# AFLATOXIGENIC FUNGI CONTAMINATING MAIZE (*Zea mays* L.) AND THE POTENTIAL OF BIOLOGICAL CONTROL USING ATOXIGENIC *ASPERGILLUS* SPECIES

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## DECLARATION AND RECOMMENDATION

### DECLARATION

This thesis is my original work and has not been submitted or presented for examination in any other institution.

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SM15/2736/10

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### RECOMMENDATION

This thesis has been submitted to the Graduate School for examination with our approval as University supervisors.

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## DEDICATION

This work is dedicated to my parents,

Mr. Wilson Odhiambo Okomba and Mrs. Millicent Anyango Odhiambo,

My Brothers and Sisters.

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**ABSTRACT**

Aflatoxin contamination of maize is a major risk to health and well-being of Kenyan people. Aflatoxin-producing moulds viz: *Aspergillus flavus*, *A. parasiticus* and *A. nomius* can infect grains from pre-harvest stages in the field through to post-harvest stages in the stores. In Kenya, deaths have resulted from dramatic outbreaks of aflatoxin poisoning. Existing strategies for control of aflatoxigenic moulds mainly based on moisture reduction have been ineffective in the warm and humid areas. Similarly, using solar driers is expensive for the small scale farmers. The aim of this study was to control the growth of aflatoxigenic *Aspergillus* species in maize and maize feeds using atoxigenic strains. *Aspergillus* moulds were isolated from 113 maize samples and 113 soil samples obtained randomly from maize farms and rural households in Makueni, Nyeri Central, Bungoma South, Moiben and Ugunja districts. Out of 174 isolates 82.3% originated from maize and 17.7% from soil. Makueni district had the highest *Aspergillus* incidence at 58.1% followed by Nyeri Central (12.6%), Moiben (10.3%), Ugunja (10.3%) and Bungoma South (8.6%). The isolates were identified to species level using cultural, morphological and microscopic characteristics resulting to 10 species namely; *Aspergillus flavus* (78.5% occurrence), *A. versicolor* (8.0%), *A. parasiticus* (3.4%), *A. clavatus* (2.3%), *A. sydowii* (2.3%), *A. fumigatus* (2.3%), *A. glaucus* (1.1%), *A. nidulans* (1.1%), *A. candidus* (0.6%) and *A. wentii* (0.6%). Their toxigenicity was established through yellow pigment formation in PDA media and colour changes upon exposure to 27% ammonia solution in YES media. A total of 6.9% of the isolates were aflatoxigenic while 93.1% were atoxigenic. The atoxigenic strains were screened for antimould activity against aflatoxigenic strains and those found to be inducing the largest inhibition zones and therefore most effective were; UM082 *A. parasiticus* (19.9 mm), NM084 *A. flavus* (18.2 mm), UM127 *A. flavus* (17.8 mm), BM071 *A. flavus* (17.7 mm), BM092 *A. fumigatus* (17.5 mm), and NM083 *A. flavus* (17.3 mm). These atoxigenic strains were most effective on five aflatoxigenic *Aspergillus* strains namely; T-RS013 *A. flavus,* T-RM023-2 *A. flavus,* T-RS162 *A. versicolor,* T-RS016 *A. versicolor* and T-BS203 *A. versicolor.* These results show that atoxigenic *Aspergillus* species can inhibit the growth and sporulation of aflatoxigenic moulds thereby minimizing subsequent aflatoxin production in maize. These atoxigenic strains or their products can be used in food preservation systems to minimize aflatoxin poisoning.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of Variance |
| CPA | Cyclopiazonic Acid |
| CZ | Czapek Dox Agar |
| LVB | Lake Victoria Basin |
| LSD | Least Significant Difference |
| PDA | Potato Dextrose Agar |
| TLC | Thin Layer Chromatography |
| UNICEF | United Nations International Children's Emergency Fund |
| UV | Ultra Violet |
| YES | Yeast Extract Sucrose |

# CHAPTER ONE
# INTRODUCTION

## 1.1 Background information

Aflatoxin is one of the widely studied mycotoxins produced by several species of moulds including *Aspergillus flavus* and *A. parasiticus* in a wide variety of agricultural commodities such as grains (maize), legumes and nuts (Patten, 1981). The main aflatoxin producing fungi *A. flavus*, *A. parasiticus* and *A. nomius* can infect maize from pre-harvest stages in the field through to post-harvest stages in the stores. The species of *Aspergillus* are almost ubiquitously present in soils of tropical areas (Ranajit *et al*., 2005). Although most species of *Aspergillus* are not of much consequence in agriculture, some of them are found in plant products, particularly oil-rich seeds. Contamination of seeds with poisonous aflatoxins results from the presence of aflatoxigenic strains of four species of *Aspergillus* that includes; *A. flavus*, *A. parasiticus*, *A. nomius* and *A. bombycis*, each producing a combination of different types of aflatoxins (Ranajit *et al*., 2005).

In Africa, aflatoxins have an impact on human and animal health and on trade. People in rural areas may have no option but to consume contaminated crops on a daily basis (Shephard, 2003). This moderate, chronic intake of aflatoxin via food can lead to severe pathological conditions, including liver cancer, immune system deficiency and impaired development of children (Williams *et al.,* 2004). Malnutrition, a common condition in rural Africa, increases disease prevalence and further reduces the ability of the human body to cope with aflatoxin exposure. Chronic aflatoxin poisoning reduces life expectancy (Anonymous, 2004). In various animal models, in addition to being hepatotoxic, aflatoxin causes significant growth faltering and is strongly immune-suppressive at weaning (Wild *et al.,* 1992). Similar effects have been reported in human population in a few African countries such as Ghana and it has been recently shown that 99% of all children weaned from mother's milk to maize-based diets in Benin and Togo had aflatoxin in their blood, indicating ingestion of aflatoxin-contaminated food (Ranajit *et al*., 2005).

Acute aflatoxin poisoning is caused by ingestion of high levels of the toxin in contaminated foods. Immediate consequences are severe liver damage, acute jaundice and hepatitis, which

subsequently may result in death (Bennett and Klich, 2003). Although on a global basis deaths from acute aflatoxin poisoning are rare, Kenya has experienced dramatic outbreaks of mycotoxin poisoning resulting in loss of lives. In 2004, an acute aflatoxicosis outbreak occurred in Machakos, Kenya resulting in 125 deaths, while cases of liver cancer have been linked to high levels of aflatoxins in the Lake Victoria Basin (LVB) (Anonymous, 2004). Previously there has been extensive use of chemical preservatives for control of aflatoxins, and this has led to the appearance of resistant micro-organisms, leading to the occurrence of emerging food borne diseases (Kaur and Arora, 1999). Due to this, there is an increasing interest to obtain alternative antimicrobial agents to use in food preservation systems.

The use of bioagents with antimould activities to control aflatoxin production would provide a technology to ensure that maize and maize feeds are free of aflatoxigenic moulds and aflatoxins (Ranajit *et al*., 2005). Products from such strains of recognized antimicrobial spectrum could appear in food preservation systems as main antimicrobial compounds or as adjuvants to improve the action of other antimicrobial compounds (Kaur and Arora, 1999). Bioagents are living organisms that have the ability to control other living organisms of undesired effects by out-competing them for food or even directly attacking them and thereby eliminating them (Cotty, 1994). Recent studies have shown that the use of atoxigenic *A. flavus* L strains as bio-agents considerably reduces aflatoxin contamination in cottonseed in the USA (Cotty, 1994). In addition to characterizing atoxigenic *Aspergillus* species associated with maize and soil, this study aimed at identifying atoxigenic *Aspergillus* strains that can be used to control the growth of aflatoxigenic moulds in maize, therefore inhibiting the production of associated aflatoxins in the grains. These strains or their products can be used by farmers, food processors and consumers as cheap, affordable and environmentally friendly antimould products for food preservation systems. This would help alleviate great losses of maize grains encountered due to aflatoxigenic moulds and prevent aflatoxin poisoning outbreaks in the country.

### 1.2 Statement of the problem

The ubiquitous nature of aflatoxigenic moulds, their ability to colonize diverse substrates and lack of effective control measures against mould food contamination, has contributed to the high

incidences of aflatoxigenic moulds and aflatoxin contamination in maize and maize based feeds. There have been reported cases of aflatoxin poisoning and deaths resulting from acute aflatoxin poisoning in Eastern province and in the Lake Victoria basin. One of the available methods of controlling aflatoxigenic moulds is the use of synthetic chemical preservatives like sodium nitrate which have been the cause of appearance of resistant micro-organisms, leading to occurrences of emerging food borne diseases. Furthermore, other methods such as the use of solar driers are expensive for the small scale farmers. Inadequately dried maize provides favourable conditions for the growth, sporulation and aflatoxin production by the aflatoxigenic moulds. Apart from these, drying maize in some regions of Kenya is not effective because of erratic rainfall and too high humidity resulting in maize which is not properly dried, and is prone to contamination by moulds and associated mycotoxins.

## 1.3 Objectives

### 1.3.1 General objective

To evaluate fungal isolates from maize and soil samples for their ability to control the growth of aflatoxigenic *Aspergillus* species.

### 1.3.2 Specific objectives

1. To isolate and characterize different strains of *Aspergillus* spp. from grain and soil samples from five different maize growing regions in Kenya.
2. To screen the isolated *Aspergillus* spp. for atoxigenic and aflatoxigenic strains in the laboratory.
3. To characterize the atoxigenic and aflatoxigenic strains based on their sporulation characteristics.
4. To evaluate the effectiveness of the atoxigenic strains in inhibiting growth and sporulation of aflatoxigenic strains in culture.

### 1.4 Hypotheses

1. Strains of *Aspergillus* species from grain and soil samples from the five different maize growing regions in Kenya are not significantly different in diversity.
2. There are no atoxigenic and aflatoxigenic strains of *Aspergillus* species in maize and soil samples from the selected regions.
3. The atoxigenic and aflatoxigenic strains have no significant differences in their sporulation characteristics.
4. The identified atoxigenic strains are not effective in inhibiting growth and sporulation of the aflatoxigenic strains.

### 1.5 Justification

Prevention of aflatoxin production in maize, which is a major staple food for Kenyans, is an urgent task and there is need to design strategies for the reduction or elimination of the aflatoxin producing organisms. Knowledge about the fungal sources of aflatoxins is necessary. Hence the need to identify aflatoxin producing moulds in maize and their control. To avoid the loss of lives due to acute aflatoxicosis outbreaks, there is need to improve food and health security through postharvest maize protection. One way of achieving this is by screening, evaluating and use of inhibitory bioagents occurring in the region. Atoxigenic *A. flavus* strain BN030D has been evaluated in Benin and was found to considerably reduce aflatoxin contamination in maize in several *in vitro* tests. Similarly atoxigenic *A. flavus* strains have been used to reduce aflatoxin contamination in cottonseed in the USA. Therefore, there is need of obtaining atoxigenic *Aspergillus* spp. strains from the region in order to control the high incidences of aflatoxigenic moulds and aflatoxin contamination in maize and maize products. The use of atoxigenic strains in maize preservation would provide an environmentally friendly and affordable technology that ensures maize is free from aflatoxigenic moulds and aflatoxins.

# CHAPTER TWO
# LITERATURE REVIEW

## 2.1 History of aflatoxin poisoning

The existence of mycotoxins was never documented until 1960 (Christensen, 1975). However, just as in the case of diseases, the concept that mouldy food could lead to illness in people or domestic animals was long suspected before their existence was demonstrated by science. Before 1900, in Italy, researchers there believed consumption of mouldy maize by children led to the development of illness. Some experiments done at that time, included the isolation and growth of the suspected fungus in pure culture, and isolation of toxic compounds from the fungus that the researchers believed to be the cause of the illness (Christensen, 1975). Nevertheless, it appeared that there was a correlation between the illness and consumption of mouldy maize, but this did not eliminate the possibility that it was the fungus itself that caused the disease, which most people believed to be the case. It was also possible that there were other reasons for the illnesses that were observed. Burnside *et al.* (1957) studied an extensive outbreak of mouldy maize disease in the southeastern United States in the early 1950's where hundreds of wild pigs foraging in cultivated maize fields became ill and many died. Teams of veterinarians and mycologists collaborated to determine the cause of the deaths of these pigs. They isolated a number of different fungi from the mouldy maize and inoculated each fungus on moist maize that had been sterilized and then fed them to pigs. The consumption of maize inoculated with *A. flavus* caused outward signs and inward lesions found in other cases of the so called mouldy maize disease. However, since there was no toxin isolated, there was little attention paid to the article since it still seemed like old news, i.e. domestic animals poisoned by eating mouldy maize (Matossian, 1989).

It was not until 1960, when approximately 100,000 turkeys and a lesser number of other domestic birds died in England, causing losses of approximately several hundred thousand dollars, before the first mycotoxin was isolated and identified (Christensen, 1975). Initially, the disease was thought to be caused by a virus and the syndrome was named "turkey-X disease". The "X" here indicated that the cause of the disease was unknown. However, with a great deal of detective work, on the parts of the researchers, soon the cause of the disease was

traced to the feed that was produced by Oil Cake Mills Ltd (Matossian, 1989). The oil cake feed was composed mostly of groundnut. However, it seemed unlikely that the groundnut meal itself was toxic, since groundnut meal had long been used as a feed ingredient and was known to be an excellent source of protein. Thus, it was reasoned that something must have been added to the groundnut meal to make it toxic, and one possibility that was investigated was that groundnuts had been made toxic by fungi growing in them. From their isolations, the investigators identified *A. flavus*, the same fungus that was isolated by Burnside and his research team (Matossian, 1989). The isolated fungus was again inoculated into the feed and fed to the turkeys. Shortly after feeding, the turkeys died with external signs and internal lesions identical to those observed in the birds that had previously died in the field (Matossian, 1989).

Unlike Burnside *et al.* (1957), chemists were also employed in this investigation, and they were able to isolate and identify the toxin from the oil cake feed. The mycotoxin isolated was named aflatoxin, the "a" from *Aspergillus* and "fla" from *flavus* (Matossian, 1989). Feeding test of food containing aflatoxin, with various laboratory animals, demonstrated that to varying degrees, all animals tested were sensitive to aflatoxin. Even consumption of extremely small amounts of aflatoxin damaged various internal organs and could induce development of cancer to the liver (Wild and Turner, 2002). This was of great concern among the nutritionists and those concerned with problems of public health, including The Food and Drug Administration (FDA). There was great concern domestically since groundnuts and groundnut products were of economic importance. It was also of international significance since groundnuts at that time were being lauded as an excellent source of protein for developing countries by United Nations International Children's Emergency Fund (UNICEF) and other such organizations (Hunter, 1989).

**2.2 Biology of aflatoxigenic fungi, associated aflatoxins and their economic implications**

Moulds generally derive energy from the organic matter in which they live (Ryan and Ray, 2004). Typically, aflatoxigenic moulds secrete hydrolytic enzymes, mainly from the hyphal tips. These enzymes degrade complex biopolymers such as starch, cellulose and lignin into simpler substances which can be absorbed by the hyphae (Kung'u, 2005). Most *A. flavus* and *A. parasiticus* can produce polypeptide-derived secondary metabolites called aflatoxins, which are highly toxic, mutagenic and carcinogenic to animals (Wild, 2007). Many aflatoxigenic moulds

secrete aflatoxins and other mycotoxins which, together with hydrolytic enzymes, inhibit the growth of competing microorganisms. Although aflatoxigenic moulds grow on dead organic matter, soil and grains, their presence is only visible to the unaided eye when mould colonies form (Shephard, 2003). Most fungi producing aflatoxins are aerobic and are found almost everywhere in varying quantities. They consume organic matter wherever humidity and temperature are ideal (Wild, 2007). In artificial environments like buildings, humidity and temperature are often stable enough to favour the growth of aflatoxigenic mould colonies, commonly seen as a downy or furry coating growing on food or other surfaces. Aflatoxigenic moulds are ubiquitous in nature, and their spores are a common component of grain stores and workplace dust (Kaaya *et al.*, 2006). However, when mould spores are present in large quantities, they can present a health hazard to humans, potentially causing allergic reactions and respiratory problems. Aflatoxins, when in high doses, can be detrimental to human and animal health. Exposure to high levels of mycotoxins can lead to neurological problems and in some cases death. Prolonged exposure, such as daily workplace exposure, can be particularly harmful (FEMA, 2005).

Aflatoxin is one of the mycotoxins produced by some strains of *A. flavus*, *A. nomius* and *A. parasiticus*. *Aspergillus* spp. belongs to the division *Ascomycota. Aspergillus flavus* and the closely related subspecies *parasiticus* have a world-wide distribution and normally occur as saprophytes in soil and on many kinds of decaying organic matter. These fungi readily grow on several important crops such as maize, cottonseed, groundnuts, and tree nuts. Although some *A. flavus* cause disease or food spoilage, atoxigenic isolates defined as isolates that do not produce any aflatoxin have been identified as biological control agents directed at competing with and displacing aflatoxin producers (Atehnkeng *et al*., 2008a). *Aspergillus flavus* is commonly found in soil and crop debris, which acts as the principal source of primary inoculum for infecting maize (Horn, 2007). Isolates of *A. flavus* vary greatly in aflatoxin production, with some producing copious amounts and others none (Dorner, 2004). Aflatoxigenic moulds cause massive losses to the economy every year and in addition, they cause adverse effects on human and animal health (Wild, 2007). Postharvest diseases caused by aflatoxigenic moulds destroy 10 to 30% of the total yields of maize and in some perishable crops, especially in developing

countries, they destroy more than 30% of the crop yields (Horn, 2007). As moulds grow they contaminate both maize products (Plate 2.1) and air. They produce enzymes that break down the food resulting to spoilage (Kung'u, 2005).

**Plate 2.1:** *Aspergillus flavus* growth on damaged maize kernels.
Source: http://www.*aspergillusflavus*.org/*aflavus*/

The presence of aflatoxins in human foods can cause acute and chronic health effects (aflatoxicoses) including immune-system suppression, growth retardation, cancer and death (Wild and Turner, 2002). Aflatoxins are carcinogens and genotoxins that directly influence the structure of DNA (Williams *et al.*, 2004) and as a result, occurrence of aflatoxins in human foods is strictly regulated to very low concentrations in developed countries. Indeed, in developed countries the exposure of domestic animals, even pets, is of both regulatory and economic concern. Deaths of pets due to aflatoxins in U.S.A pet foods have had international economic impact in terms of both trade and litigation (Anonymous, 2006). Thus, in developed countries, the drive to abate aflatoxin contamination is due to loss in crop value resulting from stringent government regulations on maximum permissible levels in crops and crop related products used as foods or feeds.

In crops intended for human consumption, maximum permitted aflatoxin levels range from 2 ng/g in the European Union to 20 ng/g in the United States (van Egmond, 2004). Aflatoxins are

readily transferred from feed to milk resulting in similarly stringent regulations on feed intended for dairies (van Egmond, 2004). Maximum permissible levels of aflatoxins in milk are 0.05 ng/g in the European Union and 0.5 ng/g in the United States of America (van Egmond, 2004). In Kenya, the maximum allowable level of aflatoxins in maize that is intended for human consumption is 10 ppb. This is similar to that allowed by the World Food Programme (Aflacontrol, 2010). The requisite destruction of highly contaminated agricultural products combined with reduced value for products with lower contamination levels makes aflatoxin economically expensive in developed countries. Acute aflatoxin poisoning is caused by ingestion of high levels of the toxin. Immediate consequences are severe liver damage, acute jaundice and hepatitis, which subsequently may result in death (Shephard, 2003).

Although on a global basis deaths from acute aflatoxin poisoning are rare, Kenya has experienced such episodes repeatedly for at least 25 years (Anonymous, 2004). In 2004, 317 cases of acute aflatoxicosis were reported, resulting in 125 deaths, thus a case fatality rate of 39%, with additional cases probably unreported (Azziz-Baumgartner *et al.*, 2005). This epidemic was caused by ingestion of maize with aflatoxin concentrations of up to 4,400 ng/g. Unfortunately in Kenya and other African countries, crops from small scale farmers frequently pass from field to storage to consumption with no regulatory oversight and without a test of the extent of aflatoxin contamination (Wilson and Payne, 1994). Aflatoxin contamination often is mysterious to farmers because the extent of contamination is not readily evident and because it appears unrelated to crop yield or quality. Indeed, complex and expensive sampling and analyses often are required to estimate the extent of contamination (Whitaker and Johansson, 2005). The fungi that produce aflatoxins grow best under warm conditions and therefore, aflatoxins are of greatest concern in warm agricultural production areas especially during dry periods (Cotty, 1994). Such areas of high vulnerability are common in Kenya and other parts of Africa where subsistence farmers frequently rely on contaminated maize and groundnuts as life sustaining staples (Egal *et al*., 2005). Aflatoxin contamination varies in most areas and crops (Wilson and Payne, 1994). This variation has been attributed to climatic factors, especially drought and high temperature with increased contamination being associated with reduced rainfall (Widstrom, 1996).

Among 18 different types of aflatoxins identified, major members are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) (Luciana and Eugenia, 2001). Aflatoxin B1 is normally predominant in amount in cultures as well as in food products. Pure AFB1 is pale-white to yellow crystalline, odourless solid. Aflatoxins are soluble in methanol, chloroform, acetone and acetonitrile. *Aspergillus flavus* typically produces AFB1 and AFB2, whereas *A. parasiticus* produce AFG1 and AFG2 as well as AFB1 and AFB2. Four other aflatoxins M1, M2, B2A, and G2A which may be produced in minor amounts have been subsequently isolated from cultures of *A. flavus* and *A. parasiticus*. A number of closely related compounds namely aflatoxin GM1, parasiticol and aflatoxicol are also produced by *A. flavus*. Aflatoxin M1 and M2 are major metabolites of aflatoxin B1 and B2 respectively, found in milk of animals that have consumed feed contaminated with aflatoxins (Martins *et al*., 2001). Aflatoxin B1 is one of the most potent hepato-carcinogens known and hence levels of aflatoxins in the diet are an important consideration for human health (Yin *et al.*, 2008).

