THE IMPACT OF PUSH-PULL TECHNOLOGY ON INCIDENCE AND SEVERITY OF MAIZE EAR ROTS AND MYCOTOXINS IN WESTERN KENYA

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EGERTON UNIVERSITY

MAY, 2018

DECLARATION AND RECOMMENDATION

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

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DEDICATION

I dedicate this work to Mr John Okinyi Okal, my father-in-law.

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ABSTRACT

Studies show there is less ear damage by insect pests and increased soil fertility from maize/legume intercropping system. These are two main benefits with pre-harvest potential on ear rots incidence and severity which result into mycotoxins reduction. Therefore, in a two season study, impact of push-pull technology (PP), maize desmodium intercrop edged by Brachiaria or Napier grass, was assessed on incidence and severity of four common ear rots: Fusarium, Gibberella, Diplodia and Aspergillus in the Push-Pull (PP) and Maize Monocrop (MM) fields in Vihiga, Butere, Siaya and Kisumu sub-counties of western Kenya. A total of 78 symptomatic (rotten) and asymptomatic (clean) ears samples were analyzed for Zearalenone (ZEA), Deoxynivalenol (DON), total Aflatoxins (AF) and Fumonisins (FB) using indirect Enzyme-Linked Immunosorbent Assay (ELISA) method. The distribution of *Aspergillus* and *Fusarium* ear rot fungal species was determined after cultural identification of *Aspergillus* and *Fusarium* ear rot causal fungi in 120 soil samples from PP and MM fields. Further identification of species in *Fusarium* section *Liseola* were done by molecular methods using species-specific primers (Translation Elongation Factor-1 alpha). The result showed low incidence of ear rot in PP (7.3%) than MM (20.8%). Similar pattern was observed on severity. The respective ear rots severities under PP and MM were: diplodia (1.15 and 1.85), gibberella (0.62 and 0.84), aspergillus (0.09 and 0.25), fusarium (0.19 and 0.68) and penicilium (0.03 and 0.05). Result showed high proportion of ZEA (100%), AF (93.3%), DON (80.0%) and FB (65.9%) in symptomatic samples compared to proportion of ZEA (90.3%), DON (51.6%), FB (38.7%) and AF (3.2%) in asymptomatic samples. The density ($CFUg^{-}1$) of *Aspergillus* and *Fusarium* species in soil from PP (2,282.8) and MM (2,516.6) was insignificant (P=0.86) showing no difference in fungal distribution. However, *Aspergillus* (80%) had high distribution in soil compared to *Fusarium* (4.4%). These findings suggest potential of PP in managing ear rots and ultimately limiting mycotoxins. However, potential exposure to aflatoxins from the field was seen by high distribution of *Aspergillus* in soil from both PP and MM. These studies also suggest likelihood of aerial infection from external sources as observed in low *Fusarium* distribution in soil samples compared to high incidence of fusarium and gibberella ear rot from fields where samples were taken. Additionally, there were increased other mycotoxins such as ZEA and DON. These findings are vital for formulation of management of different ear rots and their mycotoxins.

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LIST OF ABBREVIATIONS AND ACRONYMS

AF: Aflatoxin

AFB_1: Aflatoxin B_1

AFB_2: Aflatoxin B_2

AFPA: Aspergillus Flavus Parasiticus Agar

CDC: Centre of Disease Control

CFU: Colony Forming Unit

CTAB: Cetyltrimethylammonium bromide

CZ: Czapek Dox Agar

DNA: Deoxyribonucleic Acid

DON: Deoxynivalenol

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-Linked Immunosorbent Assay

EU: European Union

FAO: Food Agriculture Organization

FB: Fumonisin B

FB_1: Fumonisin B_1

FB_2: Fumonisin B_2

FB_3: Fumonisin B_3

FDA:	Food and Drug Administration
GDP:	Gross Domestic Product
GLM:	General Linear Model
ICIPE:	International Centre of Insect Physiology and Ecology
IITA:	International Institute of Tropical Agriculture
IPM:	Integrated Pest Management
JECFA:	Joint FAO/WHO Expert Committee on Food Additives
KBS:	Kenya Bureau of Standards
MM:	Maize Monocrop
PDA:	Potato Detrose Agar
PMTDI:	Provisional Maximum Tolerable Daily Intake
PP:	Push-Pull Technology
SNA:	Spezieller Nahrstoffarmer Agar
SSA:	Sub-Sahara Africa
TEF-1:	Translation Elongation Factor- 1 alpha
WHO:	World Health Organization
ZEA:	Zearalenone

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background information

Maize (*Zea mays* L.), amongst other cereals, is a major staple food and calorie source in Africa (FAO, 2010). The crop is produced in most parts of Africa under rain-fed agriculture, largely by smallholder farmers who are responsible for about 75% of the total maize production (Nyoro *et al.,* 1999). Despite increased demand for maize, supplies are low due to low productivity of the crop, with insect pests, changes in climate, diseases, poor soil fertility, and low inputs being the key constraints responsible. Among the key pests that severely contrain maize production in the continent are lepidopteran stemborer, with the indigenous species *Busseola fusca* Füller (Noctuidae) and the exotic *Chilo partellus* Swinhoe (Crambidae) being some of the most important (Kfir *et al*., 2002). The key diseases frequently encountered due to stemborer damage on maize ear are ear rots, with associated mycotoxins. Presently, incidence of mycotoxin contamination has significantly increased in maize, attributable to pre-harvest contamination (Munkvold, 2003) whose primary source is maize ear rot infections which result in low maize quality and yield (Kommedahl and Windals, 1981).

Ear rots are fungal diseases characterized by entire or localized discoloration of kernels on the ears. These infections mostly begin in the field prior to harvest, with post-harvest development being dependent on grain handling and storage (Bigirwa *et al.,* 2007). Several types of ear rots exist, but four are agriculturally significant and include gibberella ear rot, pink; fusarium ear rot, white to lavender; aspergillus ear rot, greenish yellow or black (Xiang *et al.,* 2010), and diplodia ear rot, white (Bigirwa *et al.,* 2007). The effects of ear rots include: reduced ear weight, taste, nutritional value, and increased mycotoxin contaminations (Gxasheka *et al.,* 2015). These effects result into significant economic losses and health risks (Bigirwa *et al.,* 2007; Zain, 2011; Gxasheka *et al.,* 2015).

Occurrence of ear rots and mycotoxins are mainly driven by environmental and biophysical factors. High rainfall and cool temperature favour incidence and severity of diplodia and gibberella ear rots (Miller, 2001). On the other hand, drought and insect damage predispose maize to fusarium and aspergillus ear rots (Munkvold and Hellmich, 2000). Other factors responsible for incidence of ear rots and mycotoxins include: insect damage (Schulthess *et al.*, 2002), organic matter (Alakonya *et al.,* 2008), maize phenotype (Odvody *et al.,* 1997), type of fungal strains (Probost *et al.,* 2009) and cultural practices (Brun, 2003; Mutiga *et al.,* 2015).

In management of ear rots and mycotoxins, inoculum source and host exposure are targeted to limit interactions of hosts with ear rot fungi (Bruns, 2003). Cultural practices including proper residue management, early planting and harvesting, crop rotation, tillage practice, irrigation, intercrop and addition of organic amendments have been shown to limit incidence and severity of ear rots and mycotoxins (Bruns, 2003; Munkvold, 2003). Application of atoxigenic species for exclusion of toxigenic species in maize fields has also been a strategic approach in management of mycotoxins (Dorner, 2009). In Bt-maize, reduction of ear rot and mycotoxin infections has been realized through control of insect pests (Gxasheka *et al.,* 2015). Studies show that control of insect damage limited spore access and dispersal to the kernels which is impactful on ear rots and mycotoxin incidence and severity (Ajanga and Hillocks, 2000; Schulthess *et al.,* 2002). However, the use of Bt-maize is currently not a widely practiced approach for management of insect damage in maize in Africa, except in South Africa. Similarly, application of insecticides is practiced only by few smallholder farmers in the region.

Generally, intercropping system show low insect pest incidence compared to monocropping system (Songa *et al.,* 2007). Thus maize/legume intercop is potentially capable for management of ear rot and mycotoxin reduction. However, most intercropping system are not designed for pest management. Thus through innovative research, a companion cropping-based approach, push-pull technology (PP), was developed as a pest and soil management strategy to control stemborers and restore soil health in western Kenya region (Khan *et al.,* 2011). The technology involves intercropping maize with stemborer moth repellent crops (push) such as legumes in the genus *Desmodium,* and planting an attractive border crop (pull) such as Napier grass (*Pennisetum purpureum* Schumach) or Brachiaria cv mulato around this intercrop. The intercrop repels stemborer moths that are subsequently attracted to the trap plant.

Desmodium the main companion crop in PP also suppresses the parasitic weeds in the genus *Striga* (Orobanchaceae), through a range of mechanisms, while at the same time improving soil health through nitrogen fixation, organic matter improvement, carbon sequestration and moisture conservation (Khan *et al.*, 2010, 2014). A number of studies have reported effective control of stemborers and striga weeds and significant improvements in soil fertility through the push-pull technology (Khan *et al.*, 2010, 2014). These benefits imply potential benefit of the technology in controlling ear rots and mycotoxins, the basis upon which the current study was conducted.

1.2 Statement of the problem

Maize ear rots are known to cause both quality and quantity losses in maize. Through maize ear rots, discoloration, reduced weight, deterioration in nutritional value and taste of maize are apparent. Furthermore, mycotoxins associated with these maize ear rots are hazardous to both human and animal health. The health concerns pull a string for stringent legislation on levels of mycotoxin concentrations in maize and its products. This affects trade further impoverishing smallholder farmers engaged in maize cultivation in Sub-Sahara Africa. Control methods currently advanced for management of maize ear rots and mycotoxins are not easily adopted by smallholder farmers in the region due to socio-economic and technical reasons. There is therefore need for continued efforts to develop and/or adopt cropping systems to effectively manage the predisposing factors for ear rots and associated mycotoxins and therefore provide a solution to the menace to the resource poor farmers. It has been demonstrated that effective control of pest attacks in maize results in reduced ear rot and mycotoxin incidence and severity, and that the push-pull technology provides effective control of stemborers, the key pests of maize in the region. However, potential contribution of the technology in management of ear rot and mycotoxin infections has not been determined.

1.3 Objectives

1.3.1 General objective

To determine potential role of push-pull technology in management of ear rots and mycotoxins in maize.

1.3.2 Specific objectives

1. To determine impact of push-pull technology on incidence and severity of maize ear rots.
2. To quantify mycotoxins levels in symptomatic and asymptomatic maize ear samples.
3. To determine distribution of aspergillus and fusarium ear rot causative fungi in soils from push-pull cropping and monocrop systems.

1.4 Hypotheses

1. Push-pull technology has no impact on incidence and severity of maize ear rots.
2. Symptomatic and asymptomatic maize ears have similar mycotoxin levels.
3. Push-pull technology has no influence on distribution of ear rot fungal species in the soil.

1.5 Justification

Insect pests are a key predisposing factor in ear rot and mycotoxin contamination in maize. However, their management remains a challenge to smallholder farmers in SSA. Chemical control of stemborer pests in maize is not only expensive but also pose significant environmental and health risks, while use of transgenic maize with insect control function is not available for smallholder farmers in much of SSA. Thus potential impact of push-pull technology on stemborers and soil health may be helpful to farmers for management of ear rots and mycotoxins on the farms. The potential impact of the technology as a component in an integrated management approach for ear rots and mycotoxins requires assessment and this is linely to have a direct impact on both trade and subsistence.

