Pipette 100µL of Stop Solution into each microwell. The color should change from blue to yellow.
- Read the wells with a microwell reader using a 450 nm filter and print out or record the optical density (OD) readings for each microwell.
- Use the OD values to construct a dose response curve using the five standards and since the amount of aflatoxin in each standard is known, the unknowns can be deduced by interpolation from this standard curve.

- If a sample contains aflatoxin levels higher than the highest standard (>20 ppb), the filtered extract should be further diluted in 70% methanol such that the diluted sample results are in a range of 2.0 - 20 ppb and reanalyzed to obtain accurate results. The dilution factor must be included when the final result is calculated.

Appendix III: Fungal DNA Extraction Protocol (Mahuku, 2004)

- Transfer fresh mycelium (150mg) to a clean mortar containing 300µL of TES extraction buffer (0.2M Tris-HCL [pH8], 10mM EDTA [pH8], 0.5M NaCl, 1% SDS) and acid washed, sterilized sea sand and macerate for 2min.
- Transfer to 1.5mL micro-centrifuge tubes and vortex samples for 30s and add an additional 200µL of TES extraction buffer containing Proteinase K (final concentration 50µg/µL)
- Vortex to thoroughly mix and place tubes in a water bath at 65°C for 30min.
- Add one half volumes (250µL) of 7.5M ammonium acetate.
- Mix and incubate the samples on ice or at -5°C in the refrigerator for 10min.
- Centrifuge for 15min at 13,000rpm.
- Transfer the supernatant to a new tube and add an equal volume (500µL) of ice-cold isopropanol.
- Incubate tubes at -20°C overnight.
- Centrifuge for 10min at 13,000rpm to pellet the DNA.
- Decant the supernatant and wash DNA pellet with 800uL of cold 70% ethanol.
- Turn tubes upside down on a clean sterile paper towel for 6hr to air dry DNA
- Dissolve the DNA in 50 µl of sterile nuclease free water.
- Add 5µL of RNAse A (20mg/µl) and incubate at 37°C for 60min, and then at 65°C for 15min to terminate the RNase activity.
- Quantify DNA concentration using a Nano drop and dilute to a desired concentration.
- Store the DNA at -20°C till further use.

Appendix IV: Gene elute gel extraction protocol (Richards *et al.*, 2005)

- Excise the DNA fragment of interest from an agarose gel using a clean, sharp scalpel and place it in a 1.5ml eppendorf tube. Weigh the excised gel slice.
- Add three gel volumes of the gel solubilization solution to the slice (for example for 100mg of gel added 300ml of gel solubiliation solution).
- Incubate the gel mixture at 60°C until the gel is completely dissolved.
- Prepare binding columns by placing them in 2ml collection tubes, add 500µl of column preparation solution to each of them and centrifuge at 12000rpm for one minute.
- Add one gel volume of isopropanol in to the solubilized gel mixture and pipette this mixture into a prepared binding column. Spin at 12000rpm for one minute. Remove the binding column from the 2ml collection tube and discard the flow through liquid.
- Return the binding column into the collection tube and add 700µl of wash solution, spin at 12000rpm for one minute and discard the flow through liquid too.
- Lastly transfer the binding column to a fresh collection tube and add 50µl of elution solution at the center of the membrane, incubated for one minute at room temperature and centrifuge at 12000rpm for one minute.
- Quantify the DNA contained in the flow through using a Nanodrop and then store it at -20°C for later use.

Appendix V: dsRNA Transcription Protocol (Manual, 2009)

Thawing the frozen reagents

- Place the RNA Polymerase Enzyme Mix on ice (it is stored in glycerol and will not be frozen at –20°C).
- Vortex the 10X Reaction Buffer and the 4 ribonucleotide solutions (ATP, CTP, GTP, and UTP) until they are completely in solution.
- Once thawed, store the ribonucleotides on ice, but keep the 10X Reaction Buffer at room temperature while assembling the reaction.
- Microfuged all reagents briefly before opening to prevent loss and/or contamination of material that may be present around the rim of the tube.

Assembling transcription reaction at room temperature

- Mix an equal amount of the four ribonucleotides (ATP, CTP, GTP, and UTP).
- To 7µL nuclease free water, add 8µL of the ribonucleotides mixture, 2 µL of 10X reaction buffer, 1µL of linear template and 2 µL of enzyme mix.
- Mix thoroughly and incubate at 37°C, for 4 hours.
- Add 1µL TURBO DNase, mix well and incubate for 15 min at 37°C.
- Lastly incubate at 65°C for 30 minutes to in activate the TURBO DNase enzyme

Appendix VI: Groundnut Sample Collection Data Sheet

Date of collection…….

Farmer's Name……..

GPS READINGS

Way Point……

Altitude……

Co-ordinates: E…….

N…….

Source of Sample (garden/granary)……

Amount collected……..

Groundnut Variety……

Village…….

Sub-county……..

District…….

Country……

Name of collector……