**2.3 Control of aflatoxigenic moulds**

One of the available methods of controlling moulds is the use of synthetic chemical preservatives which has led to appearance of resistant micro-organisms, leading to occurrences of emerging food borne diseases (Cotty, 2001). Other methods such as use of solar driers are expensive for small scale farmers due to the high costs of purchasing the equipment. Proper drying of maize denies the aflatoxigenic moulds the favourable conditions for their growth, sporulation and aflatoxin production (Kaaya *et al.,* 2006). To avoid the loss of lives due to aflatoxicoses outbreaks, there is need to improve food and health security through post-harvest food protection. This can be achieved by screening and evaluating alternative antimicrobial agents from micro-organisms based sources such as atoxigenic strains of *A. flavus* and *A. parasiticus.* Prevention or management of aflatoxin contamination may be directed at both the process of contamination and the fungi causing contamination. The contamination process can be divided into two phases based on crop maturity (Cotty, 2001). The first phase occurs during crop development and is generally associated with physical damage to the crop typically by either physiological stress or insect activity (Russell, 1982). Crop components contaminated during the first phase often fluoresce bright green-yellow as a result of kojic acid production in crop tissue by the aflatoxin producing fungi (Zeringue *et al.*, 1999).

After maturation, the crops remain vulnerable to contamination, providing a window during which a second phase of contamination may occur (Bock and Cotty, 1999). Exposure of the mature crop to both high humidity and temperatures conducive to aflatoxin producing fungi can result in both new crop infections and increase in the aflatoxin content of crop components already infected (Russell *et al.,* 1976). The second phase may occur prior to harvest in the field or after harvest during transportation, storage, or at any point until the crop is consumed. Hot dry conditions during crop development favour the first phase of contamination, whereas rain and high humidity with warm temperatures after crop maturation favour the second phase. Reliable management practices must address both phases. Improving the resistance of cultivars to contamination is one method of simultaneously addressing both phases of contamination. Although proper cultivar selection and crop management can limit vulnerability to both phases, environmental changes can affect even the best management practices and result in a highly contaminated crop (Cotty, 2001).

When management procedures fail to prevent accumulation of unacceptable levels of aflatoxins, there are still options for the utilization of the contaminated crops. These options include detoxification (Bailey *et al.,* 1994). Chemical detoxification is a viable option for even very highly contaminated crops, with ammoniation being the detoxification method currently in widest use. Ammoniation inactivates aflatoxins by hydrolysis of the lactone ring, which is followed by further breakdown. Ammoniation has been used in North America, Europe, and Africa on crops including maize, cottonseed, and peanut meal (Bailey *et al.*, 1994). Following detoxification by ammoniation, the treated crop products are nutritionally valuable for domestic animals, but are not suitable for human consumption. However, the detoxification procedures are expensive and beyond the reach of the larger population (Bailey *et al.*, 1994).

### 2.4 Biological control of aflatoxigenic *Aspergillus* strains

In the U.S.A, biological control has been used to reduce aflatoxin contamination in various crops such as cotton (Cotty, 1994), groundnut, and maize (Dorner *et al*., 1998). This technique involves the application of atoxigenic biological control strain of *A. flavus* or *A. parasiticus* to soil resulting in a high population density that allows the biological control strain to effectively compete with the native aflatoxigenic strains during invasion under conditions favourable for

aflatoxin contamination. Invasion of a seed in soil as in the case of groundnut solely by the biological control agent would be expected because of its high density relative to the wild type strain in the soil (Dorner *et al*., 1998). This would result in reduced aflatoxin contamination. For maize and cottonseed, the high population of the atoxigenic biological control strain in soil produces abundant spores on the soil surface that become airborne to infect grains and seeds (Ranajit *et al*., 2005).

Biological control using the competitive exclusion mechanism has been successfully implemented on cottonseed in Arizona (Ranajit *et al*., 2005). Natural population of *A. flavus* consists of aflatoxigenic strains that produce copious amount of aflatoxin and atoxigenic strains that lack the capacity to produce aflatoxin. In the competitive exclusion mechanism, introduced atoxigenic strains outcompete and exclude aflatoxigenic strains from colonizing grains thereby reducing aflatoxin production in contaminated grains (Ranajit *et al*., 2005). The potential to reduce aflatoxin contamination in maize using the biological control strategies mentioned above has been evaluated in Benin, where 90% of the *Aspergillus* species are *A. flavus*. The atoxigenic strains of *A. flavus* (BN22 from Benin and AF36 from the USA) were tested against aflatoxigenic strains of *A. flavus* (BN40 from Benin and AF13 from the USA) and *A. parasiticus* (BN48) *in vitro* (Cotty, 2001). For these *in vitro* trials, maize kernels were dipped in $1\times10^6$ conidia $ml^{-1}$ suspension of one *Aspergillus* species or strain and allowed to dry before repeating the dip with either the same or another strain. Maize kernels were incubated for 5 days at 30 $^oC$ in a saturated environment, and then dried for 5 days at a temperature of 40 $^oC$. The dried maize was crushed, aflatoxins extracted in acetone and quantification done using thin-layer chromatography (TLC) and a scanning densitometer. All atoxigenic isolates significantly reduced toxin production by the African *A. parasiticus* isolate BN48. The American atoxigenic isolate AF36 was effective against the American aflatoxigenic isolate AF13, but not the aflatoxigenic African S-strain, BN40 suggesting that there may be specificity of action of some atoxigenic strains. The African atoxigenic L-strain BN30 was the only isolate that reduced toxin production by the aflatoxigenic African S-strain, BN40. Further field tests confirmed the potential of atoxigenic strains to reduce aflatoxin production by aflatoxigenic strains (Ranajit *et al*., 2005). Biological control holds promise of offering a long-term solution for reducing

aflatoxin contamination in maize.

# CHAPTER THREE
# MATERIALS AND METHODS

## 3.1 Source of maize and soil samples

A total of 113 maize and 113 soil samples obtained randomly from maize farms and rural households in Makueni, Nyeri Central, Bungoma south, Moiben and Ugunja districts were used in this study (Figure 3.1). Makueni, Nyeri and Siaya counties were selected due to the fact that aflatoxin poisoning cases have been previously reported in those counties, while Bungoma County lies on a transit route where exchange of maize from other countries such as Uganda is possible. Uasin Gishu County was selected due to the fact that there is high maize production in the county every year. Most of these areas are in mid-altitude agro ecological zones with warm and humid conditions such as Ugunja while Nyeri Central and Moiben districts have high relative humidity thus mould invasion is primarily due to inadequate drying and improper storage (Pitt, 2000). These factors favour development of moulds and production of mycotoxins (Kaaya *et al.,* 2006). These areas have unpredictable rainfall patterns making it difficult for small scale farmers to efficiently dry their produce. In Nyeri Central, Bungoma south, Moiben and Ugunja districts 20 maize samples (500g each) and 20 soil samples (100g each) each were collected. However, in Makueni district 33 maize samples (500g each) and 33 soil samples (100g each) were collected. This higher number of samples collected from Makueni district was attributed to the fact that the district had several reported cases of aflatoxin poisoning. Each district apart from Makueni was divided into four sub-regions and five maize and five soil samples each were collected randomly from farmers in those sub-regions. Makueni district had seven sub-regions and five maize and soil samples each were collected in all but one where three maize and three soil samples each were collected due to scarcity of farmers with available maize reserves. Soil samples were collected from floors of maize stores, maize farms and the bare ground in the homestead where maize is usually dried. The samples were collected in properly labeled khaki paper bags to minimize saprophytic fungal contamination and transported in a cool box to the laboratory and stored at 4 $^{o}$C until further analysis.

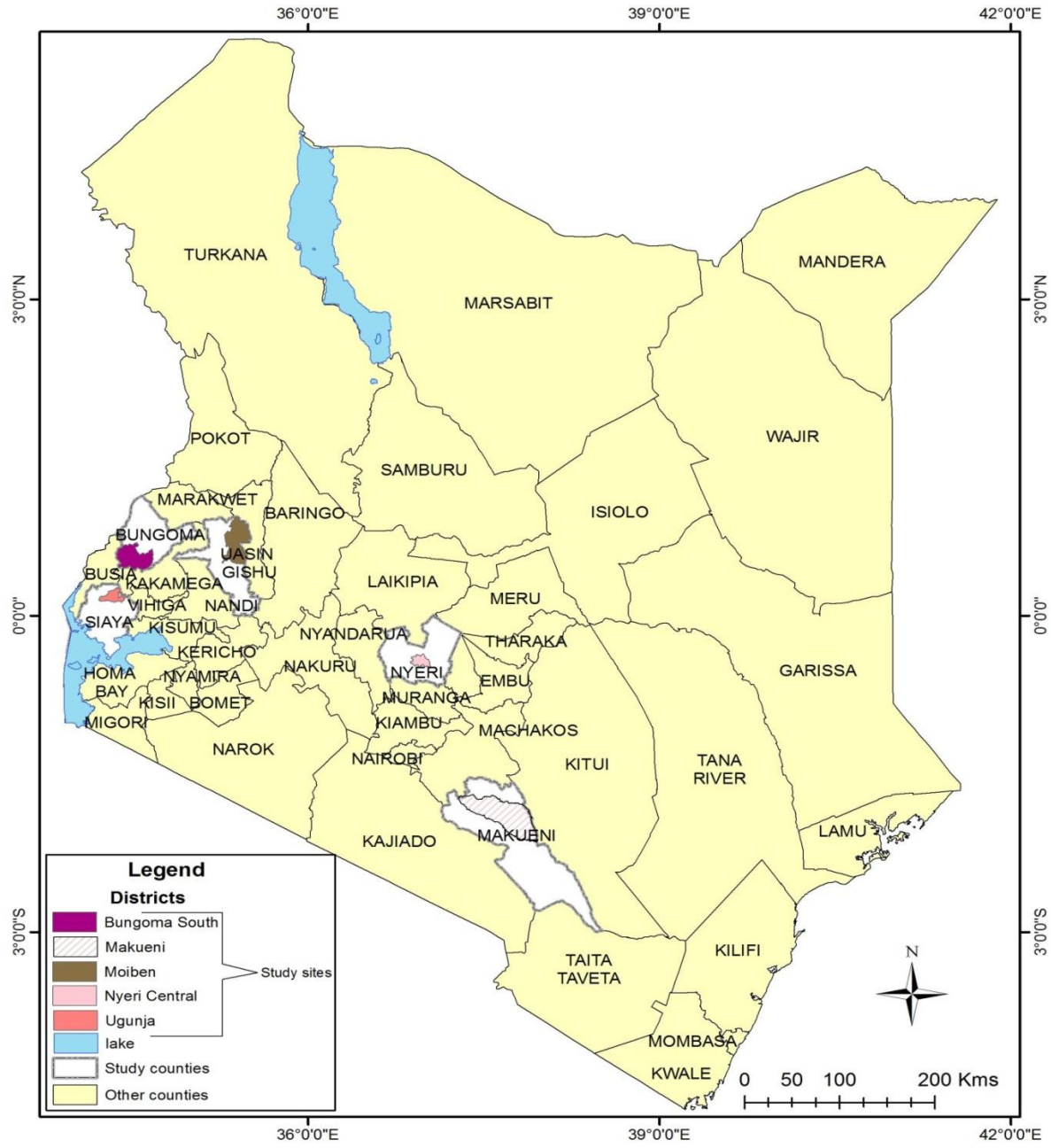



**Figure 3.1:** Map of the study area

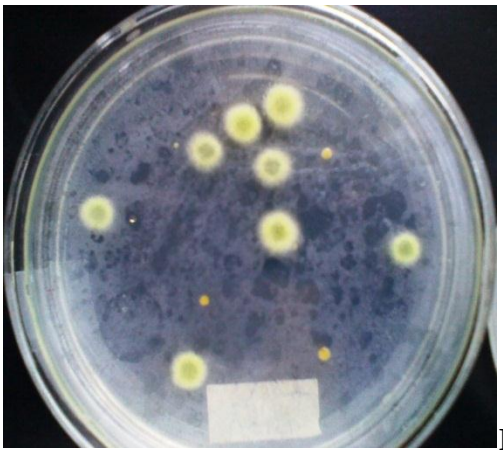
Map source: Cartographer, Department of Environmental Science, Egerton University (2013).

KEY: Weather data during the sampling period (August 2011): Bungoma (Mean Temp: 20 $^{o}$C, RH: 68-76%), Makueni (Mean Temp: 22.5 $^{o}$C, RH: 48-57%), Uasin Gishu (Moiben) Mean Temp: 17.5 $^{o}$C, RH: 75-80%), Nyeri (Mean Temp: 16 $^{o}$C, RH: 64-83%) and Siaya County (Mean Temp: 23.5 $^{o}$C, RH: 53-60%). Source of weather data: Kenya meteorology website.

### 3.2 Isolation of *Aspergillus* species from maize and soil

The maize grains were surface sterilized in 2% sodium hypochlorite for one minute and rinsed three times with sterile distilled water. A total of 20 grains were randomly picked per sample and plated (five per plate) on Czapek Dox Agar (CZ) medium amended with 50 mg of streptomycin and 50 mg of penicillin. The soil samples were first serially diluted before plating. One gram of the soil sample was suspended in 9 ml sterile distilled water and serially diluted to $10^{-4}$. One millilitre of the $10^{-3}$ and $10^{-4}$ dilutions were plated in the CZ medium amended with 50 mg of streptomycin and 50 mg of penicillin. The plates were then incubated at 28 $^{o}$C for 7 days and the number of kernels showing growth of *Aspergillus* species in each Petri dish was counted (Plate 3.2a) while for the dilution plates the number of *Aspergillus* colonies per plate was counted (Plate 3.2b). *Aspergillus* colonies were sub-cultured on Potato Dextrose Agar (PDA) and incubated at 28 $^{o}$C for 7 days. Treatments were replicated four times and the experiment was done in a complete randomized design.

**A**



**B**

**Plate 3.2:** (a) *Aspergillus* species growth on maize kernels cultured on CZ media. (b) *Aspergillus* species colonies growth from soil serial dilutions cultured on CZ media.

### 3.3 Identification and characterization of *Aspergillus* isolates

The resulting *Aspergillus* cultures were identified to species level based on cultural and morphological characteristics like colony diameter, colony colour on agar and reverse, colony texture and zonation (Rodrigues *et al*., 2007). Morphological features were studied under the microscope and the major and remarkable microscopic features that were considered were conidiophores, conidial shape, phialides and metulae, presence and shape of vesicles (Larone,

1995). Contemporary diagnosis of the *Aspergillus* species was based on the descriptions (Appendix 1) and keys by Klich (2002).

### 3.4 Screening for atoxigenic and aflatoxigenic strains

Yellow pigment formation in medium (PDA) was recorded after 7 days of growth at 28 ºC. Five-day-old yeast extract-sucrose (10 g yeast extract powder, 10 g sucrose and 15 g of agar in one litre of sterile distilled water) cultures were exposed to 27% ammonium hydroxide (Sigma, St. Louis, Mo.) for 30 minutes, and colour changes on the undersides of the cultures after exposure to ammonia vapour was recorded. Ammonia vapour can detect aflatoxin production by changing the colour of aflatoxigenic colonies from yellow to pink upon exposure (Saito and Machida, 1999). For this method a single colony was grown in the center of a Petri dish containing yeast extract-sucrose medium. The dish was inverted and 2 drops of concentrated ammonium hydroxide solution was placed on the inside of the lid. The undersides of aflatoxin-producing colonies quickly turned plum-red after the bottom of the Petri dish had been inverted over the lid containing the ammonium hydroxide. Essentially no colour change occurred on the undersides of colonies that were not producing aflatoxins.

### 3.5 Characterization of the atoxigenic and aflatoxigenic strains based on their sporulation and colony characteristics

The atoxigenic and aflatoxigenic *Aspergillus* strains were characterized based on their sporulation and colony characteristics. The sporulation and colony characteristics were compared in two different types of media; PDA and CZ to assess the effect of media type on growth and sporulation of the atoxigenic and aflatoxigenic strains. Sporulation characteristic studied in the two media types was the rate of sporulation classified as poor, moderate or heavy sporulation. The major colony characteristics that were studied and compared in the two different media types included; colony diameter, colony character (texture, colour and reverse colour) and zonation.

### 3.6 Selection of atoxigenic strains for evaluation as bioagents

A set of atoxigenic strains was selected from each of the five districts based on their sporulation characteristics for further evaluation as bioagents. Atoxigenic strains with heavy sporulation, and large colony diameters were selected and evaluated as bioagents.

### 3.7 Evaluation of the effectiveness of atoxigenic strains in inhibiting growth and sporulation of aflatoxigenic fungi

Atoxigenic strains were tested for their antimould activity on aflatoxigenic *Aspergillus* strains. The paper disc diffusion inhibition test was used for the screening of antimould activity of atoxigenic *Aspergillus* strains as described by Souza *et al*. (2005). Aflatoxigenic strain suspension of 0.2 ml with approximately $10^6$ spores was uniformly spread on sterile czapek dox agar in a petri dish. Sterile filter paper discs (Whatman No.1, 6mm in diameter) were soaked with 0.02 ml suspension with approximately $10^3$ spores of the atoxigenic *Aspergillus* strain and placed at the centre of the inoculated culture plates. The lower concentration of the atoxigenic strains was used in order to assess the competitiveness in growth of the atoxigenic strains against the aflatoxigenic strains. The plates were incubated at 28 $^oC$ for 7 days. At the end of the incubation period, the diameter of the growth inhibition zone was measured in mm and recorded. Nystatin with a concentration of 100 µg was used as the reference standard and as the positive control for the moulds. Sterile filter paper disk (Whatman No.1, 6mm in diameter) soaked with 0.02 ml sterile distilled water was used as a negative control for the moulds. The experiment was laid down in a completely randomized design with three replicates. The most effective atoxigenic strains were selected based on the size of zone of inhibition.

### 3.8 Data analysis

SAS version 9.0 was used in data analysis and mean comparisons done using Least significant difference (LSD) at P=0.05. One way analysis of variance (ANOVA) was used to test whether the *Aspergillus* strains obtained from the five districts were significantly different from each other based on the type and number of aflatoxigenic and atoxigenic strains found in each district. LSD was used to determine which maize and from which districts were highly contaminated with *Aspergillus* spp. ANOVA was used to test the differences among the atoxigenic strains to ascertain which one had the greatest effect in inhibiting growth and sporulation of the aflatoxigenic strains. Separation of means for antimould activity was done using LSD and the most effective atoxigenic *Aspergillus* strains were selected based on the size of inhibition zones.

# CHAPTER FOUR
# RESULTS AND DISCUSSION

## 4.1 *Aspergillus* isolates obtained from maize and soil and their incidences across the districts

A total of 174 *Aspergillus* isolates were obtained from the five districts from which samples were taken. Out of these, 144 (82.3%) were isolated from maize samples while 30 (17.7%) were from soil samples (Table 4.1). Makueni district had the highest number of *Aspergillus* isolates at 101 (58.1%), Nyeri Central 22 (12.6%), Moiben 18 (10.3%), Ugunja 18 (10.3%) and Bungoma South 15 (8.6%). The ratio of maize to soil *Aspergillus* isolates in each district was as follows: Makueni 99:2 (98.0%: 2.0%), Nyeri Central 17:5 (77.3%: 22.7%), Moiben 7:11 (38.9%: 61.1%), Ugunja 14:4 (77.8%: 22.2%) and Bungoma South 7:8 (46.7%: 53.3%). In Makueni, Nyeri Central and Ugunja districts, there was a higher number of maize isolates than soil isolates, while in Moiben and Bungoma South districts there was slightly more soil isolates than maize isolates. The high number of isolates in maize than in soil may be due to on-farm maize grain processing as this has been reported to be a common practice among the farmers, especially of eastern Kenya (Makueni) (Strosnider *et al*., 2006). Results obtained by Muthomi *et al.* (2012) showed that maize and maize products sampled at farm level had a higher risk of contamination by *Aspergillus* spp. and aflatoxins. These results show that maize and soils across all the five districts are highly contaminated with fungi of the genus *Aspergillus*. The frequency of the *Aspergillus* isolates across all the five districts analyzed through ANOVA, was not significantly different (P= 0.5489). This was expected because the ubiquitous nature of these *Aspergillus* spp. enables them to grow on dead organic matter everywhere in nature, their presence is only visible to the unaided eye when mould colonies form and they derive energy from the organic matter in which they live (Ryan and Ray, 2004).