CHAPTER TWO

GENERAL LITERATURE REVIEW

2.1 Economic importance and constrains in maize production

Maize (*Zea mays* L.) is one of the most important cereal crops grown worldwide. Much (66%) of worldwide production is used as animal feed while the rest for human consumption (25%) and industrial (9%) use (Romain, 2001). In Africa, especially sub-Sahara, maize has the highest human maize consumption rates, making it a major staple and calorie source for approximately 1.2 billion people (IITA, 2009). The SSA economies, particularly Kenya, maize contributes invaluably to 12% of agricultural Gross Domestic Product (Nyoro *et al.,* 1999). These contributions are constrained by several factors amongst them diseases. In most cases, maize diseases cause pre-harvest losses. However, fungal ear rot diseases have post-harvest effects on maize ears resulting in both deterioration of maize and contamination with deleterious toxins (mycotoxins) (Kaaya and Kyamuhangire, 2006).

2.2 Maize ear rots

Maize ear rots are fungal infections with distinct discolorations and infection route (Figure 1) that affect several plant parts including: roots, stalks and ears in the field (White, 2000). Infected maize ears are deleterious due to mycotoxin contamination, but on appearance are mainly manifested as deterioration, on weight, taste and nutritional value. This invites stringency in phytosanitary regulations thus reducing trade in cereals (Dohlman, 2003). Different types of ear rots exist, but those of agricultural importance are mainly: gibberella, fusarium, aspergillus (Xiang *et al.,* 2010) and diplodia (Julian *et al.,* 1995; Bigirwa *et al.,* 2007).

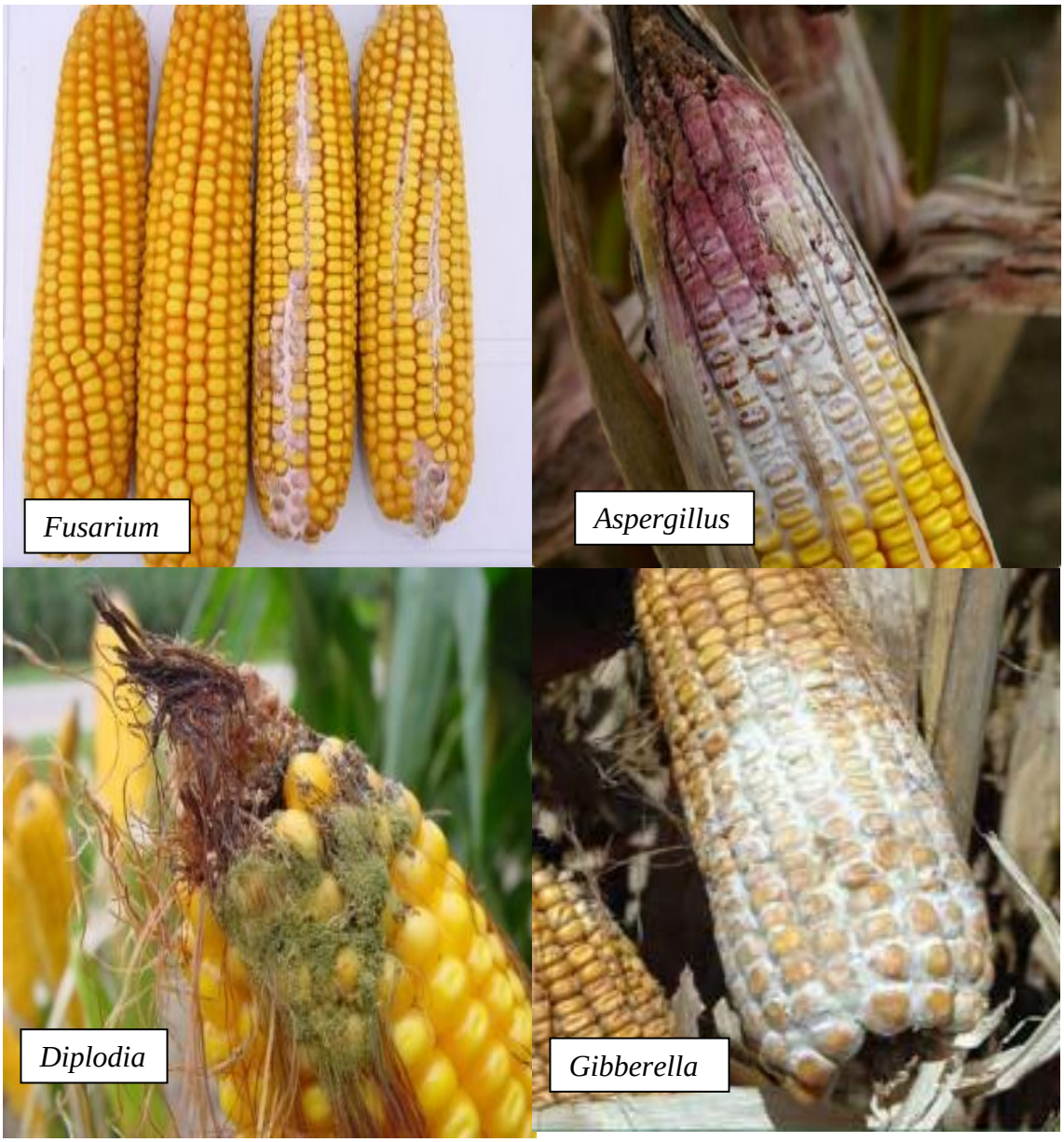

Figure 1: Maize ear rots of agricultural importance

Source: www.pioneer.com

2.2.1 Gibberella ear rot

Gibberella ear rot is a disease of maize caused by the fungus *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein) Petch) (Nelson *et al.,* 1983). The fungus infects by either asexual (microconidia) or sexual (ascospores). Colonized plant debris usually act as the principal inoculum for infections and their spores are dispersed by wind (for ascospores) or splashing and wind driven rain (for microconidia) (Sutton, 1982). Maize plants are more susceptible at silking stage with more infections manifested via silk route (Munkvold, 2003). Characteristic red or pink mycelia bodies which run from the surface tip downward to base of maize ear is observed on dehusked infected maize cob. Influence of cool and wet weather or high rainfall (precipitation) increases incidences of gibberella infection (Bigirwa *et al.,* 2007; Julian *et al.,* 1995). Natural factors including maize phenotype equally promote infection. For instance, hybrid maize varieties or landraces with ears that are tightly-husked and remain upright after maturity tend to retain or increase moisture content which is favorable for infection, unlike the loosely-husked and bent ears (Munkvold and Steve, 2004).

Gibberella ear rots result in reduced yield and nutritional value and most severely mycotoxin contamination. This ear rot is associated with two key mycotoxins: deoxynivalenol, also known as vomitoxins, and zearalenone (ZEA), which are hazardous to human and animal life. Ingestion of deoxynivalenol **(**DON) contaminated feed and foodstuff results into several complications such as: gastrointestinal diarrhea, nausea, dizziness, fever and abdominal pain in human beings (JECFA, 2001). In animals, food refusal, anorexia and vomiting are manifested (Bonnet *et al.,* 2012). On the other hand, ingestion of ZEA results into reproductive problems in animals with female sheep and pig more severly affected. In pigs the reproductive effects of ZEA are manifested as hyperestrogenic effects in female pigs, ovarian atrophy in young pre-pubertal pigs, persistent corpus luteum, prolonged oestrus, implantation failure, decreased fertility, still birth, pseudopregnancy and weak piglets (Kuiper-Goodman *et al.,* 1987). Ewes also manifest reduced ovulation rate during oestrous (Smith *et al.,* 1986), while premature breast development (thelarche) in girls has been reported in populations exposed to ZEA (Saenz *et al.,* 1985).

2.2.2 Fusarium ear rot

This is the most common fungal disease in maize caused by several *Fusarium* species in section *Liseola*. The species involved are *F. verticillioides* (Sacc.) Nirenberg, formerly *F. moniliforme* J. Shield (teleomorph: *Gibberella fujikuroi* (Sawada) Ito in Ito and Kimura) and other anamorph of *Gibberella fujikuroi, F. proliferatum* (T.Matsushima) Nirenberg and *F. subglutinans* (Wollenweb. and Reinkings) P.E Nelson, T.A. Toussoun and Marasas (Nelson *et al.,* 1983). Fusarium ear rot is manifested as localized and scattered infections on kernels across maize ears. Infected kernels display either kernel damaged or split (starburst and physical damage) with symptoms of whitish-pink to lavender cottony fungal growth. Wound infection of ears is more predominantly observed than systemic infection (Munkvold *et al.,* 1997; Sobek and Munkvold, 1999).

The incidence and severity of fusarium ear rot infection is increased by factors which compromise kernel integrity. They include: drought (water stress), insect damage, temperature and maize phenotype (Miller, 2001). Experiments have shown positive correlation between insect damage and cob rots (Schulthess *et al.,* 2002); with Flett and Van Rensberg (1992) reporting an increased *F. verticillioides* incidence in maize from *B. fusca* infestation. Kernel integrity can also be compromised by water stress resulting into silk-cut at silking stage. For instance, hot and dry weather which increase soil and air temperature (>28^{o}C) was found to be favorable for occurrence of fusarium ear rot (Shelby *et al.,* 2004). Odvody *et al.* (1997) also found manifestations of silk-cuts more on open-tipped hybrids and landraces than loose-husked ears.

Fusarium ear rots are associated with fumonisin contaminations (Munkvold, 2003), which cause toxicological effects like: equine leukoencephalomalacia (Kellerman *et al.,* 1990), porcine pulmonary edema (Haschek *et al.,* 2001), esophageal cancer in human (Marasas, 2001), liver cancer (Ueno *et al.,* 1997) and neural tube defects in children (Marasas *et al.,* 2004) when ingested with food or feed. Therefore, group provisional maximum tolerable daily intake (PMTDI) of 2 µg/Kg for FB_1, FB_2 and FB_3, alone or in combination has been allotted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (WHO, 2002) to regulate food commodity exposure.

2.2.3 Diplodia ear rot (stenocarpella ear rot)

Diplodia ear rot caused by *Stenocarpella maydis* (Berkeley) Sutton is an important disease whenever maize is grown worldwide (Rossouw *et al.,* 2009). Infection of maize stalks occurs through their roots and at the back of leaf sheaths causing root and ear rots, with young plants showing seedling blight (Roussouw *et al.,* 2009). This infection is typified by an entire appearance of white cottony mycelia between kernels, beginning from ear base and progressively spreading up to ear tip. An external brown and dry husk when maize ears are still green is also symptomatic of this ear rot. The infecting spores of this disease reside in residues of corn stalk, cobs and fallen kernels. Thus besides humid conditions (Roussouw *et al.,* 2009), incidence and severity of diplodia ear rot are increased by conservation tillage system and continuous maize cultivation (Flett *et al.,* 2001). In South Africa, high repeated occurrence of stenocarpella ear rot was reported in conservation tillage system (Flett *et al.,* 1998). The the susceptible hybrids also contribute to diplodia ear rot. The importance of stenocarpella ear rots is low quality kernels which are light in weight, discolored in appearance and low in nutrition (Wicklow *et al.,* 2011), while diplodiosis also affect animals (Roussouw *et al.,* 2009).