**Table 4.1:** Frequency of *Aspergillus* isolates from maize and soil samples across the five districts

| | Districts | | | | | | |
|---|---|---|---|---|---|---|---|
| | Makueni | Nyeri Central | Moiben | Ugunja | Bungoma South | **Total** | **% Total** |
| **Source** | | | | | | | |
| Maize isolates | 99 | 17 | 7 | 14 | 7 | **144** | **82.29** |
| Soil isolates | 2 | 5 | 11 | 4 | 8 | **30** | **17.71** |
| **Total** | **101** | **22** | **18** | **18** | **15** | **174** | **100** |
| **% Total** | **58.1** | **12.6** | **10.3** | **10.4** | **8.6** | **100** | |

The high occurrence of *Aspergillus* spp. in the maize and soil samples in the various districts can be attributed to factors such as warmth and humidity in the LVB region (Ugunja), high relative humidity with low temperatures in Nyeri Central, Bungoma South and Moiben districts leading to improper drying of the maize and high temperatures with drier conditions in Makueni district. These conditions predispose the maize to moulds at the pre-harvest stage in the field and post-harvest stage in storage (Okoth *et al.,* 2012). The fungi form sclerotia that allows for saprophytic survival for extended periods in the soil, maize residue and maize-cobs (Wagacha and Muthomi, 2008). The propagules in the soil and crop debris act as the primary source of contamination, infecting maturing maize crops (Atehnkeng *et al.,* 2008b).

These results were anticipated in the Lake Basin Region (Ugunja), because of the relatively high temperature and relative humidity which provided optimum growth conditions for the *Aspergillus* spp. (Anonymous, 2004). Higher numbers of *Aspergillus* spp. isolates were recorded in grain samples from the semi-arid Makueni district than those from the humid regions in Moiben, Nyeri Central, Bungoma South and Ugunja. These results are in agreement with the findings of Muthomi *et al.* (2012) where higher *Aspergillus* spp. isolation frequencies were recorded in grain samples from the semi-arid eastern region than those from the humid North Rift regions. Mechanical damage during and after harvest, facilitates entry of the fungal spores either in maize cobs or grains (Pitt, 2000). This could explain why very high quantities of the *Aspergillus* spp. were isolated from the maize samples because some of them had damaged grains that might have predisposed the grains to the fungal infection.

### 4.2 Morphological, cultural and microscopic characterization of the *Aspergillus* species

Morphological, cultural and microscopic features of the isolates were studied and recorded as shown in Table 4.2 and the 10 different *Aspergillus* spp. characterized are shown in plate 4.3. Most of the isolates of the same species, despite originating from different districts, showed similarities in their morphological and cultural characteristics in PDA media. *Aspergillus flavus* strains EM324, EM244, EM182 and EM1112 from Makueni; NM091, NM083 and NM084 from Nyeri Central; BM071 and BS116 from Bungoma South UM127 from Ugunja, and RS013, RM023-2 from Moiben, all had similar surface colour of olive green with whitish margins and reverse colour of creamish to yellow on PDA. Similarly, there were only slight variations in their colony diameters which ranged between 37 – 42 mm. For *A. glaucus*, strains RS024 from Moiben and EM211 from Makueni also had less variable colony colour of green with yellow areas and reverse colour of creamish yellow, and the colony diameter ranged between 26 – 29 mm.

*Aspergillus parasiticus* strain UM082 showed characteristics similar to *A. flavus* strains apart from the colony colour which was conifer green (Plate 4.3 (3a and 3b)). *Aspergillus versicolor* strain BS203 from Bungoma South had a colony diameter of 17.0 mm which differed with other *A. versicolor* strains RS016 and RS162 from Moiben which had colony diameters ranging from 32 – 34 mm. *Aspergillus clavatus* strains were from Moiben district and all were similar in their morphological characteristics. *Aspergillus sydowii* strains from Makueni, Nyeri Central and Ugunja were not different from each other in their morphological characteristics. *Aspergillus fumigatus* from Makueni, Nyeri Central and Bungoma South districts also did not show major differences in their morphological characteristics. *Aspergillus nidulans* strains from Makueni and Nyeri Central were not different from each other morphologically. *Aspergillus wentii* and *A. candidus* occurred singly from Bungoma South and Ugunja districts respectively and had no strains to be compared with.

Identification of *A. flavus* is not an easy task due to its similarities with *A. parasiticus* and *A. nomius*. However, the other *Aspergillus* spp. are distinctly different from each other, and with the

help of the descriptions (Appendix 1) and keys by Klich (2002), it was possible to achieve a reliable identification and discrimination of the various isolates of *Aspergillus* species.

**Table 4.2:** Cultural, morphological and microscopic characterization of *Aspergillus* isolates

| Group No. | Isolate code | Colony colour on PDA | | Colony size (mm) | Conidiophore | Conidial heads | Shape of Vesicles | Seriation | Conidial shape | Isolate name |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **Conidia** | **Reverse** | | | | | | | |
| **G3** | EM324 | Olive green with whitish margin | Yellowish with grey margin | 41.3±3.1 | Short, finely roughened wall and colourless | Radiate | Subclavate | Uniseriate | Spherical | *A. flavus* |
| **G7** | NM091 | Olive green with dirty white margin | Cream centre with alternating grey and cream periphery | 35.3±5.1 | Short, smooth walled and colourless | Radiate | Subclavate | Uniseriate | Spherical | *A. flavus* |
| **G8** | NM083 | Olive green with white margin | Creamish | 34.7±0.6 | Slightly long, rough walled and colourless | Radiate | Subclavate | Biseriate | Spherical | *A. flavus* |
| **G9** | EM244 | Olive green with white margin | Cream centre with alternating grey and cream rings | 34.0±4.0 | Short, finely roughed walled and colourless | Radiate | Subclavate | Uniseriate | Spherical | *A. flavus* |
| **G13** | NM084 | Olive green with cream margin | Cream center with alternating grey and cream concentric rings | 37.7±2.5 | Short, smooth walled and colourless | Radiate | Subclavate | Uniseriate | Spherical | *A. flavus* |
| **G16** | EM182 | Olive green with | Creamish | 32.3±3.2 | Short, smooth | Columnar | Subclavate | Uniseriate | Spherical | *A. flavus* |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | whitish margin | | | walled and colourless | | | | | |
| **G17** | BM071 | Olive green with cream margin | Cream center with alternating grey and cream concentric rings | 36.7±2.3 | Short, rough walled and colourless | Columnar | Subclavate | Uniseriate | Spherical | *A. flavus* |
| **G18** | EM1112 | Olive green with whitish margin | Cream with grey margin | 35.0±1.0 | Short, smooth walled and colourless | Radiate | Globose | Uniseriate | Spherical | *A. flavus* |
| **G19** | UM127 | Olive green with whitish margin | Cream with grey margin | 28.3±1.5 | Slightly long, smooth walled and colourless | Radiate | Subclavate | Uniseriate | Spherical | *A. flavus* |
| **G21** | BS203 | Grey with a yellow at the middle | Greenish centre with creamish margin | 17.0±2.7 | Short, finely roughened wall and colourless | Columnar | Subclavate | Biseriate | Spherical | *A. versicolor* |
| **G22** | RS016 | Creamish centre with green to sulphur yellow margins | Sulphur yellow | 34.0±1.0 | Short, finely roughened wall and colourless | Radiate | Globose | Biseriate | Spherical | *A. versicolor* |
| **G31** | RS024 | Green with sulphur margin | Yellow | 26.7±2.1 | Long, smooth walled and colourless | Radiate | Subclavate | Uniseriate | Spherical | *A. glaucus* |
| **G32** | BS023 | Sulphur yellow centre with alternating grey and sulphur yellow concentric rings | Sulphur yellow | 26.0±2.0 | Short, smooth walled and colourless | Columnar | Subclavate | Biseriate | Spherical | *A. wentii* |

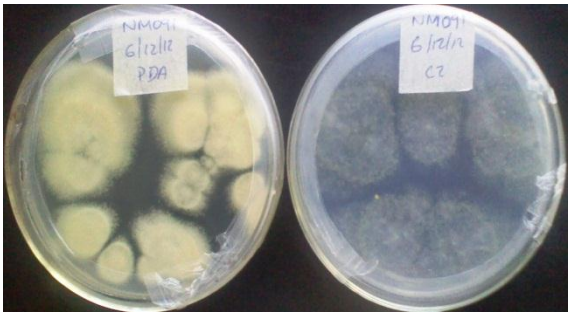
| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **G34** | RS162 | Grey centre with yellowish margin | Yellow | 32.7±1.2 | Short, smooth walled and colourless | Radiate | Globose | Biseriate | Spherical | *A. versicolor* |
| **G38** | US098 | Greyish blue with white margin | Yellow | 29.0±1.0 | Slightly long, smooth walled and colourless | Radiate | Globose | Biseriate | Spherical | *A. sydowii* |
| **G41** | RS013 | Olive green with cream margin | Yellow centre with cream margin | 36.0±2.0 | Long, smooth walled and colourless | Radiate | Globose | Uniseriate | Spherical | *A. flavus* |
| **G49** | UM082 | Conifer green with cream margin | Yellow centre with cream margin | 26.7±0.6 | Slightly long, smooth walled and colourless | Radiate | clavate | Uniseriate | Spherical | *A. Parasiticus* |
| **G50** | BM092 | Grey centre with green margin | Cream centre with grey margin | 41.3±3.1 | Slightly long, smooth walled and colourless | Radiate | Globose | Uniseriate | Spherical | *A. fumigatus* |
| **G58** | US184 | White | Deep yellow | 15.7±1.5 | Short, finely roughened wall and colourless | Radiate | Globose | Uniseriate | Spherical | *A. candidus* |
| **G64** | RM143 | Bluish green with white margin | Brown center with alternating cream and brown concentric rings | 31.7±1.5 | Short, finely roughened wall and brownish | Radiate | Subclavate | Uniseriate | Spherical | *A. clavatus* |
| **G68** | ES042 | Green | Deep red | 23.7±2.1 | Short, smooth walled and | Columnar | Subclavate | Biseriate | Spherical | *A. nidulans* |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | colourless | | | | | |
| **G71** | BS116<br><br>RM023-2 | Olive green | Creamish yellow | 36.0±2.0 | Short, smooth walled and colourless | Columnar | Subclavate | Uniseriate | Spherical | *A. flavus* |
| **G72** | EM211 | Green centre with alternating yellow and green concentric rings | Cream | 29.0±1.7 | Slightly long, smooth walled and colourless | Columnar | Subclavate | Uniseriate | Spherical | *A. glaucus* |

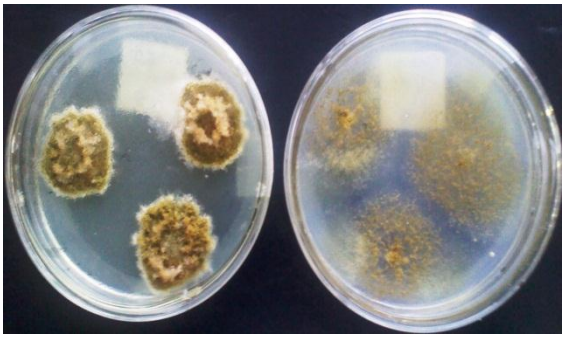
Key: E – Eastern (Makueni), R – Rift Valley (Moiben), B – Bungoma South, N – Nyeri Central, U – Ugunja.

M – Maize, S – Soil, (01-32) – Sample number in each district, (1 -12) - Isolate number from each sample, (-2) – Purification number.

**3a (i)** ***Aspergillus flavus*** **(Surface)**

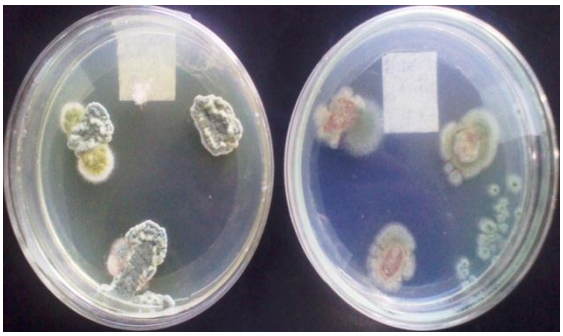


**3a (ii)** ***Aspergillus flavus*** **(Reverse)**

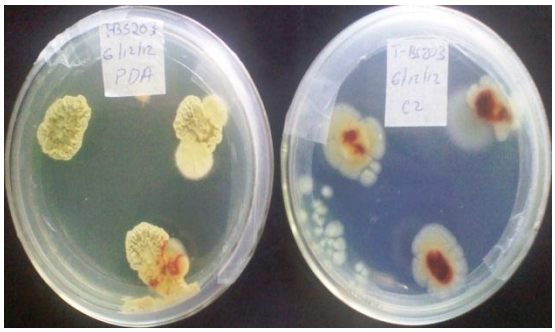


**3b (i)** ***Aspergillus parasiticus*** **(Surface)**

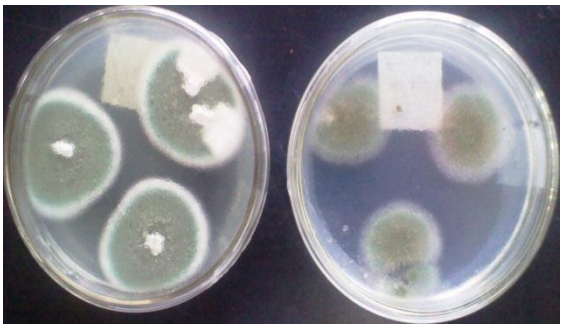
**3b (ii)** ***Aspergillus parasiticus*** **(Reverse)**



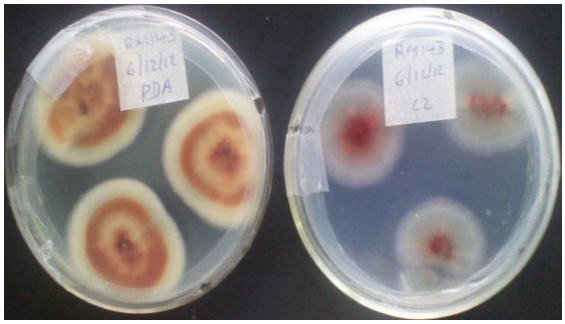
**3c (i)** ***Aspergillus versicolor*** **(Surface)**



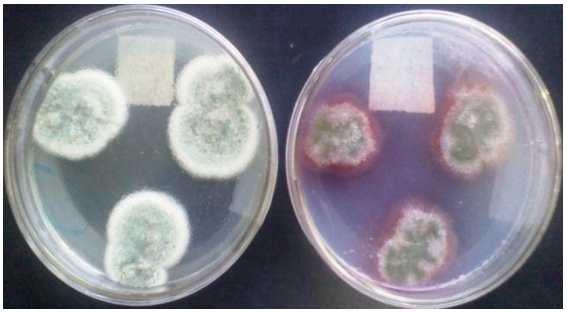
**3c (ii)** ***Aspergillus versicolor*** **(Reverse)**



**3d (i)** ***Aspergillus clavatus*** **(Surface)**



**3d (ii)** ***Aspergillus clavatus*** **(Reverse)**



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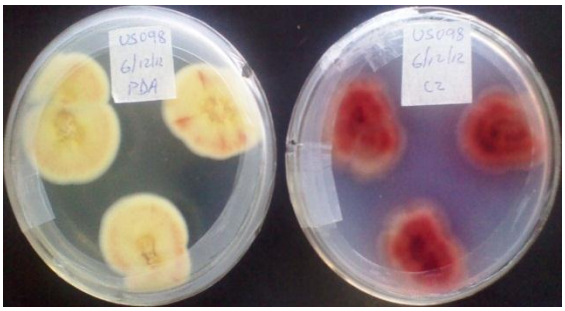
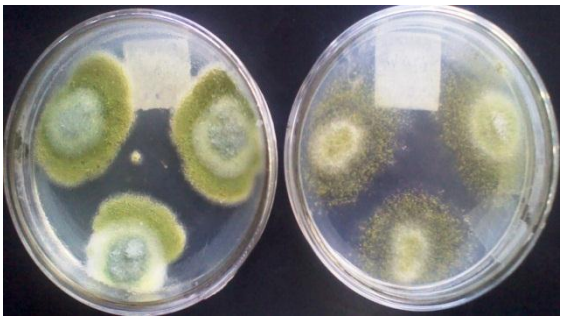
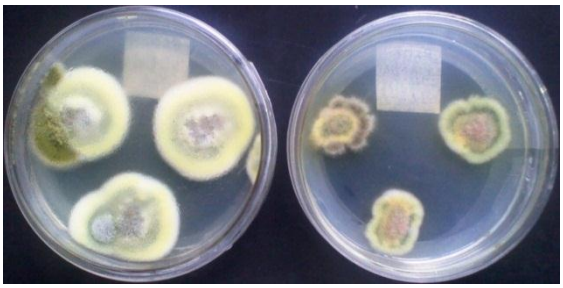


**3e (ii)** ***Aspergillus sydowii*** **(Reverse)**

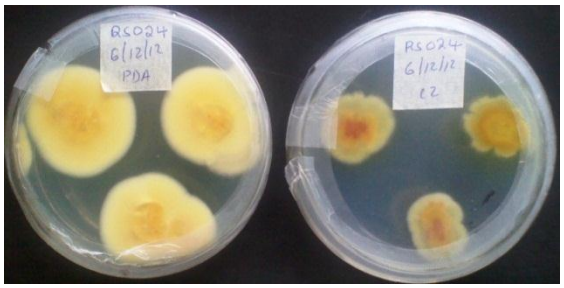


**3f (i)** ***Aspergillus fumigatus*** **(Surface)**

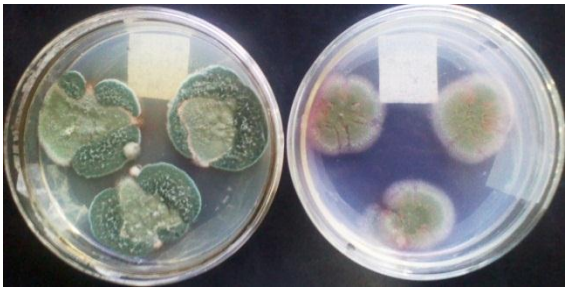
**3f (ii)** ***Aspergillus fumigatus*** **(Reverse)**



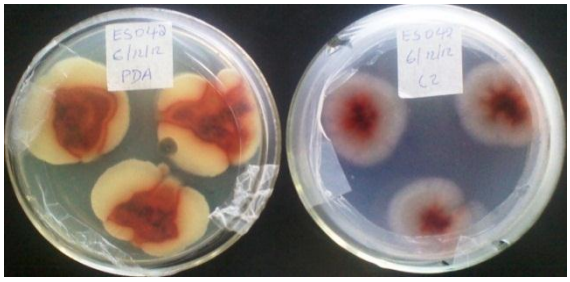
**3g (i)** ***Aspergillus glaucus*** **(Surface)**



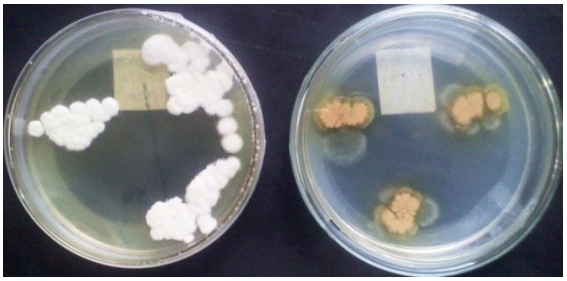
**3g (ii)** ***Aspergillus glaucus*** **(Reverse)**



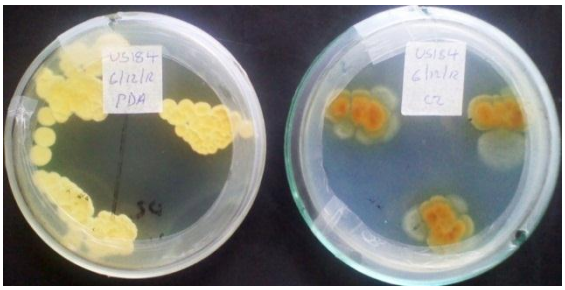
**3h (i)** ***Aspergillus nidulans*** **(Surface)**



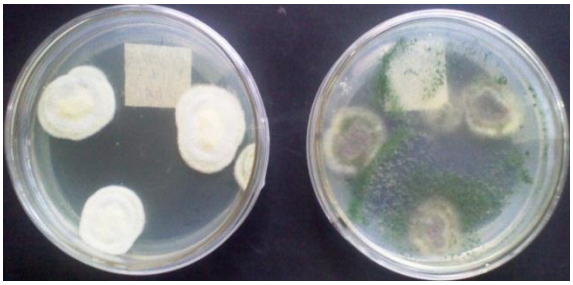
**3h (ii)** ***Aspergillus nidulans*** **(Reverse)**



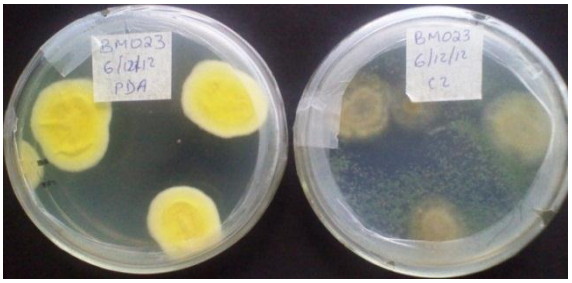
**3i (i)** ***Aspergillus candidus*** **(Surface)**



**3i (ii)** ***Aspergillus candidus*** **(Reverse)**

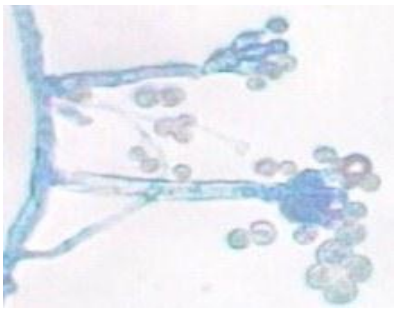


**3j (i)** ***Aspergillus wentii*** **(Surface)**



**3j (ii)** ***Aspergillus wentii*** **(Reverse)**

**Plate 4.3:** Morphological, cultural and microscopic characteristics of the 10 *Aspergillus* species growing on PDA and CZ media respectively after seven days of growth at 28 ºC.



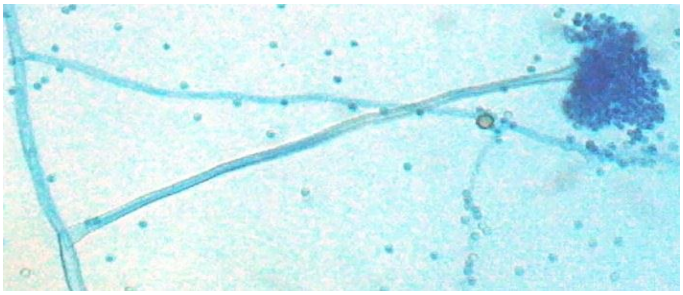


**Plate 3k (i)** **Plate 3k (ii)**

**Plate 3k (i):** Uniseriate conidial head with subclavate vesicle of *Aspergillus flavus* strain EM244 (Mg = ×400). **Plate 3k (ii):** Radiate conidial head shape attached to a long stipe of *Aspergillus parasiticus* strain UM082 (Mg = ×400).

### 4.3 Characterization of the *Aspergillus* isolates and their incidence in the five districts

The *Aspergillus* isolates were identified to species level based on cultural and morphological characteristics and their distribution pattern in each district was recorded as shown in Table 4.3. The characterization process identified 10 different *Aspergillus* spp. Out of 174 *Aspergillus* isolates, 136 (78.5 %) were *A. flavus*, 14 (8.0%) *A. versicolor*, six (3.4 %) *A. parasiticus*, four (2.3%) *A. clavatus*, four (2.3%) *A. sydowii*, four (2.3%) *A. fumigatus*, two (1.1%) *A. glaucus*, two (1.1%) *A. nidulans*, one (0.6%) *A. candidus* and one (0.6%) *A. wentii*. *Aspergillus flavus* was the most frequently occurring species in almost all the districts apart from Moiben district which had more *A. versicolor* than *A. flavus*. The distribution of *A. flavus* across the five districts was as follows: Makueni 96 (70.6%), Nyeri Central 15 (11.9%), Moiben 4 (2.9%), Ugunja 10 (7.4%) and Bungoma South 11 (8.1%). These findings are in accord with the study of Okoth *et al.* (2012) which provided for the first time, an important comparison of fungal contamination between maize sampled in Makueni County, the main region that has experienced repeated lethal human aflatoxicosis outbreaks and Nandi County, the main maize growing region in Kenya. Their study showed that *Aspergillus*, specifically *A. flavus*, is the main contaminant of maize in household storage in the two regions and *A. flavus* was the most common species. The frequency of occurrence of *A. flavus* in Nandi and Makueni was the same regardless of the differences in mean temperatures (20 $^{o}$C and 24 $^{o}$C respectively) and rainfall (900–1800 and 950–1500 mm). High temperatures and drier conditions are known to favour

infection by *A. flavus* but this was not the case in the study by Okoth *et al.,* (2012). Similar results have also been observed in Nigeria (Atehnkeng *et al*., 2008a).

The distribution of *A. versicolor* was as follows: Makueni 0 (0%), Nyeri Central two (14.3%), Moiben 8 (57.1%), Ugunja 2 (14.3%) and Bungoma South 2 (14.3%). For *A. parasiticus* distribution; Nyeri Central and Bungoma South districts had none, Makueni 1 (16.7%), Moiben 1 (16.7 %) and Ugunja 4 (66.7%). For *A. clavatus,* only Moiben had 4 (100%) isolates, the rest of the districts had none. Distribution of *A. sydowii* was as follows; Moiben and Bungoma South districts had none, Makueni 1 (25%), Nyeri Central 2 (50%) and Ugunja 1 (25%). For *A. fumigatus;* Moiben and Ugunja did not have any isolates, Makueni 1 (25%), Nyeri Central 2 (50%) and Bungoma South 1 (25%). For *A. glaucus;* Makueni 1 (50%) and Moiben 1 (50%), the other districts had none. For *A. nidulans;* Makueni 1 (50%) and Nyeri Central 1 (50%), other districts had none. For *A. candidus,* only Ugunja district had 1 (100%) isolate while the rest had none. *Aspergillus wentii* was only present in Bungoma South which had 1 (100%) isolate while all the other districts had none. These results are evidence that *A. flavus* is the major contaminant of maize and soil across all the five districts under this study as evidenced in table 4.3. Statistically, these results further showed that there was no significant difference (P= 0.0699) in the frequency of occurrence among the various *Aspergillus* species found in all the five districts. These results resonates with the study of Muthomi *et al.* (2012) in which the specific *Aspergillus* spp. isolated from whole and unprocessed maize grain and soil from North Rift and Eastern regions were; *A. flavus*, *A. niger*, *A. fumigatus, A. versicolor*, *A. terreus*, *A. clavatus* and *A. ochraceus*. The most frequently isolated were *A. flavus* and *A. niger*, while *A. clavatus* was the least frequently isolated *Aspergillus* species and was mainly isolated in samples from the humid North Rift region. Similarly in this study *A. flavus* had the highest incidences in all the districts apart from Moiben. Moiben (North Rift) had the highest incidence of *A. clavatus* as shown in Table 4.3 and this echoes the findings of Muthomi *et al*. (2012) where *A. clavatus* was predominantly isolated from the humid North rift region.

**Table 4.3:** Distribution of various *Aspergillus* species across the five districts

| | Districts | | | | | | |
|---|---|---|---|---|---|---|---|
| | Makueni | Nyeri Central | Moiben | Ugunja | Bungoma South | **Total** | **% Total** |
| ***Aspergillus* species** | | | | | | | |
| *A. flavus* | 96 | 15 | 4 | 10 | 11 | **136** | **78.5** |
| *A. versicolor* | 0 | 2 | 8 | 2 | 2 | **14** | **8.0** |
| *A. parasiticus* | 1 | 0 | 1 | 4 | 0 | **6** | **3.4** |
| *A. clavatus* | 0 | 0 | 4 | 0 | 0 | **4** | **2.3** |
| *A. sydowii* | 1 | 2 | 0 | 1 | 0 | **4** | **2.3** |
| *A. fumigatus* | 1 | 2 | 0 | 0 | 1 | **4** | **2.3** |
| *A. glaucus* | 1 | 0 | 1 | 0 | 0 | **2** | **1.1** |
| *A. nidulans* | 1 | 1 | 0 | 0 | 0 | **2** | **1.1** |
| *A. candidus* | 0 | 0 | 0 | 1 | 0 | **1** | **0.6** |
| *A. wentii* | 0 | 0 | 0 | 0 | 1 | **1** | **0.6** |
| **Total No.** | **101** | **22** | **18** | **18** | **15** | **174** | **100** |
| **% Total** | **58.0** | **12.6** | **10.4** | **10.4** | **8.6** | | **100** |

The pervasive nature of *Aspergillus* species, their high ability to colonize diverse substrates and lack of effective control measures (Souza *et al*., 2005) could have contributed to their high occurrences in maize and soil from the five districts. *Aspergillus* spp. are more commonly associated with cereals during drying and storage. *Aspergillus flavus* and *A. parasiticus* have a particular affinity for cereals and can be recognized by yellow-green or grey green colour on maize kernels in the field and in storage (Varga *et al*., 2011). This study found out that *Aspergillus flavus* was the most prevalent *Aspergillus* spp. in all the districts except for Moiben district where *A. versicolor* was the predominant species.

The dominance of *A. flavus* over other *Aspergillus* spp. in all the districts is clearly demonstrated in Figure 4.1. A study by Muthomi *et al*. (2012) showed that grain samples collected from farmers in the semi-arid eastern region (Makueni) during the short rainy seasons had higher incidences of *A. flavus,* of up to 14% compared to grain harvested during the long rainy seasons. This supports the high levels of *A. flavus* in Makueni district since sampling in this study was done immediately after the short rainy season maize harvest in all the districts. Additionally, variations in fields' cropping history, cultivation practices, sowing dates, seed varieties planted and/or soil types can differ greatly in aflatoxigenic fungi and aflatoxin contamination (Munkvold

*et al*., 2009). These dynamics may explain the variances in the incidences and type of *Aspergillus* spp. isolated from the five districts. Similarly, damaged maize kernels also favour the growth of *A. flavus* compared to any other *Aspergillus* species. This could be the reason why the most frequently isolated *Aspergillus* species in the five districts was *A. flavus.*

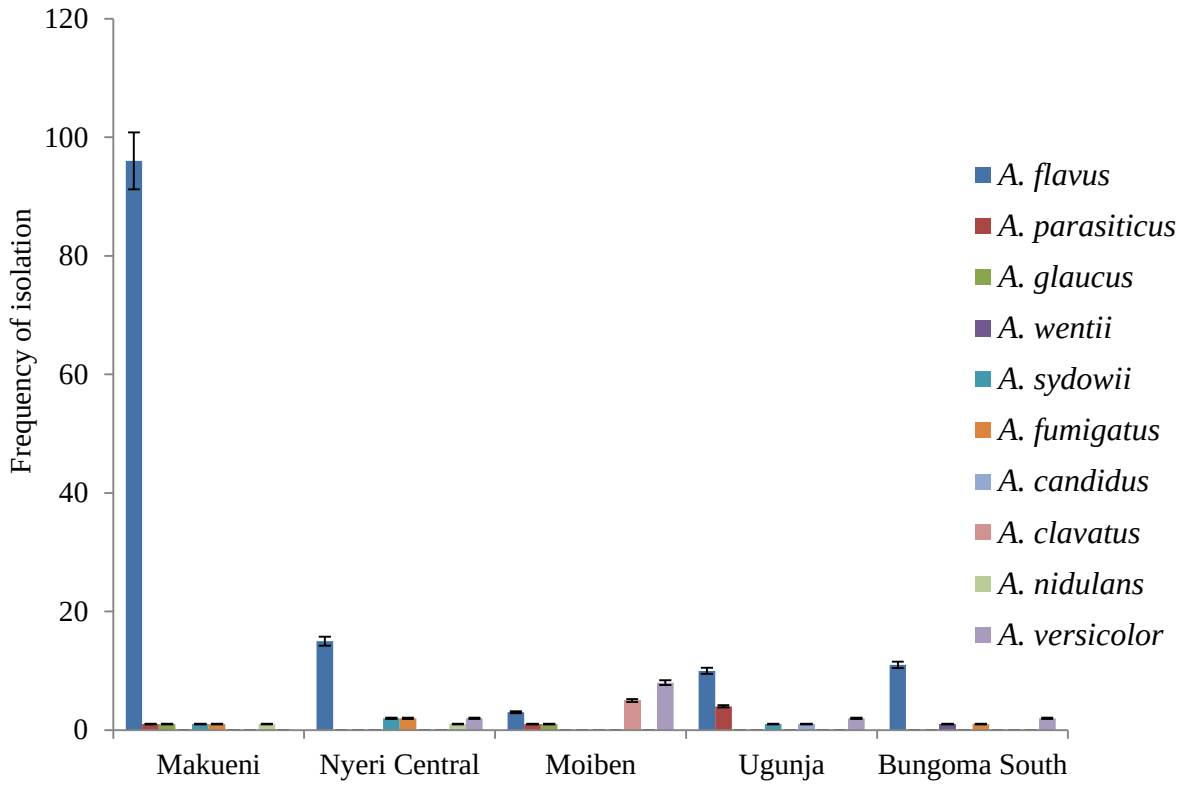



**Figure 4.2:** Comparison of distribution of different *Aspergillus* species across the five districts.

### 4.4 Screening of the isolated *Aspergillus* species for atoxigenic and aflatoxigenic strains in the laboratory

*Aspergillus* spp. isolates BS203, RS016, RS024, RS162, RS013, US184, ES042 and RM023-2 each representing a specific group (G) exhibited yellow pigment production in PDA when cultured at 28 ºC for a period of 7 days (Table 4.4, Plate 4.4). However not all these isolates producing yellow pigment in PDA media indicated toxin production when grown in YES media and exposed to 27% ammonium hydroxide, by changing their various under-sides' colour to plum-red or pink as reported by Saito and Machida in 1999 (Plate 4.5).

**Table 4.4:** Toxigenicity of various *Aspergillus* strains grown on YES media upon exposure to concentrated ammonia vapour and yellow pigment production by the strains in PDA media

| | | | | Reverse colour change upon exposure to conc. ammonia vapour on YES media | | |
|---|---|---|---|---|---|---|
| **Group No.** | **Isolate code** | **Isolate name** | **Yellow pigment production in PDA** | **Before** | **After** | **Inference** |
| **G3** | EM324 | *A. flavus* | - | Light brown | Light brown | A |
| **G7** | NM091 | *A. flavus* | - | Light brown | Light brown | A |
| **G8** | NM083 | *A. flavus* | - | Cream | Cream | A |
| **G9** | EM244 | *A. flavus* | - | Light brown | Light brown | A |
| **G13** | NM084 | *A. flavus* | - | Light brown | Brown | A |
| **G16** | EM182 | *A. flavus* | - | Light brown | Light brown | A |
| **G17** | BM071 | *A. flavus* | - | Light brown | Light brown | A |
| **G18** | EM1112 | *A. flavus* | - | Light yellow | Light yellow | A |
| **G19** | UM127 | *A. flavus* | - | Light brown | Light brown | A |
| **G21** | BS203 | *A. versicolor* | + | Brown | Plum red | T |
| **G21** | RS193 | *A. versicolor* | + | Brown | Brown | A |
| **G22** | RS016 | *A. versicolor* | ++ | Light yellow | Plum red | T |
| **G22** | RS043 | *A. versicolor* | + | Yellow | Brown | A |
| **G31** | RS024 | *A. glaucus* | + | Cream | Light brown | A |
| **G32** | BS023 | *A. wentii* | - | Cream | Cream | A |
| **G34** | RS162 | *A. versicolor* | + | Yellow | Plum red | T |
| **G38** | US098 | *A. sydowii* | - | Light brown | Light brown | A |
| **G41** | RS013 | *A. flavus* | ++ | Light yellow | Plum red | T |
| **G49** | UM082 | *A. parasiticus* | - | Brown | Brown | A |
| **G50** | BM092 | *A. clavatus* | - | Light brown | Light brown | A |
| **G58** | US184 | *A. candidus* | + | Yellow | Yellow | A |
| **G64** | RM143 | *A. clavatus* | - | Brown | Brown | A |
| **G68** | ES042 | *A. nidulans* | + | Brown | Brown | A |
| **G71** | RM023-2 | *A. flavus* | + | Light yellow | Plum red | T |
| **G71** | BS116 | *A. flavus* | - | Light brown | Light brown | A |
| **G72** | EM211 | *A. glaucus* | - | Cream | Cream | A |

**Key:**
E – Eastern (Makueni), R – Rift Valley (Moiben), B – Bungoma South, N – Nyeri Central, U – Ugunja. M – Maize, S – Soil, (01-32) – Sample number in each district, (1 -12) - Isolate number from each sample, (-2) – Purification number, **(-)** No Yellow pigment, (+) Less intense yellow pigment, (++) Intense yellow pigment, A – Atoxigenic, T – Aflatoxigenic.


A


B

**Plate 4.4:** (a) Aflatoxigenic *Aspergillus* spp. producing yellow pigment in PDA (b) No pigment production by atoxigenic *Aspergillus* spp. in PDA


A

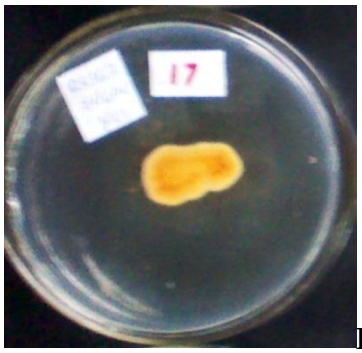

B

**Plate 4.5:** (a) Aflatoxigenic *Aspergillus* spp. before exposure to concentrated ammonia vapour (b) Aflatoxigenic *Aspergillus* spp. after exposure to concentrated ammonia vapour for 30 minutes.

Isolates US184 (*A. candidus*), ES042 *(A. nidulans)*, RS193 and RS043 (*A. versicolor*) despite producing yellow pigmentation in PDA, did not turn out to be aflatoxigenic as expected when they were exposed to 27% ammonium hydroxide. On the other hand, all the other isolates that produced yellow pigmentation in PDA indicated toxin production by changing the colour of their undersides from either yellow or brown to plum red when they were grown in YES medium and exposed to 27% ammonium hydroxide as stipulated by Saito and Machida in 1999 (Table 4.4). Saito and Machida (1999) observed the greatest colour change in colonies grown on yeast extract-sucrose and coconut media, a less intense colour change on potato dextrose agar, and the least colour change on glucose-mineral salts media, all of which favour aflatoxin production. However, no colour change was observed on peptone mineral salts and Czapek solution media, which do not support aflatoxin production.

All the *Aspergillus* spp. isolates that did not exhibit yellow pigment production in PDA as expected also did not indicate toxin production when grown in YES media and exposed to 27% ammonium hydroxide. This was a confirmatory result that *Aspergillus* spp. that do not produce yellow pigment in PDA are most likely non-toxin producers, as these results are in agreement with the findings of Abbas *et al.* (2011) where *A. flavus* strains that produced yellow pigmentation in PDA turned pink/plum red on exposure to ammonia and had the aflatoxin gene cluster functional. On the other hand the *A. flavus* strains that did not show yellow pigmentation on PDA did not show any colour change when reacted with ammonia and additionally the aflatoxin gene cluster was deleted, mutated or unknown.

### 4.5 Distribution of aflatoxigenic and atoxigenic *Aspergillus* strains from maize and soil samples across the districts

A total of 12 aflatoxigenic isolates were identified through the ammonia test. Two of the aflatoxigenic isolates were *A. flavus* while the remaining 10 were *A. versicolor*. Only one aflatoxigenic isolate (RM023-2 *A. flavus*) was obtained from maize while the remaining 11 aflatoxigenic isolates were obtained from soil as shown in Table 4.5. Distribution of the aflatoxigenic *Aspergillus* spp. across the districts was as follows; Moiben six (50 %), Bungoma South two (16.7%), Nyeri Central two (16.7%) and Ugunja two (16.7%). Makueni district had no aflatoxigenic isolates though it was leading in the number of *Aspergillus* spp. isolates which all turned out to be atoxigenic at 101 (62.3%) isolates, followed by the other districts in the following order; Nyeri Central 20 (12.4%), Ugunja 16 (9.9%), Bungoma South 13 (8.0%) and Moiben 12 (7.4%).

**Table 4.5:** Distribution of aflatoxigenic and atoxigenic *Aspergillus* strains from maize and soil samples across the districts

| | Districts | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Bungoma South** | | **Makueni** | | **Nyeri South** | | **Moiben** | | **Ugunja** | | |
| | Aflatoxigenic | Atoxigenic | Aflatoxigenic | Atoxigenic | Aflatoxigenic | Atoxigenic | Aflatoxigenic | Atoxigenic | Aflatoxigenic | Atoxigenic | Total |
| **Maize isolates** | 0 | **7** | 0 | **99** | 0 | **17** | 1 | **6** | 0 | **14** | 144 |
| **Soil isolates** | 2 | **6** | 0 | **2** | 2 | **3** | 5 | **6** | 2 | **2** | 30 |
| **Total** | 2 | **13** | 0 | **101** | 2 | **20** | 6 | **12** | 2 | **16** | 174 |
| **% Total** | 16.7 | **8.03** | 0 | **62.3** | 16.7 | **12.4** | 50.0 | **7.4** | 16.7 | **9.9** | |

Statistically, there was no significant difference (P= 0.3791) in the distribution of aflatoxigenic and atoxigenic *Aspergillus* species from maize and soil samples across the five districts within a 95% confidence limit. In this study only 6.9% of the *Aspergillus* spp. isolates were aflatoxigenic while 93.1% were atoxigenic. Growth and development of *Aspergillus* spp. are factors favoured by soil temperatures of 35 – 40 $^{o}$C (Riley and Norrad, 1999), which is also the optimum temperature for aflatoxin production by the fungus (Schmidt-Heydt *et al.,* 2010). This explains the results obtained in this study in which most of the aflatoxigenic isolates originated from soil and not maize. Schmidt-Heydt *et al.* (2010) showed that a high temperature of about 37 $^{o}$C was the key parameter for the production of $AFB_1$ by *A. parasiticus*. These results contradict the findings of Okoth *et al.* (2012) where aflatoxigenic strains of *A. flavus* were more prevalent than the atoxigenic strains of *A. flavus* in Nandi and Makueni counties. This could be attributed to the fact that in this study only good maize perceived by the farmer as fit for human consumption was collected as opposed to other studies which included bad maize in their analysis. The other possible explanation for this difference could be because most of the maize samples were collected in the month of August, 2011 which was immediately or just about after harvesting, when the storage period was still very short hence the favourable conditions for aflatoxigenic mould attack had not set in. Another reason that explains these results could be that good maize handling procedures at harvesting and post-harvest practices could have been implemented by

the farmers across all the districts especially in Makueni district. This could be as a result of adequate farmer education and training on proper handling of maize at harvesting and during storage, and creation of awareness among the small-scale maize farmers as this practice has been shown to reduce the level of aflatoxin contamination and poisoning (Azziz-Baumgartener *et al.*, 2005).