2.2.4 Aspergillus ear rot

Aspergillus flavus, greenish yellow fungus, is the principal causative agent of aspergillus ear rot. Other causal fungi include: *A. niger* and *A. glaucus,* which are characterized by black and green discolouration on maize ears or kernel, respectively (Jacobsen, 2007; Palencia *et al*., 2010). Aspergillus ear rot is promoted by drought conditions, occurence of cracks or silk-cuts on kernels, high temperature range between 27 and 38^{o}C and relative humidity (85%) during grain filling (DuPioneer, 2010). The stemborer larval feeding also created wounds and vector fungal spores to the host in both field and storage conditions. The open-tip husked maize variety is also a source of exposure to *Aspergillu*s fungal spores (Connel, 1956).

Aspergillus ear rot contaminates ears with aflatoxins thus ingestion of aflatoxin contaminated food and feed affect humans and animals. The chronic exposure to AFB_1 results into human hepatocellular carcinoma, immunomodulation and poor growth in children (Gong *et al.,* 2002) with human deaths occurring on acute exposure (CDC, 2004).

Effects of aflatoxin in dairy animals are reduced appetite, loss in body weight and suppressed lymphocyte (Paul *et al.,* 1977), while low egg production and hepatomegaly reported in poultry (Lubulwa and Davis, 1994). Due to aflatoxin carcinogenicity, different markets have specified concentration of aflatoxin. In Kenya, the tolerable limit for total aflatoxin is set at 10 ppb by Kenya Bureau of Standards (KEBS).

2.3 Challenges and strategies for controlling ear rots and mycotoxins

The control of stemborer damage has been achieved significantly by either chemical pesticide or growing of Bt-maize. Studies show that these methods also significantly reduce some ear rot and mycotoxins in maize (Munkvold *et al.,* 1997; Blandino *et al.,* 2008). However, the cost of chemical pesticides for poor smallholder farmers and low adoption of biotechnology are perceived impediments in SSA. Against this backdrop, lepidopteran stemborers, especially *B. fusca* and *C. partellus*, remain main biotic constraints in maize production in the region, with their incidence resulting into losses ranging from 10% to 88% (Kfir *et al*., 2002; Mgoo *et al.,* 2006). Nonetheless, other insect larval control strategies are also available and range from good agricultural practices and biological control to biotechnology.

2.3.1 Good agricultural practices

The measures that control pre-harvest mycotoxin contamination have high correlation with those that improve crop yields (Hells and Mutegi, 2011). These include tillage, crop rotation, early planting, weed control, fertilizer application, plant variety, insect control and timely harvesting (Bruns, 2003). In South Africa, incidence of stenocarpella was 7.7% on moldboard plow season compared to 20.6% on V-blade plow which ensured reduced tillage (Flett *et al.,* 1991). Suppression of pathogenic fungal establishment in the soil has also been realized with addition of organic matter (Alakonya *et al.* 2009). Experiment show low pest-density in intercropping of non-host crops with cereals (Songa *et al,* 2007) which reduce ear rot infection and mycotoxin contamination. Elsewhere, use of chemical insecticides and early planting showed 25% and 76% reduction of fusarium ear rot severity and fumonisins, 25% and 49% similar reduction, respectively (Blandino *et al.,* 2008). Decline of ear rots and mycotoxins by early harvesting too, shows positive results (Kaaya *et al.,* 2006).

Studies have also reported open-tipped and loosely husked hybrids, and landraces as more prone to wound-related ear rots (Odvody *et al.,* 1997), while tightly husked and upright hybrids as more susceptible to diplodia and gibberella ear rot. Thus phenotypic traits of hybrid and landrace could be exploited in control of ear rots and mycotoxins.

2.3.2 Biological control methods

The use of friendly organisms that reduce colonization of invasive organism has been used to inhibit maize ear rots and mycotoxigenic fungi. In aflatoxin control, applications of non-toxigenic fungi show positive results in exclusion of toxigenic species (Munkvold, 2003). "Afla-guard" and "afla-safe" are two commercially available products which comprise non-aflatoxigenic fungal species for use in aflatoxin control in peanut and maize production (Dorner, 2009). Significant reduction of aflatoxin levels by 70 to 99% in both laboratory and field trials has been reported on application of afla-safe (Atehnkeng *et al.,* 2008).

2.3.3 Biotechnology

Through biotechnology, development of resistant maize lines are being exploited for control of ear rot and mycotoxins. Some aflatoxin-resistant lines of maize have been developed, but are of poor commercially acceptable agronomic qualities (Brown *et al.,* 1999). Host resistance to insect larvae through transgenesis has been effectively integrated in ear rot and mycotoxin control. Munkvold *et al.,* (1997) reported significant reduction of ear rots and mycotoxins on transgenic than isogenic maize cultivars. Findings of Force *et al.,* (2010) also reported 90% reduction of FB_1 on *Bt*-Maize. This observation corroborates those made on transgenic cotton (Cotty *et al.,* 1997) which is also a host to *Aspergillus* infection and aflatoxin contamination.

2.3.4 Integrated Pest Management System (IPM)

More ear rot and mycotoxins is encouraged by high incidence of lepidopteran stemborers in SSA thus effective insect control is required. This can be achieved effectively by chemical pesticides or growing of *Bt*-maize, but affordability of these methods remains a challenge, combined with restrictions on usage.

Through intercropping, a traditional practice amongst small-scale farmers in Africa (Songa *et al.*, 2006), intercrop of non-host crops such as legumes and cassava with maize had significant impact on stemborer reduction (Chabi-Olaye *et al.*, 2005). Under similar environment of crop diversification, 'push-pull' cropping system has shown significant effects in stemborer control (Cook *et al.*, 2007).

2.3.5 Push-Pull Technology

Resistance and reliance on insecticides for control of *Helicoverpa spp*. in cotton, led to conception of push-pull concept as insect-pest management strategy thirty years ago in Australia. Simultaneous use of repellent and attractive stimuli for manipulation and distribution of this pest from cotton were attempted (Pyke *et al.*, 1987). This was followed by a number of attempts to develop 'stimulo-deterrent' based pest management approaches for a range of crops, including onion and maize. The most effective, and indeed the only one widely practiced 'stimulo-deterrent' based pest management approaches is the PP for integrated management of pests in cereals crops in Africa. Behavioral manipulation and habitat diversification strategies form the basis of pest-management in PP system as explained by Cook *et al.* (2007).

2.3.5.1 Design and rationale of push-pull technology in maize

Behavioral manipulation and habitat diversification strategies form the basis of pest-management in PP (Cook *et al.*, 2007). The plant species of the genus *Desmodium* is used as repellant intercrop producing deterrent semiochemicals which drive away (push) months to oviposit on host crop (maize or sorghum) in the field. Meanwhile, at the field edges, the trap plants, Napier grass (*Pennisetum purpureum*) or Mulato II (*Brachiaria*) releases strong volaties than host plant, thus trapping the months and promote their oviposition. Consequently, eggs are oviposited on these trap plants, leaving the target cereal crop protected. However, most (>80%) of the resultant larvae from the eggs die due to the trap plants' inherent features including production of sticky sap that entangle the larvae, predation and poor nutritive value of the trap plants (Midega *et al.*, 2015) . Consequently, eggs oviposited on these trap plants are broken besides elimination of hatched larvae by hairs on the trap plants.

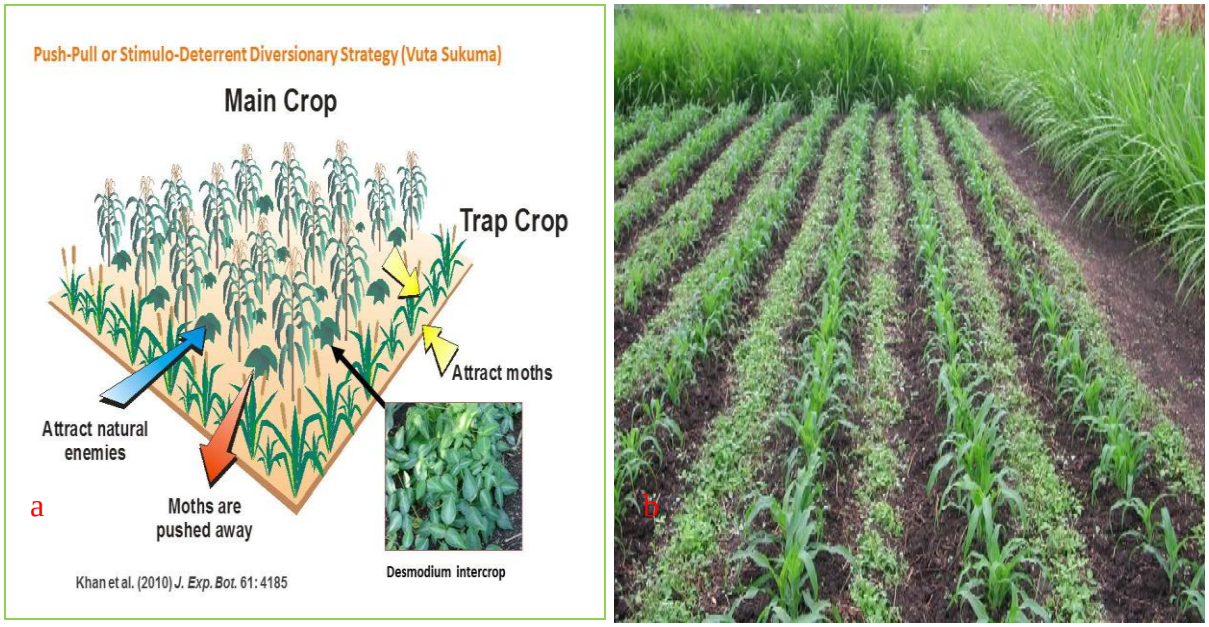

Figure 2: Diagrammatic presentation (a) and (b) showing push-pull rationale and design.

Source: www.push-pull.net

2.3.4.2 Other benefits of PP significant to ear rot and mycotoxin control

Several beneficial services of PP system exclusive of control of insect damage have been reported by Khan *et al.*, (2011). These services include nitrogen fixation, control of the parasitic striga weed, *Striga hermonthica* (Del.) Benth., (Orobanchaceae), addition of organic matter and good vegetation cover. All these benefits strengthen plant resilience to fight infections. Besides, these plants are periodically cut and fed to animals, thus further contributing to reductions in stemborer populations in the field.

CHAPTER THREE

IMPACT OF PUSH-PULL TECHNOLOGY ON INCIDENCE AND SEVERITY OF MAIZE EAR ROTS AND MYCOTOXINS IN BUTERE, KISUMU, VIHIGA AND SIAYA SUB-COUNTIES.

3.1 Abstract

Mycotoxins are harmful to health and mainly arise from ear rots affecting maize in the field. This study investigated the effect of the cropping system on ear rot and in turn mycotoxins. The incidence and severity of ear rots were studied in four sub-counties (districts) of western Kenya, Butere, Kisumu, Siaya and Vihiga. Plots comprising maize planted either as pure stand or in mixture with legumes predominantly common bean, considered as "Maize Monocrop" (MM) were used as control for those of climate-smart push-pull (PP) strategy. Symptomatic and asymptomatic maize ear samples were analysed for Total Aflatoxin (AF), Total Fumonisins (FB), Deoxynivalenol (DON) and Zearalenone (ZEA) using Enzyme-Linked Immunosorbent Assay (ELISA). Cropping system had very high significant effect on ear rot incidence and severity. In general, low incidences were observed in PP (7.3 %) than MM (20.8 %). A similar trend was also observed on severity. The maize samples infected with ear rots (symptomatic ears) had high proportions and amount of ZEA, DON, AF and FB than those without (asymptomatic). These findings suggest potential of cropping system (PP) in managing ear rots and ultimately limiting mycotoxin exposure. The study also highlighted the need to deal with emerging mycotoxins such ZEA and DON through increased surveillance and awareness among stakeholders.

3.2 Introduction

Maize ear rots are fungal infections with worldwide distribution and presence in all agro-ecologies where maize is grown (Dragich and Nelson, 2014). Some of the key fungal genera prominent in ear rot infections in include: *Aspergillus*, *Fusarium*, *Sternocarpella* and *Penicilium* (Kapindu *et al.*, 1999). Five ear rots, aspergillus, fusarium, gibberella, diplodia, and penicilium, are common in maize fields (Gxasheka *et al.*, 2015).

Several places in SSA experience an incidence above 10% of ear rot infections (Kapindu *et al.,* 1999, Ajanga and Hillock, 2000; Bigirwa *et al.,* 2007) indicating huge losses of maize. Additionally, the ravages caused by the ear rots are aggravated by four mycotoxins, fumonisins, zearalenone, deoxynivalenol and aflatoxins (Gxasheka *et al.*, 2015). These mycotoxins, secondary metabolites produced by fungi, pose high risk to human and animal health (Zain, 2011), and attract stringent regulation in food and feed in global grain trade (Otsuki *et al.,* 2001). Therefore, management and control of maize ear rots is imperative for ensuring maize quantity and quality.

In management of ear rots and mycotoxins, understanding epidemiology of each ear rot and mycotoxins show that the contamination can start from the field and progress to storage. Thus Munkvold, (2003) asserts that mycotoxins in Africa are a pre-harvest problem. Nonetheless, most farmers in this region engage in reduction of ear rot directly at post-harvest stage by sorting. Although this may contaminate other healthy ears by contact and increase fungal inoculum for progressive stages, removal of ear rot kernel at post-harvest have resulted to mycotoxin reduction (Kimanya *et al.,* 2009; Van der Westhuizen *et al.,* 2010; Mutiga *et al.,* 2014; Balconi *et al.,* 2014; Wild *et al.,* 2016). Therefore, reduction of ear rot at pre-harvest stage by limiting factors that predisposes maize ears to fungi would be the best remedy.

Amongst several biotic and abiotic factors that predisposes maize ear to fungal rots, insect damage is the main factor (Parson, 2008; Gxasheka *et al.,* 2015). Empirically, strong correlation of ear rots with insect attack, and correlation of silk-cut symptom with incidence of immature thrips population has been reported (Ajanga and Hillocks, 2000; Parson, 2008). Thus measures that aim to control damage to crops by insect pests can contribute to management of ear rots and mycotoxin attacks in the harvested crop (Munkvold *et al.,* 1997). In some countries, insect pests are controlled by planting of genetically modified maize (Bt-maize) and application of insecticides. However, in Africa the use of Bt-maize is not widespread except in South Africa. Similarly, application of insecticides is minimal as most farmers are resource-limited. Otherwise, most farmers in Africa adopt maize intercrop systems which have been found as alternative in limiting insect attack on maize through habitat diversification (Songa *et al.,* 2007). Push-pull technology is also such an intercrop developed for the basis of reduction of insect damage.

This technology, which is in practiced by over 130,000 smallholder farmers in eastern Africa to date (Khan *et al.,* 2011), may have a potential to contribute to management of ear rots and mycotoxin contamination in maize in the region. The current study sought to determine the influence of push-pull technology on incidence and severity of maize ear rots in maize and to quantify the levels, incidence and range of mycotoxins on fungal infected (symptomatic) and clean (asymptomatic) maize ears. The findings would aid in establishing potential role of companion cropping in an integrated management approach for ear rots and mycotoxin contamination in maize, particularly for the resource-constrained smallholder farmers in Africa.

3.3 Materials and methods

3.3.1 Study site

The study was conducted in four sub-counties (districts) of western Kenya namely: Butere (0° 09′ to 0° 20′ S, 34° 29′ to 34° 33′ E), Vihiga (0° to 0° 15′ S, 34° 30′ to 35° 0′ E), Kisumu (0° 15′ to 0° 25′ S, 34° 55′ to 34° 67′ E) and Siaya (0° 26′ to 0° 18′ S, 33° 58′ to 34° 33′ E) (Figure 3). The chosen sub-counties comprise areas where there are many (157, 890) PP adopters in eastern Africa (www.push-pull.net, 2017). The study sites form part of the larger grain basket of Kenya and are characterized by bimodal rainfall pattern. The bulk of smallholder farmers grow maize largely in mixed stands with legumes and in combination with livestock (Mudavadi *et al.,* 2007; Khan *et al.,* 2011). The current study was conducted in farmers' fields during the short (September to December) rain season of 2014 and the long rain season (March to August) of 2015, with treatments comprising maize grown either in push-pull or in sole stands (monocrop).

In both plots maize was planted at inter and intra-row spacing of 75 cm and 30 cm, respectively. The push-pull treatment had maize intercropped with greenleaf desmodium (*Desmodium intortum*), with Brachiaria grown as a border crop around this intercrop at a spacing of 50 cm within and between rows. Farmers in the sample districts planted their local maize varieties, ‘Nyamula’ and ‘Jowi’ (Midega *et al.,* 2015b), with only a small proportion planting medium maturity hybrids WH505.

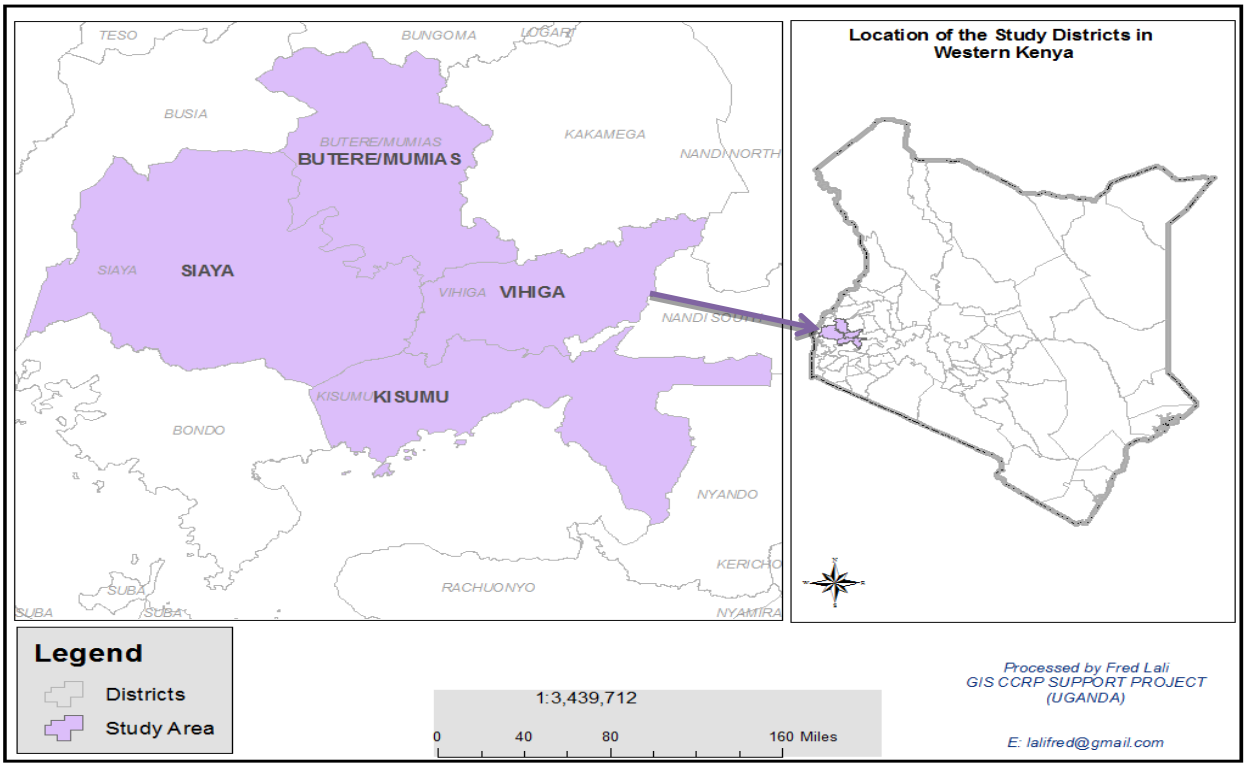

Figure 3: Map showing study districts in western Kenya

3.3.2 Sampling and determination of ear rot incidence and severity

A total random sample of 224 maize plots was picked from a sampling frame comprising push-pull and maize monocrop plots of equal number (112) in the study sub-counties. Each plot was surveyed for ear rot incidence and severity through a randomized sampling process during the end of each cropping season, short rain and long rain. Siaya sub-county was however not surveyed during the second cropping season as only a few farmers planted maize during the season.

At the beginning of harvest, 100 cobs were randomly picked per plot from which ear rot incidence was determined by physical count as described by Mutitu *et al.*, (2007). A score of severity on a scale of 0-5 where: 0= No infection, 1=1-10%, 2=11-25%, 3=26-50%, 4=51-75% and 5=76-100% infection was used to estimate severity (Jeffs, 2002). A compendium with well-illustrated photographs of maize ear rots was used and tested before identification and characterization (Hebert, 2014).

Samples of infected (symptomatic) and clean (asymptomatic) maize ear were also collected and transported in a cool box to the laboratory at the International Centre of Insect Physiology and Ecology (*icipe*) Thomas Odhiambo Campus at Mbita Point in western Kenya. These samples were further dried in an open air to moisture level of 13% measured using moisture meter (Model KM-36G, AWR Smith Process Instrumentation cc, South Africa). Samples were then hand shelled and milled before storage in a refrigerator (-4 ^{o}C) awaiting further mycotoxin analysis.

3.3.3 Mycotoxin analyses

Mycotoxin extraction and assay was conducted using ELISA commercial kit (Helica Biosystem Inc., Fullerton, CA, USA) for total aflatoxin, Cat. No.941AFLO1M-96; total fumonisin, Cat. No.951FUM01C-96; zearalenone, Cat. No.951ZEA01N-96; and deoxynivalenol, Cat. No.941DON01M-96 as described by Gutleb *et al.,* (2015). Samples were tested within the range of 1-20 ppb for total AF; 100-6,000 ppb, total FB; 15-500 ppb, ZEA; and 500-10,000 ppb, DON. The sample which had exceeded upper limit of quantification was diluted further. The final result in parts per billion (ppb) was converted to equivalent microgram per kilogram (μg/Kg).