Studies by Mutungi *et al.* (2008) found that traditional maize processing and preparation methods that use sodium salts reduced aflatoxin contamination by as much as 80%. Therefore, good agricultural practice and proper handling would be necessary to reduce this prevalence of *Aspergillus* spp. contamination in maize (Shephard, 2008). This may include the elimination of inoculum sources such as crop residue from the previous harvest to minimize infection of the crop. Studies by Strosnider *et al.* (2006) showed that individuals who received information on maize drying and storage after the 2004 aflatoxin poisoning outbreak had lower serum aflatoxin levels than those who did not receive the information (Muthomi *et al.*, 2012). Post-harvest handling of maize may positively and/or negatively affect fungal infection and mycotoxin production in maize (Fandohan *et al*., 2003).

Other factors that influence atoxigenicity or toxigenicity are broadly environmental. Environmental factors that favour *A. flavus* infection in the field include high soil and/or air temperature, high relative humidity, high rates of evapotranspiration, reduced water availability, drought stress, nitrogen stress, crowding of plants and conditions that enhance the dispersal of conidia during silking (CAST, 2003). Some of these factors have been included in a model to predict toxin contamination in groundnut systems in Mali. Weather and satellite based variables that could be used to indicate aflatoxin presence in groundnuts have been identified (Boken *et al.*, 2008). Significant correlations are in existence between agroecozones (AEZ) and aflatoxin levels, with wet and humid climates and drier regions after longer storage periods increasing aflatoxin risk (Hell *et al.*, 2000).

Additional factors that influence the incidence of fungal infection and subsequent toxin development include invertebrate vectors, grain damage, oxygen and carbon dioxide levels, inoculum load, substrate composition, fungal infection levels, prevalence of aflatoxigenic strains and microbiological interactions. Insect vectors of fungi cause damage that allows the

fungi to breach, increasing the chances of aflatoxin contamination, especially when loose-husked maize hybrids are used (Dowd, 2003). High incidence of the insect borer *Mussidia nigrivenella* Ragonot, was positively correlated with aflatoxin contamination of maize in Benin (Setamou *et al.,* 1998). Storage pests, in particular *Cathartus quadricollis* Guerin and *Sitophilus zeamais* Motschulsky, play an important role in the contamination of foods with fungi, especially those that produce toxins (Lamboni and Hell, 2009). The aflatoxigenic *Aspergillus* strains isolated, the mycotoxins they produce and health effects they cause are shown in Table 4.6.

**Table 4.6:** Aflatoxigenic *Aspergillus* strains isolated and the known toxins they produce

| **Aflatoxigenic *Aspergillus* strains** | **Major mycotoxins produced** | **Health Effects** |
|---|---|---|
| **T-RS013** ***A. flavus*** | Aflatoxin B1 | It is a probable human carcinogen. Acute levels lead to liver necrosis, hemorrhage and often death. Lower levels lead to decreased growth and impaired immune systems. Chronic exposure leads to liver cancer in animals (Diener *et al.,* 1987) |
| **T-RM023-2** ***A. flavus*** | Cyclopiazonic acid | It is frequently co-produced with aflatoxin in the field. Major effects include liver necrosis and other changes in kidney tubules, fatty acid changes and death at high doses (Pier and Richard, 1992). |
| | 3-nitropropionic acid | Has been implicated in fatal food poisoning of humans and it is toxic to mice (Golinski, 1991). |
| **T-RS162** ***A. versicolor*** **T-RS016** ***A. versicolor*** **T-BS203** ***A. versicolor*** | Sterigmatocystin | It is a precursor in the aflatoxin biosynthetic pathway. Like aflatoxin it is hepatotoxic and carcinogenic (Diener *et al.,* 1987). |

### 4.6 Characterization of atoxigenic and aflatoxigenic strains based on their sporulation, growth rate and morphological characteristics

To characterize the atoxigenic and aflatoxigenic strains based on their sporulation, growth rate and morphological characteristics, the 10 *Aspergillus* spp. were cultured in two different types of media; PDA and CZ and comparisons made as shown in plates 4.3 (3a-3j) and Table 4.7.

**Table 4.7:** Characterization of *Aspergillus* species based on their sporulation and colony characteristics in PDA and CZ media

| | | | | COLONY CHARACTER | | | ZONATION | SPORULATION |
|---|---|---|---|---|---|---|---|---|
| **ISOLATE CODE** | **ISOLATE NAME** | **MEDIA TYPE** | **COLONY DIAM (MM)** | **TEXTURE** | **SURFACE COLOUR** | **REVERSE COLOUR** | | |
| G3-EM324 | *A. flavus* | PDA | 41.3±3.1 | Floccose center with powdery periphery | Olive green with whitish margin | Yellowish with grey margin | Slightly furrowed at the centre on the reverse | Heavy |
| | | CZ | 39.3±1.2 | Powdery | Olive green | Creamish | None | Moderate |
| G7-NM091 | *A. flavus* | PDA | 35.3±5.1 | Floccose center with powdery periphery | Olive green with dirty white margin | Cream centre with alternating grey and cream periphery | Slightly furrowed at the centre on the reverse | Heavy |
| | | CZ | 33.7±1.5 | Powdery | Olive green | Greyish | None | Moderate |
| G8-NM083 | *A. flavus* | PDA | 34.7±0.6 | Powdery | Olive green with white margin | Creamish | None | Heavy |
| | | CZ | 38.0±2.0 | Powdery | Olive green with creamish margin | Slightly creamish | None | Moderate |
| G9-EM244 | *A. flavus* | PDA | 34.0±4.0 | Floccose | Olive green with white margin | Cream centre with alternating grey and cream rings | Radially furrowed at the centre with concentric zones at the reverse | Heavy |
| | | CZ | 40.7±1.2 | Powdery | Olive green | Cream | None | Moderate |
| G13-NM084 | *A. flavus* | PDA | 37.7±2.5 | Floccose center with powdery periphery | Olive green with cream margin | Cream center with alternative grey and cream concentric rings | Slightly furrowed at the centre on the reverse | Heavy |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | CZ | 38.0±2.0 | Powdery | Olive green | Greyish | None | Moderate |
| G16-EM182 | *A. flavus* | PDA | 32.3±3.2 | Powdery | Olive green with whitish margin | Creamish | Slight concentric zones on the reverse | Heavy |
| | | CZ | 40.3±2.5 | Powdery | Olive green with light creamish margin | Creamish | None | Moderate |
| G17-BM071 | *A. flavus* | PDA | 36.7±2.3 | Floccose | Olive green with cream margin | Cream center with alternative grey and cream concentric rings | Concentric zones on the reverse | Heavy |
| | | CZ | 39.3±1.2 | Powdery | Olive green | Greyish | Concentric zones on the reverse | Moderate |
| G18-EM1112 | *A. flavus* | PDA | 35.0±1.0 | Floccose center with powdery margins | Olive green with whitish margin | Cream with grey margin | Slight concentric zones on the reverse | Heavy |
| | | CZ | 40.7±2.1 | Powdery | Olive green | Cream | None | Moderate |
| G19-UM127 | *A. flavus* | PDA | 28.3±1.5 | Floccose center with powdery margins | Olive green with whitish margin | Cream with grey margin | Slight concentric zones on the reverse | Heavy |
| | | CZ | 37.0±1.7 | Powdery | Olive green | Cream | None | Moderate |
| G21-RS193 | *A. versicolor* | PDA | 17.4±3.5 | Floccose | Grey with a yellow at the middle | Greenish centre with creamish margin | Heavily wrinkled on the reverse | Moderate |
| | | CZ | 18.2±2.4 | Powdery | Red centre with green and dirty white concentric rings | Brown centre with green and cream concentric rings | Concentric zones | Moderate |
| G22- RS043 | *A. versicolor* | PDA | 36.0±1.6 | Velvety | Creamish centre with green | Sulphur yellow | Slightly wrinkled at the | Poor |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | to sulphur yellow margins | | centre on the reverse | |
| | | CZ | 28.2±0.5 | Velvety | Pink centre with green and sulphur yellow concentric rings | Red centre with green and cream concentric rings | Concentric zones | Moderate |
| G31-RS024 | *A. glaucus* | PDA | 26.7±2.1 | Velvety | Green with sulphur margin | Yellow | Slightly wrinkled at the centre on the reverse | Poor |
| | | CZ | 21.0±1.0 | Velvety | Pink centre with yellow and green concentric rings | Brown centre with yellow margin | Concentric zones on the reverse | Poor |
| G32-BS023 | *A. wentii* | PDA | 26.0±2.0 | Velvety | Sulphur yellow centre with alternating grey and sulphur yellow concentric rings | Sulphur yellow | Slightly wrinkled on the reverse | Poor |
| | | CZ | 21.0±1.0 | Velvety | Grey with dirty white margins | Grey with dirty white margin | None | Moderate |
| G38-US098 | *A. sydowii* | PDA | 29.0±1.0 | Velvety thick | Greyish blue with white margin | Yellow | Slightly radially furrowed | Poor |
| | | CZ | 27.0±1.0 | Fine | Green-blue with white margin | Deep red | Light zonation at the margin | Moderate |
| G49-UM082 | *A. parasiticus* | PDA | 26.7±0.6 | Floccose | Conifer green with cream margin | Yellow centre with cream margin | Concentric rings with slight radial furrows | Heavy |
| | | CZ | 45.0±0.0 | Floccose | Conifer green | Grey | None | Moderate |
| G50-BM092 | *A. fumigatus* | PDA | 41.3±3.1 | Powdery | Grey centre with green margin | Cream centre with grey margin | With concentric zones | Heavy |
| | | CZ | 42.3±1.5 | Powdery | Grey centre with green margin | Brown centre with creamish margin | With concentric zones | Moderate |
| G58-US184 | *A. candidus* | PDA | 15.7±1.5 | Velvety | White | Deep yellow | None | Poor |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | CZ | 19.7±1.5 | Powdery | Brownish with yellow margin | Brown with yellow margin | Slightly furrowed on the reverse | Heavy |
| G64-RM143 | *A. clavatus* | PDA | 31.7±1.5 | Fine | Bluish green with white margin | Brown center with alternating cream and brown concentric rings | Concentric zones on the reverse | Heavy |
| | | CZ | 26.0±2.0 | Powdery | Green | Red centre with dirty white margin | None | Heavy |
| G68-ES042 | *A. nidulans* | PDA | 23.7±2.1 | Fine | Green | Deep red | None | Heavy |
| | | CZ | 22.0±1.7 | Powdery | Green with dirty white margin | Plum red centre with dirty white margin | None | Heavy |
| G71-BS116 | *A. flavus* | PDA | 36.0±2.0 | Floccose | Olive green | Creamish yellow | Radially furrowed on the reverse | Heavy |
| | | CZ | 37.7±0.6 | Powdery | Olive green | Dirty white | None | Moderate |
| G72-EM211 | *A. glaucus* | PDA | 29.0±1.7 | Powdery | Green centre with alternating yellow and green concentric rings | Cream | Radially furrowed on the reverse | Heavy |
| | | CZ | 36.3±.06 | Powdery | Green centre with whitish margin | Green centre with cream margin | None | Poor |
| G21-**T**-BS203 | *A. versicolor* | PDA | 17.0±2.7 | Floccose | Grey with a yellow at the middle | Greenish centre with creamish margin | Heavily wrinkled on the reverse | Moderate |
| G21-**T**-RS022 | *A. versicolor* | | | | | | | |
| G21-**T**-NS102 | *A. versicolor* | CZ | 17.7±0.6 | Powdery | Red centre with green and dirty white concentric rings | Brown centre with green and cream concentric rings | Concentric zones | Moderate |
| G21-**T**- RS014 | *A. versicolor* | | | | | | | |
| G21-**T**- US095 | *A. versicolor* | | | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| G22-**T**-RS016 | *A. versicolor* | PDA | 34.0±1.0 | Velvety | Creamish centre with green to sulphur yellow margins | Sulphur yellow | Slightly wrinkled at the centre on the reverse | Poor |
| G22-**T**-BS042 | *A. versicolor* | | | | | | | |
| G22-**T**-NS032 | *A. versicolor* | CZ | 26.3±0.6 | Velvety | Pink centre with green and sulphur yellow concentric rings | Red centre with green and cream concentric rings | Concentric zones | Moderate |
| G22-**T**- US081 | *A. versicolor* | | | | | | | |
| G34-**T**-RS162 | *A. versicolor* | PDA | 32.7±1.2 | Velvety | Grey centre with yellowish margin | Yellow | Slightly wrinkled at the centre on the reverse | Poor |
| | | CZ | 23.3±0.6 | Velvety | Green with yellow periphery and cream margin | Green with yellow periphery and cream margin | Concentric zones | Heavy |
| G41-**T**-RS013 | *A. flavus* | PDA | 36.0±2.0 | Floccose centre with powdery periphery | Olive green with cream margin | Yellow centre with cream margin. Red pigmentation | Radially furrowed at the centre on the reverse | Heavy |
| | | CZ | 38.0±2.0 | Powdery | Green centre, red and olive green margin | Red centre and cream periphery. Red pigmentation | None | Moderate |
| G71-**T**-RM023-2 | *A. flavus* | PDA | 32.3±1.5 | Floccose | Olive green with white margin | Cream centre with alternate yellow and brown concentric rings | Slightly furrowed at the centre | Heavy |
| | | CZ | 26.7±2.5 | Powdery | Olive green with dirty white margin | Cream | None | Moderate |

Key: **G**- Group, **T**- Aflatoxigenic.

**E** – Eastern (Makueni), **R** – Rift Valley (Moiben), **B** – Bungoma South, **N** – Nyeri Central, **U** – Ugunja.

**M** – Maize, **S** – Soil, **(01-32)** – Sample number in each district, **(1 -12) -** Isolate number from each sample, **(-2)** – Purification number.

Sporulation classifications – Heavy (Densely packed conidia), Moderate (Loosely packed conidia), & Poor (Sparsely packed conidia).

In this study aflatoxigenic *Aspergillus versicolor* strain T-BS203 had moderate sporulation in both PDA and CZ media while strain T-RS016 had poor sporulation in PDA and moderate sporulation in CZ media. Only the aflatoxigenic strain T-RS162 showed heavy sporulation in CZ media and poor sporulation in PDA. Similarly the atoxigenic *A. versicolor* strains RS193 and RS043 had exactly similar sporulation characteristics as their aflatoxigenic counterparts' strains T-BS203 and T-RS016 respectively. Therefore, with this identical sporulation pattern for atoxigenic and aflatoxigenic *A. versicolor* strains, one would conclude that atoxigenic and aflatoxigenic *A. versicolor* strains have no distinct differences in their sporulation characteristics and therefore they cannot be characterized based on their sporulation.

For aflatoxigenic *Aspergillus flavus* strains T-RS013 and T-RM023-2, both had heavy sporulation in PDA and moderate sporulation in CZ media. In comparison, these sporulation characteristics exhibited by the aflatoxigenic *A. flavus* strains were exactly similar to the sporulation characteristics exhibited by all the atoxigenic *A. flavus* strains EM324, NM091, NM083, EM244, NM084, EM184, BM071, EM1112, UM127 and BS116 in both PDA and CZ media. In view of this observation, the findings of this study cannot be used to report that atoxigenic and aflatoxigenic *A. flavus* strains differ in their sporulation characteristics. Therefore based on this study, sporulation characteristics cannot be used as a reliable means of categorizing aflatoxigenic and atoxigenic strains of *A. flavus*. The other atoxigenic *Aspergillus* spp. including; *A. glaucus* RS024, *A. wentii* BS023, *A. sydowii* US098, *A. fumigatus* BM092, *A. candidus* US184, *A. clavatus* RM143, *A. nidulans* ES042 and *A. parasiticus* UM082 had no aflatoxigenic counterparts identified in this study to enable comparison of sporulation characteristics within each individual species. There is little information available on studies done on sporulation characteristics of atoxigenic and aflatoxigenic *Aspergillus* spp. and the findings of this study will contribute towards providing information on sporulation characteristics of aflatoxigenic and atoxigenic *Aspergillus* spp.

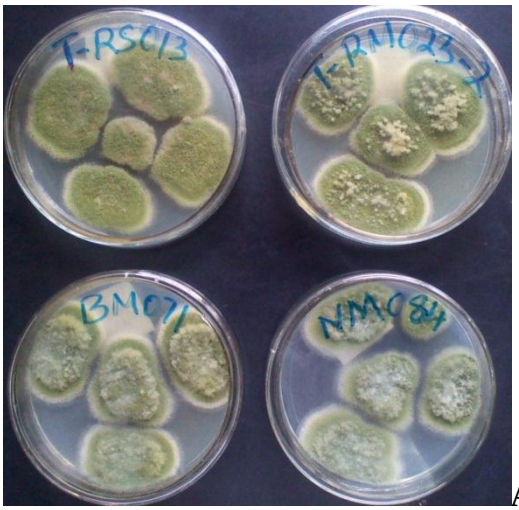
In terms of growth rate of aflatoxigenic and atoxigenic strains, there were some significant differences observed in this study. Aflatoxigenic *A. versicolor* strain (T-RS016) which had the largest radial growth among the aflatoxigenic *A. versicolor* strains, had relatively smaller colony

diameters in both PDA (34.0 mm) and CZ (26.3 mm) compared to the atoxigenic *A. versicolor* strain RS043 which had colony diameters of 36.0 mm on PDA and 28.2 mm in CZ. Similarly in comparison with other atoxigenic isolates of other *Aspergillus* spp. there was a substantial variation, for instance atoxigenic *A. parasiticus* strain UM028 had a large colony diameter of 45.0 mm in CZ media and 26.7 mm in PDA. Atoxigenic *A. flavus* strain EM324 had a colony diameter of 39.2 mm in CZ and 41.3 mm in PDA. Atoxigenic *A. fumigatus* strain BM092 had a colony diameter of 42.3 mm in CZ and 41.3 mm in PDA. In regard to the fast radial growth of the various atoxigenic strains mentioned above compared to the aflatoxigenic *A. versicolor* strains T-RS016, T-BS203 and T-RS162, one would suggest that atoxigenic strains have a fairly faster radial growth rate than the aflatoxigenic strains. Similarly, in comparing colony diameters of the above mentioned atoxigenic strains with those of the two aflatoxigenic *A. flavus* strains T-RS013 and T-RM023-2, then the same trend is repeated. The colony diameters of the atoxigenic *A. flavus* (EM324), *A. parasiticus* (UM028) and *A. fumigatus* (BM092) alongside several other atoxigenic strains were relatively larger than those of the two aflatoxigenic *A. flavus* strains (T-RS013 and T-RM023-2) in both PDA and CZ media. Aflatoxigenic *A. flavus* strain T-RS013 had a colony diameter of 38.0 mm in CZ and 36.0 mm in PDA while aflatoxigenic *A. flavus* strain T-RM023-2 had a colony diameter of 26.7 mm in CZ media and 32.3 mm in PDA media. All colony diameters were measured after 7 days of incubation at 28 $^{o}$C.

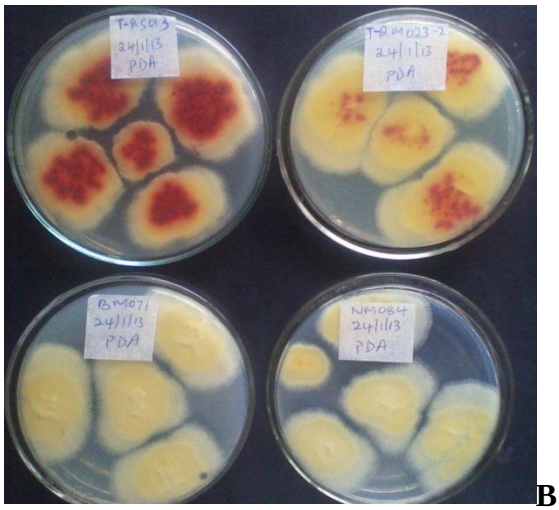
The results of this study conform to the study of Abbas *et al.* (2011) in which the phenotypic, genotypic, and chemotaxonomic characteristics of aflatoxigenic and atoxigenic *Aspergillus flavus* strains used in their study were compared in evaluating efficacy at reducing aflatoxin and cyclopiazonic acid (CPA) contamination of harvested maize kernels. In their study, the radial growth of atoxigenic *A. flavus* strains; Afla-Guard 21882 (42 mm), AF36 18543 (41 mm), K49 30797 (41 mm) and CT3 30798 (42 mm) were slightly larger compared to the aflatoxigenic *A. flavus* strains; Air 58976 (41 mm), F3W4 30796 (38 mm), Insects 58988 (41 mm), K54 58987 (39 mm), Rice 58975 (39 mm) and Soybean 58974 (39 mm). The results of this study together with that of Abbas *et al.* (2011) indicate that the growth of atoxigenic *Aspergillus* strains especially those of high potential as biological control agents is relatively higher than the growth

of the aflatoxigenic *Aspergillus* species. This therefore suggests that growth rate could be used to characterize aflatoxigenic and atoxigenic *Aspergillus* strains especially of the same species.

In terms of cultural and morphological characteristics, the aflatoxigenic *A. versicolor* strains T-BS203, T-RS162 and T-RS016 were similar to their atoxigenic counterparts' strains RS193 and RS043. Therefore, there are no clear cultural and morphological differences among the individual aflatoxigenic and atoxigenic *A. versicolor* strains that can be used to classify them. Similarly, the aflatoxigenic *A. flavus* strains (T-RS013 and T-RM023-2) were similar to their atoxigenic counterpart *A. flavus* strains (BM071 and NM084) in terms of surface colour and reverse but the atoxigenic strains were more floccose in texture on the surface on PDA media as shown in Plate 4.6 (A). On the reverse the distinct difference between the aflatoxigenic *A. flavus* strains (T-RS013 and T-RM023-2) and all the atoxigenic *A. flavus* strains (including BM071 and NM084), was the presence of plum red pigment production by the aflatoxigenic *A. flavus* strains in PDA media as shown in Plate 4.6 (B). However, further studies should be done to ascertain if the observed differences between aflatoxigenic and atoxigenic *A. flavus* strains in this study can be authenticated and used as a basis of characterizing them. There is little information available on the cultural and morphological differences between aflatoxigenic and atoxigenic *Aspergillus* spp. and the findings of this study would contribute towards making such information available.



**A**



**B**

**Plate 4.6:** (a) More floccose atoxigenic *Aspergillus flavus* (BM071 and NM084) on the surface as opposed to aflatoxigenic *Aspergillus flavus* (T-RS013 and T-RM023-2). (b) Plum red pigment production by aflatoxigenic *Aspergillus flavus* (T-RS013 and T-RM023-2) and absence

of plum red pigment production by atoxigenic *Aspergillus flavus* (BM071 and NM084) on the reverse

### 4.7 Comparison of cultural, morphological and sporulation characteristics of *Aspergillus* strains of similar species growing on PDA and CZ media

In this study, most of the atoxigenic *Aspergillus* spp. differed considerably in their morphological characteristics when cultured on two different types of media, PDA and CZ. Different culture media when used for isolation of the *Aspergillus* spp. differently influence the vegetative growth and colony morphology, pigmentation and sporulation with regard to the composition of a specific culture medium, pH, temperature, light, water availability and surrounding atmospheric gas mixture (Kuhn and Ghannoum, 2003). Distinct variations were observed in colony diameter, colony texture, zonation and sporulation. There were no major colony colour variations on both media for all the species except for *A. candidus*. *Aspergillus flavus* strains EM324 and NM091 had slightly larger colony diameters on PDA than on CZ media. However, both showed similar texture, surface and reverse colour, zonation and sporulation in the two types of media (Table 4.7). *Aspergillus flavus* strains NM083, EM244, NM084, EM182, BM071, EM1112, UM127 and BS116 had a significantly greater colony diameter on CZ media than on PDA media. All these *A. flavus* strains had similar zonations on PDA and on CZ apart from strain NM083 which had no zonation on PDA (Table 4.7). All the strains had a heavy sporulation on PDA and moderate sporulation on CZ media. The observations in this study showed that zonations in *Aspergillus* spp. colonies were influenced by the culture media used. Almost all *Aspergillus* strains were characterized with distinct radial furrows or wrinkles on the reverse in PDA with the exception of a few strains as shown in Table 4.7. In CZ media, radial furrows were less prominent or none at all was observed across the *Aspergillus* strains (Table 4.7).

*Aspergillus parasiticus* strain UM082 had a distinct larger colony diameter on CZ (45 mm) media than on PDA (26.7 mm) media. It had a floccose texture on PDA and powdery texture on CZ media. It was slightly radially furrowed on PDA but in CZ it had no zonations. Sporulation was heavy on PDA while moderate on CZ media. *Aspergillus glaucus* strain RS024 had a higher colony diameter on PDA than in CZ media, while for strain EM211 the opposite was true. Strain

RS024 had velvety texture on both media while strain EM211 had a powdery texture on both media. Strain RS024 had zonations on both the two types of media while strain EM211 had zonations on PDA media only. Strain EM211 had heavy sporulation on PDA and poor sporulation on CZ, while strain RS024 had poor sporulation on both media. *Aspergillus wentii* strain BS023 showed slightly larger colony diameter on PDA (26.0 mm) than on CZ (21.0 mm) media. Colony texture was the same in both media and it was slightly wrinkled on the reverse on PDA but no zonation on CZ media. Sporulation was poor on PDA and moderate on CZ media. *Aspergillus sydowii* strain US098 had a slightly larger colony diameter on PDA than on CZ media. It had a velvety colony texture on PDA while fine in CZ. Zonations were present in both media and sporulation was poor on PDA and moderate on CZ media. *Aspergillus fumigatus* strain BM092 had almost equal colony diameter, similar colony texture and zonation on both media. However sporulation was heavy on PDA and moderate on CZ media. *Aspergillus candidus* strain US184 had a larger colony diameter on CZ (19.7 mm) than on PDA (15.7 mm) media. It had a velvety colony texture on PDA but powdery texture on CZ media. It had a distinct change in colony colour in the different types of media. Zonation was only on CZ media and sporulation was heavy on CZ and poor on PDA media. *Aspergillus clavatus* strain RM143 had a larger colony diameter on PDA (31.7 mm) than on CZ (26.0 mm) media. Colony texture on PDA was fine but powdery on CZ media. Zonations were present in both media and sporulation was heavy in both media. *Aspergillus nidulans* strain ES042 had almost equal colony diameters in both media while colony texture was fine on PDA but powdery on CZ media. There were no zonations observed on both media and sporulation was heavy on both PDA and CZ media.

For the aflatoxigenic strains, *A. versicolor* strains T-RS016 and T-RS162 had distinct larger colony diameters on PDA than on CZ media and a velvety texture on both media, while strain T-BS203 had almost equal colony diameter on PDA (17.0 mm) and on CZ (17.7 mm) media, colony texture was floccose on PDA and powdery on CZ media. All the strains had similar zonations in both media. Sporulation of strain T-RS016 and T-RS162 was poor on PDA but moderate and heavy on CZ media respectively. Strain T-BS203 had moderate sporulation on both media types. The atoxigenic *A. versicolor* strains RS193 and RS043 besides colony

diameter, shared similar colony characteristics with those of aflatoxigenic strains T-BS203 and T-RS016 respectively.

Aflatoxigenic *A. flavus* strain T-RS013 had a slightly larger colony diameter on CZ (38.0 mm) than on PDA (36.0 mm) media, but strain T-RM023-2 on the other hand had a larger colony diameter on PDA (32.3 mm) than on CZ (26.7 mm). The two strains had similar colony texture, zonation and sporulation characteristics in both media types. Physical and chemical properties are important factors in diagnostic characteristics of fungi. Therefore, it is essential to use several media in trying to identify and characterize fungus in culture since growth and sporulation on artificial media are important biological characteristics (St-Germain and Summerbell, 1996). The results of this study revealed that culture media differentially influenced the growth, colony character and sporulation of the various *Aspergillus* strains. Between the two test media used in this study, PDA was found to be most suitable for heavy sporulation and reproduced most visible colony morphology while CZ did not favour heavy sporulation of the *Aspergillus* strains. Generally, fungal growth requirements are less stringent as compared to those required for fungal sporulation (Kumara and Rawal, 2008). The observations of this study were similar to the observations made by Sharma and Pandey (2010), where *Aspergillus* spp. exhibited higher colony growth on PDA and better sporulation than in CZ media where growth and sporulation was poor to moderate. PDA is one of the most commonly used culture media because of its simple formulation and its ability to support mycelial growth of a wide range of fungi (Saha *et al.,* 2008). Several researchers have reported PDA to be the best media for mycelial growth (Maheshwari *et al.,* 1999). Poor or moderate fungal sporulation was observed in CZ which may be due to the presence of chloride ion in the test medium (Okunowo *et al.,* 2010).

The results of this study showed that PDA was the best media to use to culture fungi of the *Aspergillus* spp. since it favours faster colony growth and sporulation. The type of culture media used and their chemical compositions considerably influences the mycelia growth rate and production of conidia of various fungi (Zhae and Simon, 2006). Furthermore, variations in spore colour are some of the major criterion commonly used for the identification and taxonomic placement especially of *Aspergillus* species, which seems to be mainly correlated to the

composition of a medium (St-Germain and Summerbell, 1996). Moreover, significant morphological and physiological variations exist particularly for filamentous fungi and thus findings for one species are not readily extrapolated to others (Meletiadis *et al.,* 2001). Mycologists have used different concepts in characterizing fungi of *Aspergillus* species, out of which morphological and reproductive stages are the typical approaches and standard to fungal taxonomy and nomenclature that are still valid (Zain *et al.*, 2009). *Aspergillus* spp. grows on diverse substrates in nature and are ubiquitous in distribution requiring a variety of specific elements for growth and reproduction. These *Aspergillus* spp. are isolated under laboratory conditions on specific culture media such as PDA and CZ, for cultivation, preservation, microscopic examination and biochemical and physiological characterization (Northolt and Bullerman, 1982).

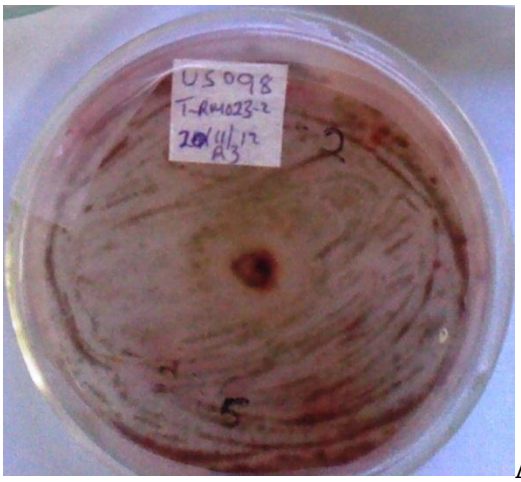
### 4.8 Evaluation of effectiveness of atoxigenic strains in inhibiting growth and sporulation of aflatoxigenic strains in culture

The paper disc diffusion inhibition test was used for the screening of antimould activity of atoxigenic *Aspergillus* strains as described by Souza *et al*. (2005). The atoxigenic *Aspergillus* strains showed a wide variation of inhibitory capabilities against the aflatoxigenic *Aspergillus* strains as evidenced by the inhibition zones (Table 4.8). A clear observation made was that there was a species specificity of the atoxigenic species in inhibiting the growth of the various aflatoxigenic *Aspergillus* species. Atoxigenic strains that were of a different species from an individual aflatoxigenic species, were leading in inhibiting the growth of that particular aflatoxigenic species, and in a few instances even better than nystatin (100 µg) which was the standard control used in this study. Atoxigenic strains that were found to be most effective in inhibiting the growth of all the five aflatoxigenic strains were predominantly strains from two *Aspergillus* species; *A. parasiticus* and *A. flavus*. The two aflatoxigenic *A. flavus* strains also exhibited a particular trend in their response to the various atoxigenic strains. The aflatoxigenic *A. flavus* strain T-RM023-2 isolated from maize was more easily out-grown by the atoxigenic strains as evidenced by larger inhibition zones as compared to the aflatoxigenic *A. flavus* strain T-RS013 isolated from soil, which had inhibition zones of smaller diameter. This particular property denotes that aflatoxigenic *A. flavus* strains from soil are probably better adapted for competition and are therefore more vigorous in growth and sporulation than aflatoxigenic *A.*

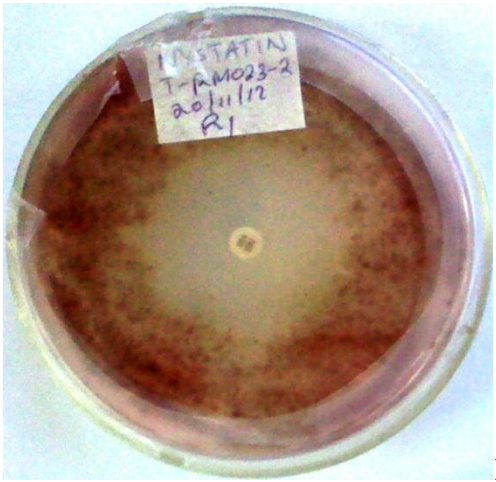
*flavus* strains from maize. However, this is speculation and further work needs to be done to ascertain this observation.

SAS version 9.0 was used to analyze the data. The inhibition zones induced by each atoxigenic *Aspergillus* strain were analyzed through ANOVA to determine if there was any significant difference in the bio-control activity of the various atoxigenic strains against the aflatoxigenic strains in culture. Statistically there was a high significant difference (P = 0.0001) in the inhibition zones induced by the various atoxigenic strains at a 95% confidence limit with an overall C.V. of 24.5% and LSD value of 2.7 (Appendix 2).

The growth of aflatoxigenic *A. flavus* strain T-RM023-2 was best inhibited by the atoxigenic *A. parasiticus* strain UM082 with a mean inhibition zone of 28.7 mm in diameter as compared to the standard control nystatin which had a mean inhibition zone of 36.0 mm (Plate 4.6 B). The next best performing atoxigenic strains that considerably inhibited the aflatoxigenic *A. flavus* strain T-RM023-2 were; *A. flavus* strain BM071 (28.3 mm), *A. flavus* strain NM084 (27.3 mm), *A. flavus* strain NM091 (24.3 mm), *A. sydowii* strain US098 (24.3 mm) (Plate 4.6 (A)) and *A. flavus* strain EM1112 (23.0 mm).



**A**



**B**

**Plate 4.7:** (a) Inhibition zone of atoxigenic *Aspergillus sydowii* strain US098 against aflatoxigenic *Aspergillus flavus* strain T-RM023-2. (b) Inhibition zone of nystatin (100 µg) against aflatoxigenic *Aspergillus flavus* strain T-RM023-2.

**Table 4.8:** Inhibition zones (in mm) induced by atoxigenic *Aspergillus* strains on aflatoxigenic *Aspergillus* strains

| | **Atoxigenic strains** | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Isolate code** | EM324 | NM091 | NM083 | EM244 | NM084 | EM182 | BM071 | EM1112 | UM127 | RS024 | BS023 | US098 | UM082 | BM092 | US184 | RM143 | ES042 | BS116 | EM211 | Positive Control | Negative Control | CV%[a] | LSD[a] |
| **Isolate name** | *A. flavus* | *A. flavus* | *A. flavus* | *A. flavus* | *A. flavus* | *A. flavus* | *A. flavus* | *A. flavus* | *A. flavus* | *A. glaucus* | *A. wentii* | *A sydowii* | *A. parasiticus* | *A. fumigatus* | *A. candidus* | *A. clavatus* | *A. nidulans* | *A. flavus* | *A. glaucus* | *Nystatin* | *Sterile distilled water* | | |
| **Aflatoxigenic strains** | | | | | | | | | | | | | | | | | | | | | | | |
| **T-RS013** ***A. flavus*** | 9.7 | 8 | 10 | 8.7 | 9.3 | 9 | 10 | 10.3 | 10 | 17.3 | 14.3 | 16.7 | 18.7 | 16 | 11.7 | 8.7 | 12 | 17.3 | 12 | 30 | 0 | 4.9 | 1 |
| **T-RS162** ***A. versicolor*** | 15.3 | 13.7 | 22.7 | 18.3 | 18.3 | 13.7 | 15 | 16 | 25 | 12 | 10 | 10 | 17 | 19.3 | 8.7 | 6 | 7 | 15.7 | 15.7 | 20 | 0 | 8.2 | 2 |
| **T-RS016** ***A. versicolor*** | 15.3 | 20 | 22.7 | 23.7 | 22.3 | 25.7 | 21.3 | 20 | 25 | 14 | 9.7 | 12 | 19.3 | 20 | 8 | 7 | 8 | 20 | 19.3 | 21.3 | 0 | 4.7 | 1.4 |
| **T-BS203** ***A. versicolor*** | 12.3 | 14 | 11 | 14.3 | 14 | 12.7 | 14 | 14.7 | 13.3 | 16.7 | 14.7 | 11.7 | 16 | 12.3 | 10 | 9.3 | 9.3 | 11 | 12 | 20.7 | 0 | 5.6 | 1.2 |
| **T-RM023-2** ***A. flavus*** | 18.7 | 24.3 | 20 | 17.7 | 27.3 | 14.3 | 28.3 | 23 | 15.7 | 18.7 | 19.3 | 24.3 | 28.7 | 20 | 14.3 | 11.3 | 19.3 | 22.3 | 17.7 | 36 | 0 | 7.9 | 2.6 |
| **CV%[b]** | 8.1 | 6 | 6.7 | 7.5 | 5.1 | 5.4 | 6 | 6.3 | 5.4 | 4.9 | 5 | 5.2 | 9.5 | 5.5 | 7.4 | 9.2 | 5.2 | 6.3 | 9.7 | 2.3 | - | | |
| **LSD[b]** | 2.1 | 1.8 | 2.1 | 2.3 | 1.7 | 1.5 | 1.9 | 1.9 | 1.8 | 1.4 | 1.2 | 1.4 | 3.5 | 1.8 | 1.4 | 1.4 | 1.1 | 2 | 2.7 | 1.1 | 0 | | |

CV%[a] & LSD[a] - Across all atoxigenic strains

CV%[b] & LSD[b] – Within a single atoxigenic strain

Aflatoxigenic *A. flavus* strain T-RS013 was best inhibited by atoxigenic *A. parasiticus* strain UM082 with a mean inhibition zone of 18.7 mm compared to the standard control nystatin which had a mean inhibition zone of 30.0 mm. Other atoxigenic strains that performed well against the aflatoxigenic *A. flavus* strain T-RS013 include; *A. flavus* strain BS116 (17.3 mm), *A. glaucus* RS024 (17.3 mm), *A. sydowii* US098 (16.7 mm), *A. fumigatus* BM092 (16.0 mm) and *A. wentii* BS023 (14.3 mm).

For aflatoxigenic *A. versicolor* strain T-RS162, the best growth inhibiting atoxigenic strain was *A. flavus* UM127 with a mean inhibition zone of 25.0 mm followed by *A. flavus* NM083 with a mean of 22 .7 mm. These two leading strains performed better than the standard control nystatin which had a mean inhibition zone of 20.0 mm. The other strains that significantly performed well were; *A. fumigatus* BM092 (19.3 mm), EM244 *A. flavus* (18.3 mm), NM084 *A. flavus* (18.3 mm) and UM082 *A. parasiticus* (17.0 mm). Aflatoxigenic strain *A. versicolor* T-RS016 was best inhibited by atoxigenic strain *A. flavus* EM182 (25.7 mm) followed by *A. flavus* UM127 (25.0 mm), *A. flavus* EM244 (23.7 mm), *A. flavus* NM083 (22.7 mm), *A. flavus* NM084 (22.3 mm) and *A. flavus* BM071 (21.3 mm). These performed better than the standard control nystatin which had a mean inhibition zone of 21.3 mm. Aflatoxigenic strains T-BS203 *A. versicolor* was best inhibited by the atoxigenic strain RS024 *A. glaucus* which had a mean inhibition zone of 16.7 mm compared to the standard control nystatin which had a mean 20.7 mm. It was closely followed by UM082 *A. parasiticus* (16.0 mm), EM1112 *A. flavus* (14.7 mm), BS023 *A. wentii* (14.7 mm), EM244 *A. flavus* (14.3 mm), NM091 *A. flavus* (14.0 mm), NM084 *A. flavus* (14.0 mm) and BM071 *A. flavus* (14.0 mm).

Overall, the best atoxigenic *Aspergillus* strain in inhibiting the growth and sporulation of the aflatoxigenic strains was strain UM082 *A. parasiticus* which had an overall mean inhibition zone of 19.9 mm as compared to the overall mean of the standard control (nystatin 100 µg) which was 25.6 mm. The other atoxigenic strains that performed best overall were the following, ranked in order of decreasing potential; NM084 *A. flavus* (18.2 mm), UM127 *A. flavus* (17.8 mm), BM071 *A. flavus* (17.7 mm), BM092 *A. fumigatus* (17.5 mm), NM083 *A. flavus* (17.3 mm), BS116 *A. flavus* (17.3 mm), EM1112 *A. flavus* (16.8 mm), EM244 *A. flavus* (16.5 mm) and NM091 *A.*

*flavus* (16.0 mm). The most effective atoxigenic strains were selected based on the best overall inhibition zone means. In this regard six atoxigenic strains that were not significantly different in their overall inhibition potential (Appendix 2) were selected and suggested for further efficacy trials in green-house and field trials. Propensity to multiply, colonize and survive are other selection criteria to make sure that few reapplications will be required once the atoxigenic strains are introduced in the environment (Ranajit *et al.*, 2005). The six were; UM082 *A. parasiticus* (19.9 mm), NM084 *A. flavus* (18.2 mm), UM127 *A. flavus* (17.8 mm), BM071 *A. flavus* (17.7 mm), BM092 *A. fumigatus* (17.5 mm), and NM083 *A. flavus* (17.3 mm).

The results of this study affirms that the most effective *Aspergillus* spp. that are most suitable for use as bioagents are atoxigenic strains of *A. flavus* and *A. parasiticus.* It is in this principle that other studies seeking to control aflatoxin contamination in maize have mainly used non toxin producing strains of either *A. flavus* or *A. parasiticus*. For instance, biological control using the competitive exclusion mechanism has been successfully implemented on cottonseed in Arizona (Ranajit *et al*., 2005). Natural population of *A. flavus* consists of aflatoxigenic strains that produce copious amount of aflatoxin and atoxigenic strains that lack the capacity to produce aflatoxin. In the competitive exclusion mechanism, introduced atoxigenic strains outcompete and exclude aflatoxigenic strains from colonizing grains thereby reducing aflatoxin production in contaminated cottonseed (Ranajit *et al*., 2005).