3.3.3.1 Mycotoxin extraction

Twenty grams of sub-samples was used for total aflatoxin extraction in 100 ml of 70% methanol made of 30 ml deionised water and 70 ml methanol (3:7 v/v). Each extraction mixture was placed into 250 ml conical flasks, swirled and blended for 3 min. The blended mixtures was allowed to settle then filtered (Whatman paper# 1). Total fumonisins (FB) was extracted from 20 gm of sub-sample of maize flour in 40 ml of 90% methanol (1:9 v/v). The mixture was blended for 1 min, stopped and allowed to settle. The settled mixture was filtered (Whatman paper # 1). An aliquot of 1.5 ml of the collected filtrate was diluted in the ratio of 1:20 in distilled water. Equal (20 gm) amount of sub-sample was used to extract zearalenone (ZEA) using 90% methanol (1:9 v/v). The mixture was blended for 3 min and allowed to settle before filtration (Whatman paper #1). An aliquot of the filtrate was then diluted with 70% methanol in the ratio of 1:10. Deoxynivalenol was extracted from 20 gm sub-sample in 100 ml distilled water. The mixture was blended for 5 min and filtered after which further dilution of filtrate with distilled water was done in the ratio of 1:10 with 70% methanol.

3.3.3.2 Mycotoxin assay

All the reagents were brought to room temperature (25±3 ^{o}C) as per manufacturer's instructions. PBS-Tween reconstituted by washing out the contents of the packet with distilled water into a 1-liter container before ELISA. The dilution wells were placed in a microwell holder for each standard and sample. Equal number of antibody coated microtiter wells were also placed in another microwell holder. One hundred microlitres of the conjugate solution A (green) were dispensed into the appropriate dilution wells followed by 100 µl of conjugate solution B (clear). Volume of 100 µl of each standard and sample were added to appropriate dilution well containing conjugate and mixed by priming pipettor 3 times. Location of each standard and sample were carefully recorded throughout the test. Using a new pipette tip for each, 100 µl of contents from each dilution well were transferred to a corresponding antibody coated microtiter wells and incubated at room temperature for 10 min. The contents from microwells were then decanted into a discard basin. For fumonisins and zearalenone, the microwells were washed by filling each with PBS-Tween wash buffer then decanting the water into a discard basin, while distilled water was used for aflatoxins and deoxynivalenol washes. The wash was repeated 3 times and the microwells (face down) tapped on a layer of absorbent towels to remove residual water. To each microwell, 100 µl of substrate reagent was added followed by incubation at room temperature for 10 min away from direct light. Afterwards, 100 µl volume of stop solution was added and optical density of each microwell on ELISA plate (Plate 1) read on microtiter plate reader under 450 nm wavelengths (*EZ* Read 400, biochrom). The samples were done in duplicate. The optical densities of the standard samples against their log transformed mycotoxin concentrations were used to construct standard curve using Graphpad Prism software version 6.5. From the standard curve, the test (unknown) samples mycotoxin concentration were interpolated. Samples below limit of quantification were considered as negative (no detectable toxin) while those above upper limits reconstituted by dilution and quantified with inclusion of the dilution factor.

Figure 4: ELISA plate ready for reading in microplate reader. The arrow on the left side of the first column show increasing concentration downward for the standards in the first 6 wells.

3.3.4 Data analysis

Effects of cropping system and season on ear rot incidence were analyzed using Generalized Linear Model, while ear rots severity was analyzed by Analysis of Variance using R software version 3.3.1 (R Core Team, 2013). Mean, frequency and percentage of samples contaminated with mycotoxins were presented by simple descriptive statistic calculated in Statistical Package for the Social Sciences version 22.0 (IBM Corp, Armonk, New York, USA).

3.4 Results

3.4.1 Maize ear rot incidence and severity in push-pull and maize monocrop

The effect of cropping system on incidence of ear rots in the study was significant (p=0.001) (Table 1). In general, low incidence of ear rot was observed in PP (7.3%) than MM (20.8%). The following was respective ear rots incidence in PP and MM: diplodia (3.33% and 7.31%), gibberella (1.30% and 4.48%), aspergillus (0.65% and 2.09%), fusarium (0.21% and 0.51%) and penicillium (0.11% and 0.40%). The severities of ear rots were also significant (p=0.001) under cropping system with low severity observed in PPT than MM. Under PPT and MM, the respective ear rots severities were: diplodia (1.15 and 1.85), gibberella (0.62 and 0.84), aspergillus (0.09 and 0.25), fusarium (0.19 and 0.68) and penicilium (0.03 and 0.05).

Table 1: Effects of cropping system, season and their interaction on percentage incidence of ear rot disease

Factor	Level	Gibberella	Fusarium	Penicillium	Aspergillus	Diplodia	Total incidence
		$\bar{x}$±SE	$\bar{x}$±SE	$\bar{x}$±SE	$\bar{x}$±SE	$\bar{x}$±SE	$\bar{x}$±SE
System	Push-Pull (PPT)	1.30±0.1	0.12±0.1	0.11±0.04	0.65±0.1	3.22±0.2	7.30±0.3
	Maize Monocrop (MM)	4.48±0.2	0.51±0.2	0.40±0.1	2.09±0.2	7.31±0.3	20.8±0.4
Season	Long rains (LR)	2.02±0.1	2.67±0.1	0.70±0.1	1.38±0.1	3.12±0.2	10.1±0.3
	Short rains (SR)	3.00±0.2	0.22±0.2	0.05±0.04	0.96±0.1	8.02±0.3	16.0±0.4
System x Season							
	PP- LR	3.21±0.2	0.20±0.1	0.22±0.1	1.58±0.15	4.7±0.2	13.9±0.4
	PP- SR	2.19±0.2	0.26±0.1	0.21±0.03	1.05±0.14	4.93±0.3	12.1±0.3
	MM –LR	2.19±0.2	0.26±0.2	0.21±0.1	1.05±0.2	4.93±0.3	12.1±0.5
	MM-SR	2.19±0.3	0.26±0.3	0.21±0.1	1.05±0.2	3.93±0.5	12.1±0.6
Source of variation							
System		***	***	***	***	***	***
Seasons		Ns	***	***	**	***	***
System x Season		Ns	Ns	Ns	Ns	Ns	Ns

LR = Long rain; SR = Short rain; MM=Maize Monocrop; PP=Push-Pull; ns=not significant; x=Interaction,

$\bar{x}$±SE, Standard error of the mean.

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05

3.4.2 Influence of seasonality on incidence and severity of maize ear rots

A significant (p=0.001) effect of seasons on ear rot incidence was observed. Short rain (SR) season experienced high (16% ±0.4) ear rot incidence compared to long rain (LR) (10.1% ±0.3) season (Table 1). Generally, effect of season on incidence of ear rots was significant (p=0.001) for fusarium, penicillium and diplodia, significant (p=0.01) for aspergillus, but insignificant for gibberella. Fusarium and aspergillus were low in SR (0.22%±0.2 and 0.96%±0.1) and high in LR (2.67%±0.3 and 1.38%±0.3). Similarly, a high incidence of diplodia was observed in SR (8.02%±0.3) than LR (3.12%±0.2). A significant difference on ear rot severity was observed only on penicilium (p<0.043).

3.4.3 Ear rots and mycotoxin incidence

Incidences of mycotoxins were high in symptomatic than asymptomatic ears samples (Table 3). The respective proportion of symptomatic samples from which ZEA, AF, DON and FB were detected was 100%, 93.3%, 80.0% and 65.9%. Similarly, the asymptomatic samples had high amount of ZEA (90.3%) and DON (51.6%), but low amount FB (38.7%) and AF (3.2%). The mycotoxin range were also wide for symptomatic samples as observed in ZEA (18.7-688 µg/Kg), AF (0.35-28.9 µg/Kg), DON (0-18,260 µg/Kg) and FB (0-8,280 µg/Kg). However, ranges were comparatively narrow in asymptomatic samples and were as follows: ZEA (0-405.8 µg/Kg), AFB (0-11.7 µg/Kg), FB (0-6,460 µg/Kg) and DON (0-4,360 µg/Kg). The high proportion of symptomatic samples had ZEA (46.7%), AF (26.7%), DON (50.0%) and FB (56.8%) exceeded maximum limit set by EU. However, asymptomatic samples had proportion of 3.2% contaminated with ZEA and AF, and 19.4% having DON and FB which exceeded ADI.

Table 2: Mean severity of ear rots disease by cropping system, season and their interaction

Factor	Level	Gibberella x̄±SE	Fusarium x̄±SE	Penicillium x̄±SE	Aspergillus x̄±SE	Diplodia x̄±SE
System	Push-pull	0.62±0.09	0.19±0.05	0.03±0.01	0.09±0.03	0.84±0.1
	Maize momocrop	1.15±0.09	0.68±0.04	0.05±0.01	0.25±0.03	1.85±0.1
Season	Long rains	0.82±0.10	0.45±0.04	0.06±0.01	0.19±0.03	0.89±0.1
	Short rains	0.95±0.09	0.41±0.04	0.01±0.01	0.14±0.03	1.79±0.1
System x Season						
	PP – LR	0.58±0.1	0.23±0.06	0.06±0.02	0.11±0.04	0.45±0.1
	PP – SR	0.66±0.1	0.16±0.06	0.004±0.02	0.06±0.04	1.22±0.1
	MM-LR	1.06±0.1	0.68±0.06	0.07±0.02	0.27±0.04	1.32±0.1
	MM-SR	1.23±0.1	0.67±0.06	0.02±0.02	0.22±0.04	2.37±0.1
Source of variation						
System		***	***	Ns	***	***
Seasons		Ns	Ns	**	Ns	Ns
System x Season		Ns	Ns	Ns	Ns	Ns

LR = Long rain; SR = Short rain; MM=Maize Monocrop; PP=Push-Pull; x=Interaction; Ns=not significant; x̄±SE, Standard error of the mean.

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05

Table 3: Mycotoxin incidence and levels on symptomatic and asymptomatic ear samples

Mycotoxin	Symptomatic ears				Asymptomatic ears			
	AF	DON	FB	ZEA	AF	DON	FB	ZEA
Total sample (N)	45	30	44	45	31	31	31	31
Positive N (%)	29 (64.3)	24 (80.0)	29 (65.9)	45 (100)	1 (3.2)	16 (51.6)	12 (38.7)	28 (90.3)
Range (µg/Kg)	0.35-28.9	0-18,260	0-8,280	18.7-688	0-11.7	0-4,360	0-6,460	0-405.8
Samples>ML	12 (26.7)	15(50.0)	25(56.8)	21(46.7)	1(3.2)	6(19.4)	6(19.4)	1(3.2)

N, Number of ears sample; (%), Percent; AFB, Total Aflatoxin; DON, Deoxynivalenol; FB, Fumonisins (Total fumonisins B1+B2+B3); and ZEA, (Zearalenone); ML, Maximum Limit of concentration for mycotoxins.

3.5 Discussion

Maize ear rots reduce grain yield and quality with some of the causative pathogenic fungi producing mycotoxins that pose a health risk to humans and livestock (Mukanga *et al.,* 2010). Such ear rots are thus an important component of the myriad factors responsible for the high rates of food insecurity and health complications among smallholder farm families in sub-Saharan Africa. There is evidence that attack of maize by the ear rots and mycotoxins begin before the crop is harvested (Mukanga *et al.,* 2011) and the attack is aggravated by grain handling and storage conditions (Mutiga *et al.,* 2015). Indeed, incidence of ear rots in the study region, pre-harvest, often exceeds 20% (Ajanga and Hillocks, 2000), as confirmed by the current study. Notably, results of the current study, which to the best of our knowledge is the first that directly relates ear rots and mycotoxins to cropping system under field conditions, demonstrated that maize grown under the push-pull cropping system suffered less ear rot than pure stand maize reducing the incidence level to 7.3%.