Similarly, the potential to reduce aflatoxin contamination in maize using the biological control strategies mentioned above has been evaluated in Benin, where 90% of the *Aspergillus* species are *A. flavus*. The atoxigenic strains of *A. flavus* (BN22 from Benin and AF36 from the US) were tested against aflatoxigenic strains of *A. flavus* (BN40 from Benin and AF13 from the US) and *A. parasiticus* (BN48) *in vitro* (Cotty, 2001). For these *in vitro* trials, maize kernels were dipped in $1\times10^{6}$ conidia $ml^{-1}$ suspension of one *Aspergillus* species or strain and allowed to dry before repeating the dip with either the same or another strain. Kernels were incubated in a saturated environment at 30 $^{o}$C for 5 days, dried at 40 $^{o}$C for 5 days, crushed, extracted in acetone and aflatoxins quantified using thin-layer chromatography (TLC) and a scanning densitometer. All atoxigenic isolates significantly reduced toxin production by the African *A. parasiticus* isolate BN48. The American atoxigenic isolate AF36 was effective against the American aflatoxigenic

isolate AF13, but not the aflatoxigenic African S-strain, BN40 suggesting that there may be specificity of action of some atoxigenic strains. The African atoxigenic L-strain BN30 was the only isolate that reduced toxin production by the aflatoxigenic African S-strain, BN40. Further field tests confirmed the potential of atoxigenic strains to reduce aflatoxin production by aflatoxigenic strains (Ranajit *et al*., 2005). Several micro-organisms have been tested for biological control capabilities against aflatoxin contamination; these include bacteria, yeasts, and atoxigenic strains of the causal organisms (Yan *et al.,* 2008) of which only atoxigenic strains have reached the commercial stage. In the United States, biological control of aflatoxin production in crops has been approved by Environmental Protection Agency and a commercial product based on atoxigenic *Aspergillus flavus* strains is being marketed (Afla-Guard®) (Abbas *et al.,* 2011). Here in Africa, two isolates of *A. flavus* have been identified as atoxigenic strains to competitively outgrow and eliminate aflatoxigenic fungi in the maize fields. These strains have been tested and proved to reduce aflatoxin concentrations in both laboratory tests and field trials, reducing toxin contamination by 70 to 99% (Atehnkeng *et al.,* 2008b). A mixture of four atoxigenic strains of *A. flavus* of Nigerian origin has gained provisional registration (AflaSafe) to determine efficacy in on-farm tests and candidate strains have been selected for Kenya and Senegal (Atehnkeng *et al.,* 2008b).

The atoxigenic *Aspergillus* isolates UM082 *A. parasiticus* (19.9 mm), NM084 *A. flavus* (18.2 mm), UM127 *A. flavus* (17.8 mm), BM071 *A. flavus* (17.7 mm), BM092 *A. fumigatus* (17.5 mm), and NM083 *A. flavus* (17.3 mm) selected from this study will be developed for use with crops and will be tested for the reduction of other mycotoxins such as cyclopiazonic acid (CPA) as recommended by Abbas *et al.,* (2011). Aflatoxins and CPA are co-produced by many aflatoxigenic strains of *A. flavus* (Chang *et al.,* 2005) and they co-accumulate in maize (Georgianna *et al.,* 2010). For that reason, control measures that are effective in preventing aflatoxin contamination are thought to be similarly effective for CPA. The levels of CPA in maize after inoculation with AF36 represent a health hazard, which seems particularly unnecessary given the availability of safer strains with similar or greater efficacy. So far, K49 and Afla-Guard®, meet these criteria (Abbas *et al.,* 2011). It is possible that these new *Aspergillus* isolates from this study will be even more useful as biological control agents. The principal objective in developing bio-control strategies is to minimize mycotoxin contamination

in crops, especially maize (Atehnkeng *et al.,* 2008b). This subject is being investigated around the world (Accinelli *et al.,* 2009). The selected atoxigenic *Aspergillus* strains; UM082 *A. parasiticus* (19.9 mm), NM084 *A. flavus* (18.2 mm), UM127 *A. flavus* (17.8 mm), BM071 *A. flavus* (17.7 mm), BM092 *A. fumigatus* (17.5 mm), and NM083 *A. flavus* (17.3 mm) for use as biocontrol agents should be evaluated for; genetic stability, consistency in growth and sporulation, adaptability, environmentally friendly properties, ability to compete with indigenous *Aspergillus* strains, and ability to control production of (CPA) and sterigmatocystin as well as aflatoxins as suggested by Abbas *et al.* (2011). The limitation of using atoxigenic *Aspergillus* strains as biocontrol agents is that the atoxigenic strains may be effective within the regions of isolation but fail to work in other regions due to species diversity. However, biological control holds promise of offering a long-term solution for reducing aflatoxin contamination in maize, and the results of this study will contribute significantly towards the realization of this noble course.

# CHAPTER FIVE
# CONCLUSIONS AND RECOMMENDATIONS

## CONCLUSIONS

The results of this study indicated that *Aspergillus* species diversity across the selected districts was not different, and "good" looking maize perceived to be safe for human consumption is contaminated with moulds of the genus *Aspergillus* with *A. flavus* being the major contaminant. All the "good" maize samples from Makueni, Nyeri Central, Moiben, Ugunja and Bungoma South districts were found to have higher *Aspergillus* spp. contaminations compared to their respective soil samples. It is evident that residents of these districts are consuming the "good" maize oblivious of the health risks that they are exposed to. According to this study, there are more aflatoxigenic *Aspergillus* strains in soil than in maize. Sporulation characteristics cannot provide a reliable means of categorizing aflatoxigenic and atoxigenic strains of *Aspergillus* species as the compared aflatoxigenic and atoxigenic strains of *A. flavus* and *A. versicolor* had similar sporulation characteristics in both PDA and CZ media. Atoxigenic *Aspergillus* strains *A. parasiticus* UM082, *A. flavus* NM084, *A. flavus* UM127, *A. flavus* BM071, *A. fumigatus* BM092, and *A. flavus* NM083 were the most effective in inhibiting the growth and sporulation of the aflatoxigenic *Aspergillus* strains and hence controlling the production of aflatoxins. They had varying levels of inhibition against the aflatoxigenic *Aspergillus* strains *A. flavus* T-RS013, *A. flavus* T-RM023-2, *A. versicolor* T-RS162, T-RS016 *A. versicolor* and T-BS203 *A. versicolor*.

## RECOMMENDATIONS

1. The atoxigenic *Aspergillus* strains effective in reducing growth and sporulation of aflatoxigenic strains should be tested for the reduction of aflatoxin and cyclopiazonic acid (CPA) in maize and other cereals.
2. Further evaluation of the selected atoxigenic *Aspergillus* strains through green-house and field trials should be conducted to determine their effectiveness in inhibiting growth and sporulation of the aflatoxigenic strains in the natural environment.
3. The effective atoxigenic strains should be developed and used as the main components or adjuvants of anti-mould products in protection of crops against

aflatoxigenic strains and their associated toxins and also in food preservation systems.

4. Molecular characterization and sequencing of the effective atoxigenic *Aspergillus* strains should be done and the bioactive compounds isolated and tested against growth of these aflatoxigenic moulds and other moulds and aflatoxin production.
5. Atoxigenic fungal isolates from other species such as *Trichoderma* and *Penicillium* should also be evaluated for inhibitory properties against the growth and sporulation of aflatoxigenic moulds.
6. Further studies on the mechanism of inhibition by the atoxigenic *Aspergillus* strains should be done to establish their mode of action.
7. There is need to educate farmers on better pre and post-harvest maize handling procedures to minimize transfer of aflatoxigenic moulds from the soil to the maize produce. After harvesting, maize should be well dried to a moisture content of 12% and stored in well ventilated granaries, to prevent buildup of favourable conditions for growth of aflatoxigenic moulds and subsequent aflatoxin production.
8. The existing regulatory policies on maize quality standards and acceptable levels of aflatoxins in maize and cereals should be strictly enforced to protect people from consuming contaminated maize cereals.

## REFERENCES


Abbas, K.H., Mark, A.W., Bruce, W.H., Ignazio, C., James, T.M., and Shier, W.T. (2011). Special Double Issue—*Aspergillus*, Aflatoxins, Cyclopiazonic Acid and Biological Control of Aflatoxins. *Toxin Reviews.* **30 (2–3):** 59–70.

Accinelli, C., Saccà , M.L., Abbas, H.K., Zablotowicz, R.M. and Wilkinson, J.R. (2009). Use of a granular bioplastic formulation for carrying conidia of a non-aflatoxigenic strain of *Aspergillus flavus*. *Bioresource Technology.* **100:** 3997–4004.

Aflacontrol (2010). The aflacontrol project: reducing the spread of aflatoxins in Kenya and Mali. *Project note 1 July 2010*. Pp 1.

Anonymous, (2004). Outbreak of aflatoxin poisoning – Eastern and Central provinces, Kenya, January-July 2004. *Morbidity and Mortality Weekly Report (MMWR).* **53:** 790-793.

Anonymous, (2006). Diamond Pet Foods narrows recall after aflatoxin tests. *Journal of the American Veterinary Medical Association.* **228:** 490.

Atehnkeng, J., Ojiambo, P.S., Donner, M., Ikotun, T., Sikora, R.A., Cotty, P.J. and Bandyopadhyay, R. (2008a). Distribution and toxigenicity of *Aspergillus species* isolated from maize kernels from three agro-ecological zones in Nigeria. *International Journal of Food Microbiology.* **122:** 74–84.

Atehnkeng, J., Ojiambo, P. S., Ikotun, T., Sikora, R. A., Cotty, P. J and Bandyopadhayay, R. (2008b). Evaluation of atoxigenic isolates of *Aspergillus flavus* as potential biocontrol agents for aflatoxin in maize. *Food Additives and Contaminants.* **25:** 1264-1271.

Azziz-Baumgartner, E., Lindblade, K., Gieseker, K., Schurz Rogers, H., Kieszak, S., Njapau, H., Schleicher, R., McCoy, L.F., Misore, A., DeCock, K., Rubin, C., Slutsker, L. and the Aflatoxin Investigative Group (2005). Case-control study of an acute aflatoxicosis outbreak – Kenya 2004. *Environmental Health Perspectives.* **113:** 1779-1783.

Bailey, G.S., Price, R.L., Park, D.L. and Hendricks, J.D. (1994). Effect of ammoniation of aflatoxin B1-contaminated cottonseed feedstock on the aflatoxin M1 content of cows' milk and hepatocarcinogenicity in the trout bioassay. *Food and Chemical Toxicology.* **32:** 707-715.

Bennett, J.W. and Klich, M. (2003). Mycotoxins. *Clinical Microbiology Reviews.* **16:** 497-516.

Bock, C.H., and Cotty, P.J. (1999). The relationship of gin date to aflatoxin contamination of cotton-seed in Arizona. *Plant Disease.* **83:** 279-285.

Boken, V.K., Hoogenboom, G., Williams, J.H., Diarra, B., Dione, S. and Easson, G.L. (2008). Monitoring peanut contamination in Mali (Africa) using the AVHRR satellite data and a crop simulation model. *International Journal of Remote Sensing.* **29:** 117-129.

Burnside, J.E., Sippel, W.L., Forgacs, J., Carll, W.T., Atrwood, M.B. and Doll, E.R. (1957). A disease of swine and cattle caused by eating mouldy corn. II. Experimental production with pure cultures of molds. *American Journal of Veterinary Research.* **18:** 817-824.

CAST, (2003). Council for Agricultural Science and Technology. Mycotoxins: Risks in plant, animal, and human systems. Ames, Iowa, USA. Task Force Report 139.

Chang, P.K, Horn, B.W. and Dorner, J.W. (2005). Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. *Fungal Genetics and Biology.* **42:** 914–923.

Christensen, C.M. (1975). Moulds, Mushrooms, and Mycotoxins. University of Minnesota Press, Minneapolis. Pp 264.

Cotty, P.J. (1994). Influence of field application of an atoxigenic strain of *Aspergillus flavus* on the populations of *A. flavus* infecting cotton bolls and on the aflatoxin content of cottonseed. *Phytopathology.* **84:** 1270–1277.

Cotty, P.J. (2001). Cotton seed losses and mycotoxins. In; Kirkpatrick, T.L. and Rothrock, C.S. (eds.) *Compendium of Cotton Diseases. Part 1. Infectious Diseases.* 2nd ed. APS Press, St. Paul, Minnesota. Pp 9-13.

Diener, U.L., Cole, R.J., Sanders, T.H., Payne, G.A., Lee, L.S and Klich, M.A. (1987). Epidemiology of aflatoxin formation by *Aspergillus flavus*. *Annual Review of Phytopathol*ogy. **25:** 249-270.

Dorner, J.W., Cole, R.J. and Blankenship, P.D. (1998). Effect of inoculum rate of biological control agents on pre-harvest aflatoxin contamination of peanuts. *Biological Control.* **12:** 171-176.

Dorner, J.W. (2004). Biological control of aflatoxin contamination of crops. *Journal of Toxicology Toxin Reviews.* **23:** 435–450.

Dowd, P.F. (2003). Insect management to facilitate preharvest mycotoxin management. In: Abbas, (ed.). Aflatoxin and Food Safety - Part 1. *Journal of Toxicology Toxins Reviews.* **22:** 327-350.

Egal, S., Hounsa, A., Gong, Y.Y., Turner, P.C., Wild, C.P., Hall, A.J., Hell, K. and Cardwell, K.F. (2005). Dietary exposure to aflatoxin from maize and groundnut in young children from Benin and Togo, West Africa. *International Journal of Food Microbiology*. **104:** 215-224.

Fandohan, P., Hell, K. and Marasas, W.F. (2003). Infection of maize by *Fusarium* species and contamination with fumonisins in Africa. *African Journal of Biotechnology*. **2 (12):** 570-579.

FEMA, (2005). Mold Health Issues. Assessed from http://en.wikipedia.org/wiki/Mold-health-issues. 19th June 2011, 11.50 a.m.

Georgianna, D.R, Fedorova, N.D, Burroughs, J.L, Dolezal, A.L, Bok, J.W, Horowitz, Brown. S., Woloshuk, C.P. and Payne, G.A. (2010). Beyond aflatoxin: four distinct expression patterns and functional roles associated with *Aspergillus flavus* secondary metabolism gene clusters. *Molecular Plant Pathology*. **11:** 213–226.

Golinski, P. (1991). Secondary metabolites (mycotoxins) produced by fungi colonizing cereal grain in storage - structure and properties. In: *Cereal Grain, Mycotoxins, Fungi and Quality in Drying and Storage.* In: J. Chelkowski (ed.). Amsterdam: Elsevier Science Publishers. Pp 355-403.

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

Horn, B.W. (2007). Biodiversity of *Aspergillus* section *flavi* in the United States: A review. *Food Additives and Contaminants.* **24:** 1088–1101.

Hunter, B.T. (1989). Mycotoxins: A Significant public health problem. *Consumer's Research.* **72 (6):** 8-9.

Kaur, J. and Arora, D. (1999). Antimicrobial activities of species. *International Journal of Antimicrobial Agents.* **12:** 257-262.

Kaaya, A.N., Kyamuhangire, W. and Kyamanwa, S. (2006). Factors affecting aflatoxin contamination of harvested maize in the three agro ecological zones of Uganda. *Journal of Applied Sciences.* **6:** 2401-2407.

Klich, M.A. (2002). Identification of common *Aspergillus* species (1st edition). Published by the Centraalbureau voor Schimaelcultures, Utrecht, The Netherlands *An institute of the Royal Netherlands Academy of Arts and Sciences.* Pp 12-106.

Kuhn, D.M. and Ghonnoum, M.A. (2003). Indoor mold, toxigenic fungi, and *Stachybotrys chartarum*: Infectious disease perspective. *Clinical Microbiology Reviews.* **16 (1):** 144-172.

Kumara, K. L.W. and Rawal, R. D. (2008). Influence of carbon, nitrogen, temperature and pH on the growth and sporulation of some Indian isolates of *Colletotrichum gloeosporioides* causing anthracnose disease of papaya (*Carrica papaya L*). *Tropical Agricultural Research and Extension.* **11:** 712.

Kung'u, J. (2005). Mould and food spoilage. http://www.moldbacteria.com/myblog/2005/07/mould- and -food- spoilage.html. Accessed Jun 19th, 2011 at 12.20 p.m.

Lamboni, Y. and Hell, K. (2009). Propagation of mycotoxigenic fungi in maize stores by post-harvest insects. *International Journal of Tropical Insect Science.* **29:** 31–39.

Larone, D.H. (1995). Medically important fungi – A guide to identification, 3rd ed. ASM press, Washington D.C. http://doctorfungus.org/thefungi/*Aspergillus* spp.htm. Accessed June 17th, 2011 at 2.40 p.m.

Luciana de Castro and Eugenia, A.V. (2001). Determining Aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ in maize using florisil clean up with thin layer chromatography and visual and densitometric quantification. *Ciênc. Tecnol. Aliment., Campinas.* **21:** 115-122.

Maheshwari, S.K., Singh, D.V. and Sahu, A.K. (1999). Effect of several nutrient media, pH and carbon sources on growth and sporulation of *Alternaria alternata*. *Journal of Mycopathological Research.* **37:** 21-23.

Martins, M.L., Martins, H.M. and Bernedo, F. (2001). Aflatoxins in spices marketed in Portugal. *Journal of Food Additives and Contaminants.* **18:** 315-319.

Matossian, M.K. (1989). Poisons of the Past: Molds, Epidemics and History. Yale University Press, New Haven. Pp 248.

Meletiadis, J., Meis, J.F.G.M., Mouton, J.W. and Verweij, P.E. (2001). Analysis of growth characteristics of filamentous fungi in different nutrient media. *Journal of Clinical Microbiology.* **39 (2):** 478-484.

Munkvold, G.P., Hurburgh, C. and Meyer, J. (2009). File: Pest Management 2-5. Iowa State University. University Extension.

Muthomi, J.W., Mureithi, B.K., Chemining'wa, G.N., Gathumbi, J.K. and Mutitu, E.W. (2012). *Aspergillus* species and Aflatoxin $B_1$ in soil, maize grain and flour samples from semi-arid and humid regions of Kenya. *International Journal of AgriScience.* **2 (1):** 22-34.

Mutungi, C., Lamuka, P., Arimi, S., Gathumbi, J.K. and Onyango, C. (2008). The fate of aflatoxins during processing of maize into muthokoi – a traditional Kenyan food. *Food Control*. **19:** 714–721.

Northolt, M.D. and Bullerman, L.B. (1982). Prevention of mold growth and toxin production through control of environmental condition. *Journal of Food Protection.* **6:** 519-526.

Okoth, S., Beatrice, N., Vincent, A., Erastus, K., Hannu, K. and Vesa, J. (2012). Toxigenic Potential of *Aspergillus* species Occurring on Maize Kernels from Two Agro-Ecological Zones in Kenya. *Toxins.* **4:** 991-1007.

Okunowo, W.O., Gbenle, G.O., Osuntoki, A.A. and Adekunle, A.A. (2010). Media studies on *Myrothecium roridum* Tode: *A potential biocontrol agent for water hyacinth. Journal of Yeast Fungal Research.* **1 (4):** 55-61.

Patten, R.C. (1981). Aflatoxins and disease. *American Journal of Tropical Medicine and Hygiene.* **30:** 442-425.

Pier, A.C. and Richard, I.L. (1992). Mycoses and mycotoxicoses of animals caused by *Aspergilli*. In: J.W. Bennett & M.A. Klich (eds.) *Aspergillus*: Biology and Industrial Applications. Pp 233-248. Boston: Butterwoth-Heinemann.

Pitt, J. I. (2000). Toxigenic Fungi and Mycotoxins. *British Medical Bulletin.* **56 (1):** 184-192.

Ranajit, B., Sebastian, K., Joseph, A., Matthias, D., Peter, J.C. and Kerstin, H. (2005). Biological control of aflatoxin contamination in maize in Africa. *Conference on International Agricultural Research for Development.*

Riley, R.T. and Norrad, W.P. (1999). Mycotoxin prevention and decontamination – A case study on maize. Agricultural Research Service, Athens, Georgia, United States.

Rodrigues, P., Soares, C., Kozakiewicz, Z., Paterson, R.R.M., Lima, N., and Venancio, A. (2007). In: A. Méndez-Vilas (ed.). Identification and characterization of *Aspergillus flavus* and aflatoxins. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*. Pp 527-534.

Russell, T.E. (1982). *Aflatoxins in Cotton Seed*. University of Arizona Cooperative Extension Service, Tucson, Arizona. Publication Q422.

Russell, T.E., Watson, T.F. and Ryan, G.F. (1976). Field accumulation of aflatoxin in cottonseed as influenced by irrigation termination dates and pink bollworm infestation. *Applied and Environmental Microbiology.* **31:** 711-713.

Ryan, K.J. and Ray, C.G. (2004). *Sherris Medical Microbiology* (4th edition). Mc Graw Hill. Pp 633-638.

Saha, A., Mandal, P., Dasgupta, S. and Saha, D. (2008). Influence of culture media and environmental factors on mycelial growth and sporulation of *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. *Journal of Environmental Biology.* **29 (3):** 407-410.

Saito, M. and Machida, S. (1999). A rapid identification method for aflatoxin-producing strains of *A. flavus* and *A. parasiticus* by ammonia vapour. *Mycoscience.* **40:** 205–211.

Schmidt-Heydt, M., Rüfer, C.E., Abdel-Hadi, A., Magan, N. and Geisen, R. (2010). The production of aflatoxin B1 or G1 by *Aspergillus parasiticus* at various combinations of temperature and water activity is related to the ratio of aflS to aflR expression. *Mycotoxin Research.* **26:** 241–246.

Setamou, M., Cardwell, K.F. Schulthess, F. and Hell, K. (1998). Effect of insect damage to maize ears, with special reference to the earborer *Mussidia nigrivenella,* on *Aspergillus flavus* infection and aftoxin production in pre-harvest maize in the Republic of Benin. *Journal of Economic Entomology.* **91:** 433.

Shephard, G.S. (2003). Aflatoxin and food safety: Recent African perspective. *Journal of Toxicology.* **22:** 267-286.