Infestation of maize by stemborer pests has been shown to predispose the grains to ear rots and mycotoxin contamination. Indeed studies by Ajanga and Hillocks (2000) reported positive and high correlation between stemborers and incidence of ear rots in maize. Additionally, an interplay of other factors such as increase of organic matter (Alakonya *et al.,* 2008), cover cropping (Tédihou *et al.,* 2012), and intercropping (Vincelli, 1997; Flett and Ncube., 2015) have been reported to reduce ear rot incidence in maize. The push-pull cropping system effectively controls stemborers in maize (Midega *et al.,* 2015a, b), improves soil organic matter content (Midega *et al.,* 2005) and provides other soil improvement benefits. Therefore the significant reduction in incidence of ear rots observed in the push-pull plots might have resulted from the multiple ecological benefits provided by the technology.

Planting seasons are important on disease forecasting and appropriate for decision by farmers (De Wolf *et al.,* 2003). Maize is grown in seasons which have varied amount of rainfall and temperature, the two major factors for ear rot incidence and severity. In Uganda, diploda was the most abundant ear rot found in areas receiving high rainfall (Bigirwa *et al.,* 2006), thus Bigirwa *et al.,* (2007) reported more ear rot during first season.

Similar observation was also made during the study in second season, but not first season which receives high rainfall. This may be due to wet conditions at silking stage favourable for diplodia and gibberella infection and progression (Miller, 2001; Woloshuk *et al.,* 2010) which was met when late rainfall cessation extended beyond silking stage in short rain (Mugo *et al.,* 2016). Similarly, push-pull cropping system could as well promote cooler conditions due to high evapotranspiration from intercrop, thereby predisposing ears to potential infection with diplodia or gibberella ear rot as observed on insignificant by slightly high gibberella and total ear rot incidence by interaction of push-pull and long rain season.

Mycotoxicosis in Africa is majorly brought by aflatoxins and fumonisin (Darwish *et al.,* 2014; Okoth, 2016). Some studies suggest that an incidence of 2% of ear rots is capable for mycotoxicosis (Ajanga and Hillocks, 2000). Thus majority of farmers invest their time in sorting of ears or kernels affected by rots to promote quality at post-harvest stage (Munkvold and Desjardins, 1997). However, the incidence of aspergillus and fusarium ear rots in this study show that both labour and quality can be conserved at pre-harvest by exploiting cultural strategies such as push-pull intercropping system.

Fusarium mycotoxins are abundant in cereals and their products (Yazar and Omurtag, 2008), and are diverse in nature where they cause food poisoning upon ingestion. Deoxynivalenol poisoning is characterized by diarrhea, vomiting, nausea, headache, dizziness and fever (Sobrova *et al.,* 2010), while zearalenone is known to cause reproductive problems mostly in pigs and sheep (Zain, 2011). These two mycotoxins have received little attention in Kenya due to their causal agents devastation mostly on wheat, and apparent presence in wheat products (Mbugua and Gathumbi, 2004; Muthomi *et al.,* 2008; Okumu *et al.,* 2016) than maize (Kirui *et al.,* 2014). Although ZEA and DON have less acute outbreaks compared to aflatoxin (Darwish *et al.,* 2014) and low incidence on maize unlike fumonisins (Mutiga *et al.,* 2015); however, a likelihood of high population exposure was evident in these studies, with most samples of symptomatic ear having high levels of ZEA and DON beyond respective acceptable limits of 375 µg/Kg and 1,725 µg/Kg for unprocessed maize by European Union (Pinoti *et al.,* 2012). Similar, observation were made in Tanzania where high average levels of zearalenone (3,663 µg/Kg) and deoxynivalenol (23,586 µg/Kg) were found on household maize samples (Degraeve *et al.,* 2016).

In conclusion, the studies show potential impact of cropping system on ear rots; and high incidence and amount of mycotoxins contributed by ear rot samples. Thus it is recommended that the PP system is worth integration with other management system for control ear rots and mycotoxins. The high incidence and amount of zearalenone and deoxynivalenol in these studies suggest need for their surveillance and sensitization of farmers on their management.

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CHAPTER FOUR

DISTRIBUTION OF *ASPERGILLUS* AND *FUSARIUM* EAR ROT CAUSATIVE FUNGI IN SOILS UNDER PUSH-PULL AND MAIZE MONOCROPPING SYSTEM IN BUTERE, KISUMU, SIAYA AND VIHIGA SUB-COUNTIES

4.1 Abstract

Soil is the primary reservoir for fungi of which *Aspergillus* and *Fusarium* species are the main causal agents of maize ear rot and mycotoxin production. Season and cropping systems are known to influence soil fungal community structure. It is imperative to establish the distribution and density of soil fungal communities as a requisite for formulating strategies for management of ear rot infections and mycotoxin contamination. The current study was carried out to investigate the distribution of *Aspergillus* and *Fusarium* fungi causing ear rots and producing mycotoxins from soil collected from maize fields under push-pull and maize monocrop systems in three sub-counties (districts) (Vihiga, Kisumu and Siaya) in western Kenya. Out of 60 fields (20 per district and 10 per cropping system), 120 soil samples were collected at silking stage of maize during the short rain (March to May) season of 2014 and long rain seasons in 2015. Cultural methods were used for identification of *Aspergillus* and *Fusarium* species, while molecular techniques were used for confirmation of *Fusarium* section *Liseola*. Detection of total aflatoxins in cultures of section *Flavi* isolates was carried out by Enzyme-Linked Immunosorbent Assay (ELISA). A total of 338 fungi were isolated; 80% were identified as *Aspergillus* and 4.4% *Fusarium*. The distribution of fungi was significant with season but not cropping systems. The frequency of occurrence was higher during the long (68.4%) than the short rainy seasons (31.6%). In cropping systems, the frequency of occurrence of *A. flavus* was high in maize monocrop systems (60.2%) than in push-pull system (39.8%). However, *A. parasiticus* was more frequent in push-pull (71.4%) than maize monocrop systems (28.6%); and during the short (78.6%) than the long rainy seasons (21.4%). Majority (81.3%) of *A. flavus* and *A. parasiticus* were toxigenic. There was low recovery of *Fusarium spps* in soil samples. These findings show that soils from both cropping systems are potential for *Aspergillus* infection and aflatoxins contamination; however, low *Fusarium* distribution in soil suggested external inoculum source for fusarium ear rot infections common in most maize fields in western Kenya.

4.2 Introduction

Fungi are part of diverse living components of soil, with several of them living as saprophytes and symbionts contributing to various soil services including structure formation, organic decomposition, recycling of major elements (e.g. Carbon, Nitrogen and Phosphorus) and toxic removal (Aislabie and Deslippe, 2013). Pathogenic fungi also exist as major causal agents of soil borne diseases affecting roots, stalks, leaves and ears of various crops including maize (Shurtleff, 1980). Nevertheless, the presence of certain non-pathogenic (mainly saprophytes) or pathogenic fungi on grains, soils and other reservoirs are potential for ear rot infection and mycotoxin production, especially species in the *Aspergillus* and *Fusarium* genera (Horn *et al.,* 1995; Pereira *et al.,* 2011).

The *Aspergillus* genus is divided into sections (or subgenus groups) of which *Flavi* is most important in agriculture as cause of ear rot diseases and producer of aflatoxins (Gnonlonfin *et al.,* 2011). Several species are classified under *Flavi*, but *A. flavus* Link, *A. parasiticus* Speare and *A. nomius* Kurtzman (Rodriguez *et al.,* 2007) are prominent isolates in maize and soil samples. Amongst these species, *A. flavus* and *A. parasiticus* are prolific producers of aflatoxins with the former being the most abundant in both air and soil (Hedayati *et al.,* 2007), hence affecting more of aerial crops like maize. On the other hand, *A. parasiticus* is mostly reserved in soils with high isolation frequency in soils from peanuts fields (Zhang *et al.,* 2017).

The filamentous fungus with equal importance in maize production is *Fusarium*. Most of its members are producers of three important agricultural mycotoxins which include: fumonisins, deoxynivalenol and zearalenone (D'Mello *et al.,* 1999). They are also causative agents of root and ear rots in maize resulting in yield losses (Sutton, 1982). Three *Fusarium* species with high frequency of isolation in maize include: *Fusarium graminearum* Schwabe, *F. verticillioides* (Sacc.) Nirenberg, *F. proliferatum* (T. Matsushima) Nirenberg and *F. subglutinans* (Wollenweb and Reinkings) P. E Nelson, T.A. Toussoun and Marasas (Nelson *et al.,* 1983).

Soil is the primary habitat for *Fusarium* and *Aspergillus* species. The population of *Aspergillus* and *Fusarium* propagules in the soil (field) increases the risk for maize infections and mycotoxin contamination (Sutton, 1982; Horn, 2003; Jaime-Garcia and Cotty, 2004).

In order to safeguard against losses, fungal distribution in food and soil ecology is imperative for effective formulation of prevention and control measures (Abbas *et al.,* 2006). In soil fungal ecology, cultural practices greatly encourage or discourage fungal distribution. For instance, rotation of susceptible crops like wheat with maize together (Schaafsma *et al.,* 2005) and sorghum with cotton (Cardwell, 2000) is inferentially associated with increase in fumonisin and aflatoxin incidence, respectively. Addition of organic matter either by cultural practice through minimum tillage, or application of organic amendments increases *Aspergilli* propagules (Zablotowicz *et al.,* 2007) while decreasing those of *Fusarium* in soil (Alakonya *et al.,* 2008).

Although, PPT is known for insect pest management, it contributes to soil health improvement which is potentially impactful on soil fungal community. The technology improves organic matter content of the soil, nitrogen fixation, overall improvement in soil macro- and micro arthropods and conservation of soil moisture (Midega *et al.,* 2008, 2009; Khan *et al.,* 2011). However, distribution of *Aspergillus* and *Fusarium* ear rot fungi in soil under push-pull remains unknown. In this context, the aim of this study was to investigate the level of soil-borne *Aspergillus* and *Fusarium* species in push-pull and maize monocrop plots in western Kenya.

4.3 Materials and Methods

4.3.1 Field survey

The study sites covered three sub-counties (districts) as described in chapter three (subheading 3.3.1). Soils in these sites are generally vertisols, ferralsol and nitosols showing a natural decline in soil fertility predominantly manifested by occurrence of purple witch weed, *Striga hermonthica* (Del.) Benth. (Orobanchaceae) (Parker, 2008), soil erosion (Mango, 1996), nitrogen and phosphorus deficiency (Shepherd *et al.,* 1997). However, heterogeneity in soil fertility exists amongst smallholder farms in the region (Tittonel *et al.,* 2005) where there is less investment in external inputs to restore soil fertility (Waithaka *et al.,* 2006). Push-pull technology has been disseminated for pest control and soil fertility improvement for over 10 years in the region (Khan *et al.,* 2011).

4.3.2 Soil Sample Collection

The sampling method of Horn and Dorner (1998) used in soil sampling. A transect which runs 5 Km from one push-pull cluster to the next was made in the four sub-counties. A total of 60 fields were sampled at maize silking period during the short and long rain seasons of 2014 and 2015, respectively. In a cluster, four push-pull and maize monocrop fields were sampled by removal of 4 subsamples of soil with a sterile trowel from the top 4-6 cm of soil at intervals of 2-4 m. The collected soil subsamples from each field were mixed and placed in a paper bag and air dried at 25 ^{o}C for 1week. The soil was then carefully mixed and sieved through a no.10 USA standard sieve (2.00mm opening) (Dual Manufacturing Company, Franklin Park, IL 60131, USA) and stored at 4 ^{o}C.

4.3.3 Isolation of Fungi

The dilution plate technique by Cotty (1994) and Leslie and Summerell (2006) were used for *Aspergillus* and *Fusarium* recovery respectively. One gram of thoroughly mixed soil samples was suspended in 9 ml of distilled water. These resultant solutions were serially diluted to 10^{-3}. One milliliter of 10^{-2} and 10^{-3} were plated in quadruplicate in Petri dish (90 x 15 mm) containing a quarter strength Potato Dextrose Agar (PDA) (HiMedia Laboratories Pvt. Ltd) amended with 30 mg Chloramphenicol. The plates were then incubated at 31 ^{o}C for 6 days in the dark for *Aspergillus* recovery, and at 25 ^{o}C for 14 days for *Fusarium*. Colonies of *Aspergillus* and *Fusarium* that grew on each plate were counted and their population determined as Colony Forming unit (CFU) per gram and calculated as follows:

Total fungal colonies=Number of colonies * Dilution factor / weight of soil (1 gram)

Colonies of *Aspergillus* and *Fusarium* were then sub-cultured on full strength PDA amended with 30 mg Chloramphenicol.

4.3.4 Morphological identification of *Aspergillus* and *Fusarium*

The colonies on PDA identified as *Aspergillus* were transferred aseptically onto Czapek Dox Agar (CZ) (Oxoid Ltd, Basingstoke, Hampshire, England) plates and incubated at 31 ^{o}C for 5 days. Their colony characteristics (colour and reverse) were observed.

Those characterized to belong to *Aspergillus* section *Flavi* were confirmed on Aspergillus Flavus Parasiticus Agar (AFPA) base (HiMedia Laboratories Pvt. Ltd) plates incubated at 25 ^{o}C for 5 days for positive orange reverse. Microscopic features such as: head serration, vesicle and conidia were observed in a compound light microscope (Carl Zeiss MicroImaging GmbH 37081, Gottingen, Germany) (Figure 4) using keys by Klich (2002) and Kurtzman *et al.* (1987).

Fusarium colonies recovered were grown on PDA plates and observed for pigmentation on both top and reverse, and on Spezieller Nahrstoffarmer Agar (SNA) for macroconidial features. Further identification using species-specific primers was used for identification of *F. verticilloides, F. proliferatum* and *F. subglutinans*.

Figure 5: A picture of compound light microscope (Carl Zeiss MicroImaging GmbH 37081, Gottingen, Germany).

4.3.5 Molecular identification of *Fusarium* section *Liseola*

4.3.5.1 DNA extraction

Fusarium isolates, 13 in total, culturally identified to belong to *Fusarium* section *Liseola* were grown as monosporing cultures on PDA plates for 7 days at room temperature. For each isolate, mycelium was harvested for total DNA extraction according to Gherbawy *et al.,* (2001). One gram of freshly harvested mycelium was ground in liquid nitrogen with a mortar and pestle into a very fine powder. Fifty milligram of the ground mycelium was transferred into 1.5 ml Eppendorf tube and mixed with 700 µl 2 x CTAB buffer. The contents of Eppendorf tube was incubated at 65 ^{o}C for 30 min before addition of 700 µl of Chloroform: Isoamyl Alcohol (24:1 v/v), and a brief mixing. The mixtures were then centrifuged at 10,000 g for 30 mins and supernatant transferred into another tube. Isopropanol, 700 µl in volume was added and mixed with the supernatant and left to chill overnight at −20 ^{o}C. This content was centrifuged again at 10,000 g for 5 min, after which the supernatant discarded and pellets washed twice in 1 ml of 70 % ethanol and left to dry under a vacuum. The pellets were afterwards resuspended in 700 µl distilled water. The quality of DNA was evaluated in 1% agrose gel electrophoresis.

4.3.5.2 Detection of *Fusarium* DNA using species -specific primers

The following primer pairs VER 1/2, PRO 1/2, and SUB 1/2 (Mule *et al.*, 2004) were used for identification of *F. verticillioides*, *F. proliferatum* and *F. subglutinans*, respectively in PCR assay according Rahjoo *et al.* (2008).

F. verticillioides, VER 1/2

(F: 5′-CTT CCT GCG ATC TTT CTC C-3′, R: 5′-AAT TGG CCA TTG GTA TTA TAT ATC TA-3′);

F. proliferatum, PRO 1/2

(F: 5′-CTT TCC GCC AAG TTT CTT C-3′, R: 5′-TGT CAG TAA CTC GAC GTG TTG-3′)

F. subglutinans, SUB 1/2

(F: 5′-CTG TCG CTA ACC TCT TTA TCC A-3′, R: 5′-CAG TAT GGA CGT TGG TAT TAT TAT ATC TAA-3′).

The PCR assay was done in a total volume of 25 µl of master mix comprising 5X buffer, 25 mM of each dNTP, 25 mM $MgCl_2$, 0.2 µl of Ampli Taq polymerase (Applied Biosystems, USA), 2.0 µL of each primer and 5 µl of fungal template DNA. Reactions were performed in Proflex PCR system thermocycler (Applied Biosystems, USA) under following conditions: denaturation at 95 ^oC for 5 min, 35 cycles of denaturation at 94 ^oC for 50 sec, annealing at 56 ^oC for 50 sec, extension at 72 ^oC for 1 min, final extension at 72 ^oC for 7 min with cooling at 4 ^oC for final recovery of the samples. The amplified products were then visualized in 1.2% agarose gels stained with ethidium bromide.

4.3.6 Test for Aflatoxigenicity

Twenty seven species identified belonging to *Aspergillus* section *Flavi* were grown on PDA at 31 ^oC for a period of 7 days and total aflatoxin extracted from their cultures according to method described by Rao *et al.* (1997). A whole sample comprising: agar, mycelia and spores was ground in a blender for 5 min. Two grams of the blended mixture was used to extract total aflatoxins in 10 ml of 60% methanol. The mixture was then filtered (Whatman #1) and the filtrate analysed by using ELISA Kits for total aflatoxin (Helica Biosystem Inc.).

4.3.7 Data Analysis

All analyses were done using R version 3.3.1 (R Core Team, 2013). The incidences of fungi were presented in counts and percentages as score of total counts. The data for total fungi, *Aspergillus* and *A. flavus* were normalized by log x+1 transformation before mean comparison. However, means of *A. parasiticus, A. fumigatus, A. niger, A. terreus, A. tamarii* and *F. verticillioides* were compared with Mann-Whitney U test or Kruskal-Wallis test.

4.4 Results

4.4.1 Distribution of fungi in soil

A total of 338 fungi in four genera were isolated from soil samples (Table 1). From these isolates, 80.0% were *Aspergillus*, 8.9% *Penicillium*, 5.6% *Trichoderma* and 4.4% *Fusarium* and 1.1% other fungi. The incidence of fungi amongst sub-counties was not statistically significant. However, the incidence of *Fusarium* was low in Kisumu (1.6%) than Siaya (6.1%) and Vihiga (6.3%). This was converse to *Penicillium* which had high (12.5%) incidence in Kisumu than Siaya (5.3%) and Vihiga (8.3%). A significant difference ($t(118) = 4.6018$, $p<0.001$) on distribution of total fungi was observed in short and long rain season. However, SR had lower (29.9%) incidence than LR (70.1%). Similarly, the incidence of *Aspergillus* was significant ($t(118) = 2.1683$, $p<0.001$) in SR and LR season. Incidence of total fungi in both PP and MM was not significant. However, *Penicillium* had high incidence in PP (12.7%) than MM (5.2%).

Table 4: Incidence of ear rot fungi recovered from maize fields in Kisumu, Siaya and Vihiga sub-counties

Variable	Fungal Genera				
	Aspergillus	*Fusarium*	*Trichoderma*	*Penicillium*	Total fungi
	n (%)	n (%)	n (%)	n (%)	n (%)
District					
Kisumu (N=40)	106 (82.8)	2 (1.6)	4 (3.1)	16 (12.5)	128 (37.9)
Siaya (N=40)	88 (77.2)	7 (6.1)	13 (11.4)	6 (5.3)	114 (33.7)
Vihiga (N=40)	80 (83.3)	6 (6.3)	2 (2.1)	8 (8.3)	96 (28.4)
Season					
SR (N=60)	85 (84.2)	2 (2.0)	6 (5.9)	8 (7.9)	101 (29.9)
LR (N=60)	189 (79.8)	13 (5.5)	13 (5.5)	22 (9.3)	237 (70.1)
Cropping system					
PP (N=60)	130 (78.3)	8 (4.8)	7 (4.2)	21 (12.7)	166 (49.1)
MM(N=60)	144 (83.7)	7 (4.1)	12 (7.0)	9 (5.2)	172 (50.9)
Total (N=120**)**	274(81.1)	15 (4.4)	19 (5.6)	30 (8.9)	

n, number of isolates; N, number of samples; SR, short rain; LR, long rain; PP, push-pull; MM, maize monocropping.

4.4.2 Identification of *Aspergillus* and *Fusarium* species

Three species belonging to *Aspergillus* section *Flavi* were identified by colony reverse on AFPA agar (Figure 6). The three species further identified on CZ based on their conidial colour and head serration were *A. flavus,* yellow green surface and numerously biseriated (Figure 7 and Figure 8); *A. parasiticus,* conifer green surface and mainly uniseriated (Figure 9 and Figure 10); and *A. tamarii,* dark green surface and abundantly uniserate (Figure 11 and Figure 12). Other *Aspergillus species* equally identified on PDA by other features were *A. terrei,* sand brown surface with columnar conidial ornamentation (Figure 13 and Figure 14); *A. fumigatus,* blue grey surface and subglobose vesicle (Figure 15 and Figure 16)*;* and *A. nigri,* black surface and brownish, relatively long and smooth conidiophore (Figure 17 and Figure 18).

There was low recovery of *Fusarium* species causing ear rots; however 13 isolates recovered were morphologically belonging to *Fusarium verticillioides* (Figure 19 and Figure 20). Out of these isolates (13), 9 were positive (Figure 21) for *F. verticillioides* after molecular characterization with TEF-gene.

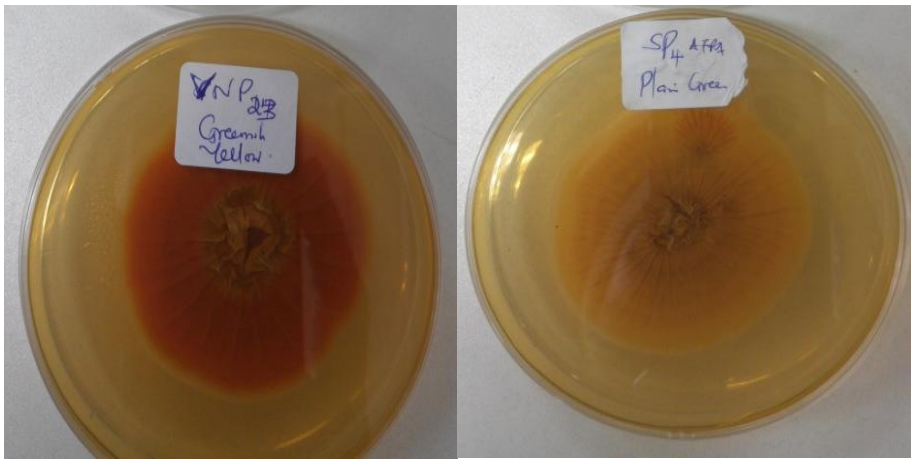

Figure 6: Colony reverses of two isolates in *Aspergillus* section *Flavi* showing bright orange and yellow colour after incubation on AFPA for 5 days at 25 ^{o}C. Orange colour is positive for *Flavi* section.