Shephard, G.S. (2008) Impact of mycotoxins on human health in developing countries. *Food Additives and Contaminants.* **25 (2):** 146–151.

Souza, E.L., Lima, E.O, Freire, K.R. and Sousa, C.P. (2005). Inhibitory action of some essential oils and phytochemicals on the growth of various moulds isolated from foods. *Brazilian Archives of Biology and Technology*. **48:** 245-250.

St-Germain, G. and Summerbell, R. (1996). Identifying Filamentous Fungi – A Clinical Laboratory Handbook, 1st Ed. Star Publishing Co., Belmont, California.

Strosnider, H., Azziz-Baumgartner, E., Banziger, M., Bhar, R.V., Breiman, R., Brune, M., DeCock, K., Dilley, A., Groopman, J., Hell, K., Henry, S.H., Jeffers, D., Jolly, C., Jolly, P., Kibata, G.N., Lewis, L., Liu, X., Luber, G., McCoy, L., Mensah, P., Miraglia, M., Misore, A., Njapau, H., Ong, C., Onsongo, M.T.K., Page, S.W., Park, D., Partel, M., Phillips, T., Pineiro, M., Pronczuk, J., Rogers, H.S., Rubin, C., Sabino, M., Schaafsma, A., Shephard, G., Stroka, J., Wild, C., Williams, J.T. and Wilson, D. (2006). Workgroup report: Public health strategy for reducing Aflatoxin exposure in developing countries. *Environ Health Perspect.* **114 (12):** 1898-1903.

van Egmond, H.P. (2004). Natural toxins: Risks, regulations and the analytical situation in Europe. *Analytical and Bioanalytical Chemistry*. **378:** 1152-1160.

Varga, J., Frisvad, J.C. and Samson, R.A. (2011). Two new aflatoxin producing species and an overview of *Aspergillus* section *Flavi*. *Studies in Mycology Journal.* **69 (1):** 57-80.

Wagacha, J.M. and Muthomi, J.W. (2008). Mycotoxin problem in Africa: Current status, implications to food safety and health and possible management strategies. *International Journal of Food Microbiology.* **124:** 1-12.

Whitaker, T.B. and Johansson, A.S. (2005). Sampling uncertainties for the detection of chemical agents in complex food matrixes. *Journal of Food Protection.* **68:** 1306-1313.

Widstrom, N.W. (1996). The aflatoxin problem with corn grain. *Advances in Agronomy*. **56:** 219-280.

Wild, C.P. (2007). Aflatoxin exposure in developing countries: The critical interface of agriculture and health. *Food and Nutrition Bulletin.* **28:** S372–S380.

Wild, C.P. and Turner, P.C. (2002). The toxicology of aflatoxins as a basis for public health decisions. *Mutagenesis.* **17:** 471-481.

Wild, C.P., Hudson, G.J., Sabbioni, G. Chapot, B., Hall, A.J., Wogan, G.N., Whittle, H., Montesano, R. and Groopman, J.D. (1992). Dietary-intake of aflatoxins and the level of

albumin-bound aflatoxin in peripheral blood in the Gambia, West Africa. *Cancer Epidemiology, Biomarkers and Prevention.* **1:** 229-234.

Williams, J.H., Phillips, T.D., Jolly, P.E., Stiles, J.K., Jolly, C.M. and Aggarwal, D. (2004). Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences and interventions. *American Journal of Clinical Nutrition.* **80:** 1106-1122.

Wilson, D.M. and Payne, G.A. (1994). Factors affecting *Aspergillus flavus* group infection and aflatoxin contamination of crops. In Eaton, D.L. and Groopman, J.D. (eds.) *The Toxicology of Aflatoxins. Human Health, Veterinary, and Agricultural Significance.* Academic Press, San Diego, California. Pp 309-329.

Yan, L.Y., Jiang, J.H. and Mao, Z.H. (2008). Biological control of aflatoxin contamination of crops. *Journal of Zhejiang University Science.* **B 9:** 787-792.

Yin, Y.N., Yan, L.Y., Jiang, J.H and Mao, Z.H. (2008). Biological control of aflatoxin contamination of crops. *Journal of Zhejiang Universal Science.* **9:** 787-792**.**

Zain, M.E., Razak, A.A, El-Sheikh, H.H., Soliman, H.G. and Khalil, A.M. (2009). Influence of growth medium on diagnostic characters of *Aspergillus* and *Penicillium* species. *African Journal of Microbiology Research.* **3 (5):** 280-286.

Zeringue, H.J.J., Shih, B.J., Maskos, K. and Grimm, D. (1999). Identification of the bright-greenish-yellow-fluorescence (BGY-F) compound on cotton lint associated with aflatoxin contamination in cottonseed. *Phytochemistry.* **52:** 1391-1397.

Zhae, S. and Simon, F.S. (2006). Effect of culture media, temperature, pH and bio-herbicidal efficacy of *Phoma exigua*, a potential biological control for salal (*Gaultheria shallon*). *Biocontrol Science and Technology.* **6:** 1043-1055.

# APPENDICES

## Appendix 1

**Diagnostic features that were used in identification and characterization of common *Aspergillus* species according to Klich (2002)**

*Aspergillus flavus*: Conidia deep green, olive green or olive, sometimes overlaid with greyish yellow to olive yellow areas; mycelium white; sclerotia, when present, dark brown to black variable in shape and size; exudate when present, uncoloured to brown; reverse uncoloured, dull brown or orangish; variable in colony texture, lanose to floccose. Conidial heads radiate to columnar. Stipe walls usually quite rough, occasionally finely roughened, generally uncoloured, sometimes very pale brown; vesicles spherical to elongate; variable in seriation, biseriate or uniseriate; metulae covering three quarters to the entire surface of the vesicle. Conidia globose to ellipsoidal with smooth to finely roughened walls.

*Aspergillus parasiticus*: Conidia dark olive or deep dark green; mycelium white, usually inconspicuous; sclerotia occasionally formed, brown to black; exudate hyaline when present; reverse uncouloured, dull pinkish-red or dull yellow, sometimes brown; colonies usually quite low, velutinous, some isolates becoming floccose. Conidial heads usually radiate; stipe walls finely roughened to very rough, colourless; vesicles spherical or slightly elongate; predominantly uniseriate, up to 20% biseriate in some isolates, metulae or phialides covering at least half of the vesicle. Conidia globose, distinctly rough-walled.

*Aspergillus versicolor*: Conidia variable in abundance, dull green to grey green; mycelium white, dull pink, buff or orange; exudate, when present, uncoloured to red brown; reverse uncoloured to shades of brown or reddish purple; soluble pigment, when present, pink to brownish orange or brown; colony texture generally velutinous, often radially sulcate. Conidial heads radiate; stipes uncoloured to yellow or slightly brownish, smooth-walled, brittle, expanding into pyriform to spathulate vesicles; biseriate; metulae covering half to the entire vesicle. Diminutive conidial heads sometimes present, resembling penicillium. Conidia mostly globose to subglobose, with finely to distinctly roughened walls.

*Aspergillus clavatus*: Conidia dull green, greyish turquoise, dark turquoise or dull green; mycelium white, inconspicuous to floccose; exudate, when present, uncoloured; reverse uncoloured or in dull yellow to brownish colours; colonies dense, plane to radially furrowed. Conidial heads radiate, or splitting into columns in age; stipes smooth-walled, colourless to slightly brown near the apices, expanding gradually into clavate vesicles, smaller vesicles sometimes pyriform, conidial zone extending from down the apices of the vesicles; uniseriate with phialides. Conidia smooth-walled, ellipsoidal, occasionally pyriform, apiculate or almost cylindrical.

*Aspergillus sydowii*: Colonies heavily sporulating in greyish turquoise or dark turquoise to dark green colours; mycelium white; exudate reddish brown to dark brown when present; reverse brown to orange brown or maroon; soluble pigment, when present coloured as reverse; colonies dense, velutinous to lanose, radially, radially sulcate. Conidial heads radiate; stipes smooth, thick-walled, colourless to pale brown, expanding into pyriform, spathulate, or almost clavate vesicles; usually biseriate. Diminutive conidial structures produced by many isolates, down to simple penicillate heads. Conidia spherical, very rough to spinose.

*Aspergillus fumigatus*: Conidia greyish turquoise or dark turquoise to dark green or dull green; mycelium white; exudate, when present, uncoloured; reverse uncoloured, yellowish, red brown or green; soluble pigment usually absent, occasionally in reddish brown colours; texture velutinous to floccose, plane or radially furrowed. Conidial heads predominantly columnar; stipes uncoloured or greyish near the apices, smooth-walled, expanding gradually into pyriform or spathulate vesicles; uniseriate; phialides cover upper half to two thirds of the vesicle, usually curving such that all phialides are parallel to each other and the stipe axis. Conidia globose to broadly ellipsoidal, smooth to finely roughened or spinose.

*Aspergillus glaucus*: Colonies are grayish-turquoise to deep green with yellow central areas due to cleistothecial production. Reverse is pale yellow to pale brown. Conidial heads are radiate to loosely columnar. Conidiophores are smooth walled and uncolored to pale brown. Vesicles are globose to subglobose, and are uniseriate. Phialides cover the upper portion of the vesicle. Conidia are globose to subglobose, finely roughened to echinulate. Cleistothecia are yellow, thin-walled, globose and are usually covered with red hyphae.

*Aspergillus nidulans*: Conidia sparse to abundant, green, deep green or dark green; mycelium white to cream or greyish; exudate dull red to brown, when present; reverse uncoloured to brownish orange or deep purple red; soluble pigment similarly coloured or pink; colony velutinous or with a floccose overlay, dense, plane or sulcate. Conidial heads radiate to loosely columnar; stipes smooth-walled, becoming brown in age, expanding into spathulate to pyriform vesicles; biseriate, metulae covering only the upper half of the vesicle. Conidia spherical, usually smooth to slightly rough.

*Aspergillus candidus*: Conidia pure white to pale yellow; mycelium white, dense; sclerotia sometimes formed, dull pinkish purple initially, becoming black in age; exudate, when present, uncoloured to yellow; reverse uncoloured, light or yellowish orange; colony dense, velutinous to lanose, plane or radially sulcate. Conidial heads radiate; stipes smooth-walled to finely roughened, uncoloured; vesicles globose to somewhat elongate; predominantly biseriate but small ones often uniseriate: metulae covering the entire surface of the vesicle: often only 2-3 phialides per metulae. Conidia globose to slightly ovoid, smooth-walled.

*Aspergillus wentii*: Conidia variable in abundance, greyish yellow to olive brown; mycelium dense, white to pale yellow; dense white to pinkish hyphal masses sometimes found; exudate, when present, uncoloured to yellow-brown; reverse uncoloured to yellow or pale brown; texture variable velutinous to floccose, plane or radially sulcate. Conidial heads radiate, often splitting into columns in age; stipes often sinuous, usually uncoloured, smooth-walled or slightly warted beneath vesicles; vesicles elongate to globose; biseriate; metulae densely packed covering most of the vesicular surface. Conidia globose to broadly ellipsoidal, surface smooth to very rough.

**Appendix 2**

**ANOVA and LSD tables for frequency of maize and soil isolates across the districts**

| Class | Levels | Values |
|---|---|---|
| Origin | 2 | Maize, Soil |
| Region | 5 | Bungoma South, Makueni, Nyeri Central, Ugunja, Moiben. |

Number of observations 10

Dependent Variable: Frequency

| Source | DF | Sum of Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Model | 5 | 4051.0 | 810.2 | 0.92 | 0.5489 |
| Error | 4 | 3535.4 | 883.85 | | |
| Corrected Total | 9 | 7586.4 | | | |

| R-Square | Coeff Var | Root MSE | Frequency Mean |
|---|---|---|---|
| 0.533982 | 170.8599 | 29.72961 | 17.40000 |

| Source | DF | Type I SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Origin | 1 | 1299.6 | 1299.6 | 1.47 | 0.2920 |
| Region | 4 | 2751.4 | 687.85 | 0.78 | 0.5930 |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Origin | 1 | 1299.6 | 1299.6 | 1.47 | 0.2920 |
| Region | 4 | 2751.4 | 687.85 | 0.78 | 0.5930 |

**LSD Table 1: Comparison of frequency distribution of maize and soil isolates across the Districts**

t Tests (LSD) for Frequency

NOTE: This test controls the Type I comparison wise error rate, not the experimentwise error rate.

| | |
|---|---|
| Alpha | 0.05 |
| Error Degrees of Freedom | 4 |
| Error Mean Square | 883.85 |
| Critical Value of t | 2.77645 |
| Least Significant Difference | 82.543 |

Means with the same letter are not significantly different.

| t Grouping | Mean | N | Region |
|---|---|---|---|
| A | 50.50 | 2 | Makueni |
| A | | | |
| A | 11.00 | 2 | Nyeri Central |
| A | | | |
| A | 9.00 | 2 | Moiben |
| A | | | |
| A | 9.00 | 2 | Ugunja |
| A | | | |
| A | 7.50 | 2 | Bungoma South |

**LSD Table 2: Comparison of frequency of isolates between the sources in all districts**

t Tests (LSD) for Frequency

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

| | |
|---|---|
| Alpha | 0.05 |
| Error Degrees of Freedom | 4 |
| Error Mean Square | 883.85 |
| Critical Value of t | 2.77645 |
| Least Significant Difference | 52.205 |

Means with the same letter are not significantly different.

| t Grouping | Mean | N | Origin |
|---|---|---|---|
| A | 28.80 | 5 | Maize |
| A | | | |
| A | 6.00 | 5 | Soil |

**ANOVA and LSD tables for frequency distribution of *Aspergillus* species across all districts**

Class Levels Values

Region 5 Bungoma South, Makueni, Nyeri Central, Ugunja, Moiben.

Species 10 A.candid A.clavat A.flavus A.fumiga A.glaucu A.nidula A.parasi A.sydowi A.versic A.wentii

Number of observations 50

Dependent Variable: frequency

| Source | DF | Sum of Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Model | 13 | 3701.96 | 284.766154 | 1.86 | 0.0699 |
| Error | 36 | 5498.52 | 152.736667 | | |
| Corrected Total | 49 | 9200.48 | | | |

| R-Square | Coeff Var | Root MSE | frequency Mean |
|---|---|---|---|
| 0.402366 | 355.1341 | 12.35867 | 3.48 |

| Source | DF | Type I SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Region | 4 | 550.28 | 137.57 | 0.90 | 0.4738 |
| Species | 9 | 3151.68 | 350.186667 | 2.29 | 0.0376 |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Region | 4 | 550.28 | 137.57 | 0.90 | 0.4738 |
| Species | 9 | 3151.68 | 350.186667 | 2.29 | 0.0376 |

**LSD Table: Comparisons of frequency distribution of *Aspergillus* species across all districts**

t Tests (LSD) for frequency

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

| | |
|---|---|
| Alpha | 0.05 |
| Error Degrees of Freedom | 36 |
| Error Mean Square | 152.7367 |
| Critical Value of t | 2.02809 |
| Least Significant Difference | 11.209 |

Means with the same letter are not significantly different.

| t Grouping | Mean | N | Region |
|---|---|---|---|
| A | 10.100 | 10 | Makueni |
| A | | | |
| A | 2.200 | 10 | Nyeri Central |
| A | | | |
| A | 1.800 | 10 | Moiben |
| A | | | |
| A | 1.800 | 10 | Ugunja |
| A | | | |
| A | 1.500 | 10 | Bungoma South |

**ANOVA and LSD tables for frequency distribution of atoxigenic and aflatoxigenic *Aspergillus* strains across the districts**

| Class | Levels | Values |
|---|---|---|
| Region | 5 | Bungoma South, Makueni, Nyeri Central, Ugunja, Moiben. |
| Source | 2 | Maize Soil |
| Toxigenicity | 2 | Atoxigenic Aflatoxigenic |

Number of observations 20

Dependent Variable: Frequency

| Source | DF | Sum of Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Model | 6 | 3150.5 | 525.083333 | 1.17 | 0.3791 |
| Error | 13 | 5833.7 | 448.746154 | | |
| Corrected Total | 19 | 8984.2 | | | |

| R-Square | Coeff Var | Root MSE | Frequency Mean |
|---|---|---|---|
| 0.350671 | 243.4900 | 21.18363 | 8.700000 |

| Source | DF | Type I SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Region | 4 | 1375.7 | 343.925 | 0.77 | 0.5657 |
| Source | 1 | 649.8 | 649.8 | 1.45 | 0.2503 |
| Toxigenicity | 1 | 1125.0 | 1125.0 | 2.51 | 0.1374 |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Region | 4 | 1375.7 | 343.925 | 0.77 | 0.5657 |
| Source | 1 | 649.8 | 649.8 | 1.45 | 0.2503 |
| Toxigenicity | 1 | 1125.0 | 1125.0 | 2.51 | 0.1374 |

**LSD Table: Comparison of frequency distribution of aflatoxigenic and atoxigenic strains across the districts**

t Tests (LSD) for Frequency

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

| | |
|---|---|
| Alpha | 0.05 |
| Error Degrees of Freedom | 13 |
| Error Mean Square | 448.7462 |
| Critical Value of t | 2.16037 |
| Least Significant Difference | 32.36 |

Means with the same letter are not significantly different.

| t Grouping | Mean | N | Region |
|---|---|---|---|
| A | 25.25 | 4 | Makueni |
| A | | | |
| A | 5.50 | 4 | Nyeri Central |
| A | | | |
| A | 4.50 | 4 | Moiben |
| A | | | |
| A | 4.50 | 4 | Ugunja |
| A | | | |
| A | 3.75 | 4 | Bungoma South |

**ANOVA and LSD tables for zones of inhibition of the atoxigenic *Aspergillus* strains against the aflatoxigenic *Aspergillus* strains**

| Class | Levels | Values |
|---|---|---|
| aflatoxigenic | 5 | T-BS203 T-RM023- T-RS013 T-RS016 T-RS162 |
| atoxigenic | 21 | BM071 BM092 BS023 BS116 EM1112 EM182 EM211 EM244 EM324 ES042 NEGATIVE NM083 NM084 NM091 POSITIVE RM143 RS024 UM082 UM127 US098 US184 |

Number of observations 315

Dependent Variable: inhibition zone

| Source | DF | Sum of Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Model | 24 | 10026.74921 | 417.78122 | 29.95 | <.0001 |
| Error | 290 | 4044.79365 | 13.94756 | | |
| Corrected Total | 314 | 14071.54286 | | | |

| R-Square | Coeff Var | Root MSE | inhibitionzone Mean |
|---|---|---|---|
| 0.712555 | 24.52393 | 3.734644 | 15.22857 |

| Source | DF | Type I SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| aflatoxigenic | 4 | 2667.606349 | 666.901587 | 47.81 | <.0001 |
| atoxigenic | 20 | 7359.142857 | 367.957143 | 26.38 | <.0001 |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| aflatoxigenic | 4 | 2667.606349 | 666.901587 | 47.81 | <.0001 |
| atoxigenic | 20 | 7359.142857 | 367.957143 | 26.38 | <.0001 |

**LSD Table: Comparisons of inhibition zone means across all the atoxigenic *Aspergillus* strains**

t Tests (LSD) for inhibitionzone

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

| | |
|---|---|
| Alpha | 0.05 |
| Error Degrees of Freedom | 290 |
| Error Mean Square | 13.94756 |
| Critical Value of t | 1.96818 |
| Least Significant Difference | 2.684 |

Means with the same letter are not significantly different.

| t Grouping | | | | | Mean | N | atoxigenic |
|---|---|---|---|---|---|---|---|
| | | A | | | 25.600 | 15 | POSITIVE |
| | | B | | | 19.933 | 15 | UM082 |
| | | B | | | | | |
| | C | B | | | 18.267 | 15 | NM084 |
| | C | B | | | | | |
| | C | B | | D | 17.800 | 15 | UM127 |
| | C | B | | D | | | |
| | C | E | B | D | 17.733 | 15 | BM071 |
| | C | E | B | D | | | |
| F | C | E | B | D | 17.533 | 15 | BM092 |
| F | C | E | B | D | | | |
| F | C | E | B | D | 17.267 | 15 | BS116 |
| F | C | E | B | D | | | |
| F | C | E | B | D | 17.267 | 15 | NM083 |
| F | C | E | | D | | | |
| F | C | E | G | D | 16.800 | 15 | EM1112 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| F | C | E | | G | D | | | | |
| F | C | E | | G | D | | 16.533 | 15 | EM244 |
| F | C | E | | G | D | | | | |
| F | C | E | | G | D | H | 16.000 | 15 | NM091 |
| F | C | E | | G | D | H | | | |
| F | C | E | | G | D | H | 15.733 | 15 | RS024 |
| F | | E | | G | D | H | | | |
| F | | E | | G | D | H | 15.333 | 15 | EM211 |
| F | | E | | G | | H | | | |
| F | | E | | G | | H | 15.067 | 15 | EM182 |
| F | | | | G | | H | | | |
| F | | | | G | | H | 14.933 | 15 | US098 |
| | | | | G | | H | | | |
| | | | | G | | H | 14.267 | 15 | EM324 |
| | | | | | | H | | | |
| | | | I | | | H | 13.600 | 15 | BS023 |
| | | | I | | | | | | |
| | J | | I | | | | 11.133 | 15 | ES042 |
| | J | | | | | | | | |
| | J | | | | | | 10.533 | 15 | US184 |
| | J | | | | | | | | |
| | J | | | | | | 8.467 | 15 | RM143 |
| | | | | | | | | | |
| | | | K | | | | 0.000 | 15 | NEGATIVE |