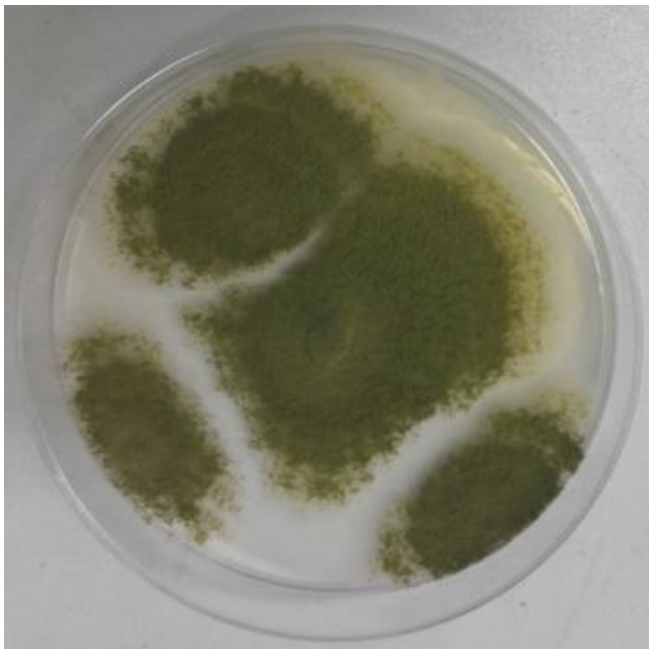

Figure 7: *Aspergillus flavus* greenish yellow surface on CZ.

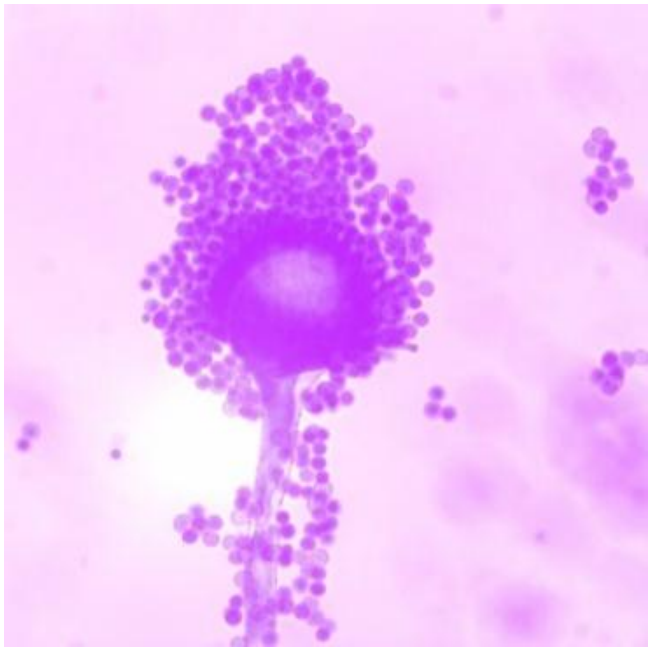

Figure 8: A biserated conidial head with a globose vesicle of *Aspergillus flavus* (Mg=500x).

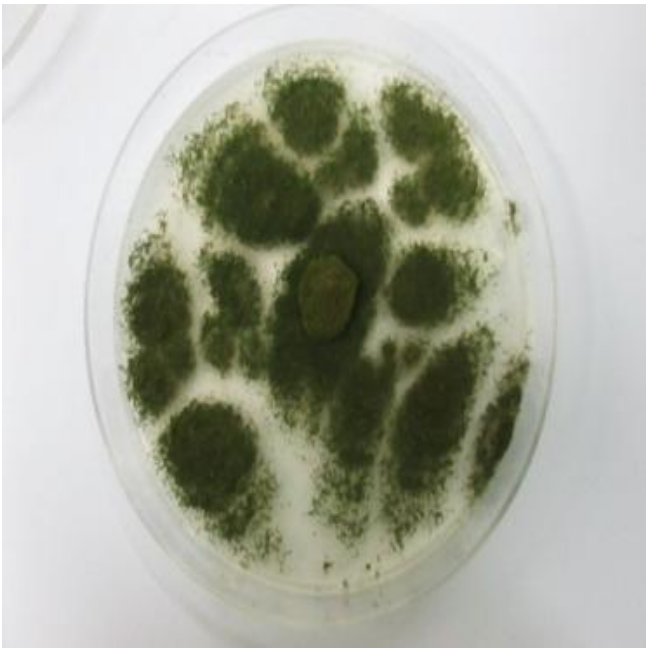

Figure 9: *Aspergillus parasiticus* ivy green surface on CZ.

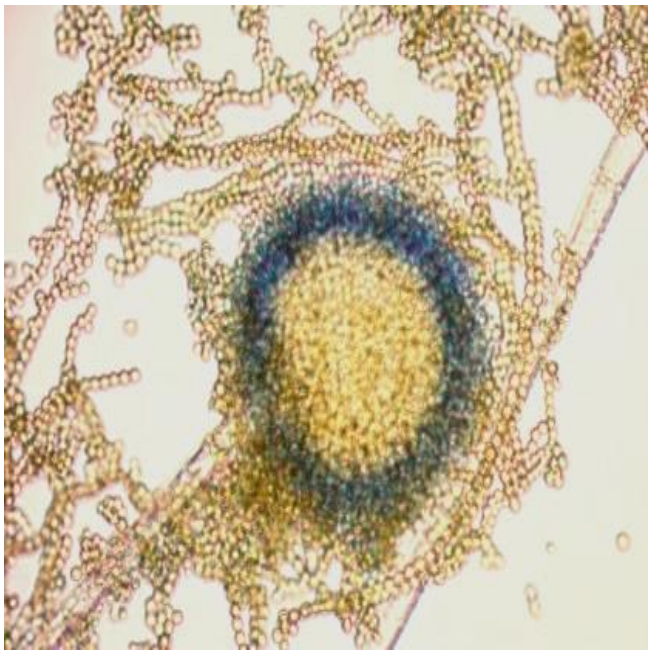

Figure 10: *Aspergillus parasiticus* with uniserated, globose and conidia in chains (Mg=1000x).

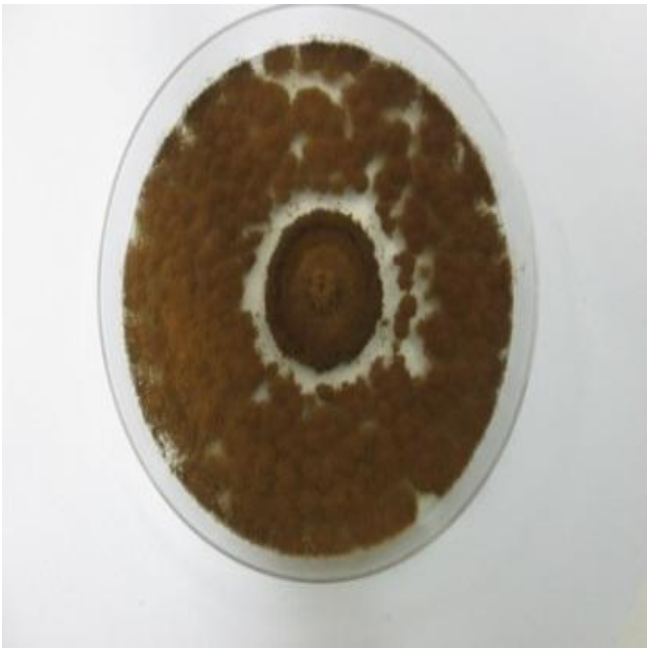

Figure 11: *Aspergillus tamarii* dark brown surface on PDA.

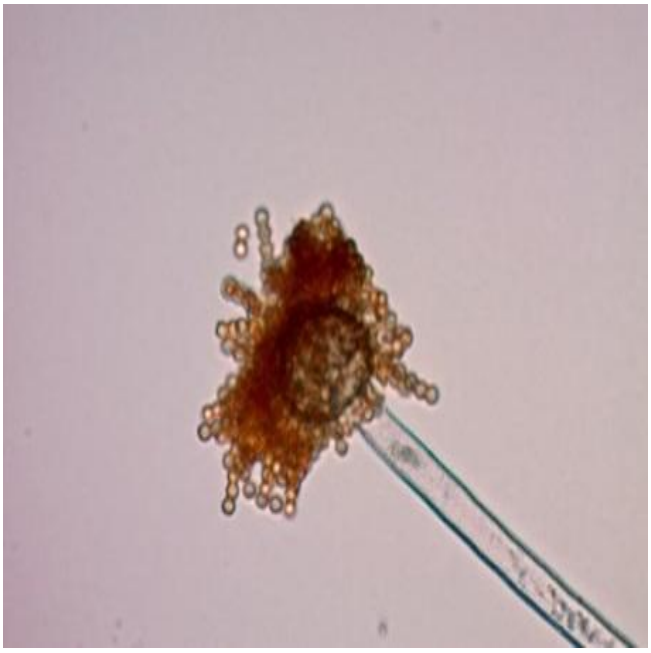

Figure 12: A globose vesicle of *Aspergillus tamarii* (Mg=1000×).

Figure 13: *Aspergillus terreus* sand brown surface on PDA.

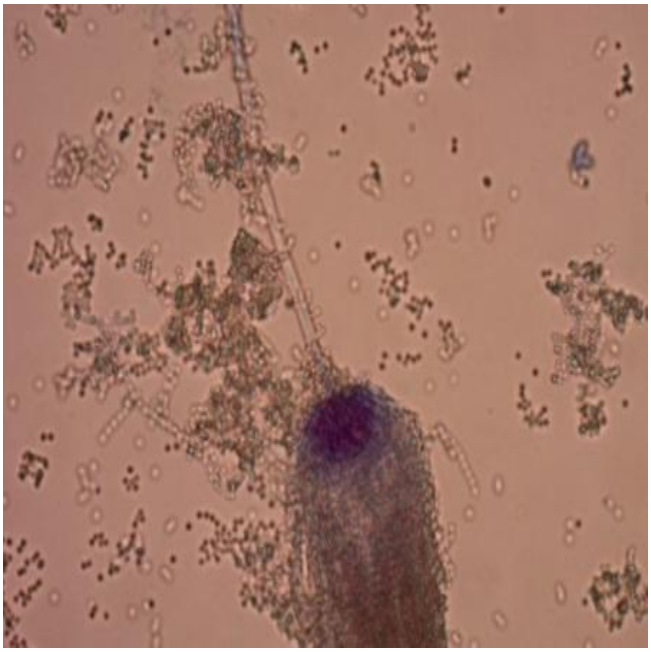

Figure 14: A columnar conidial ornamentation in *Aspergillus terreus* (Mg=500×).

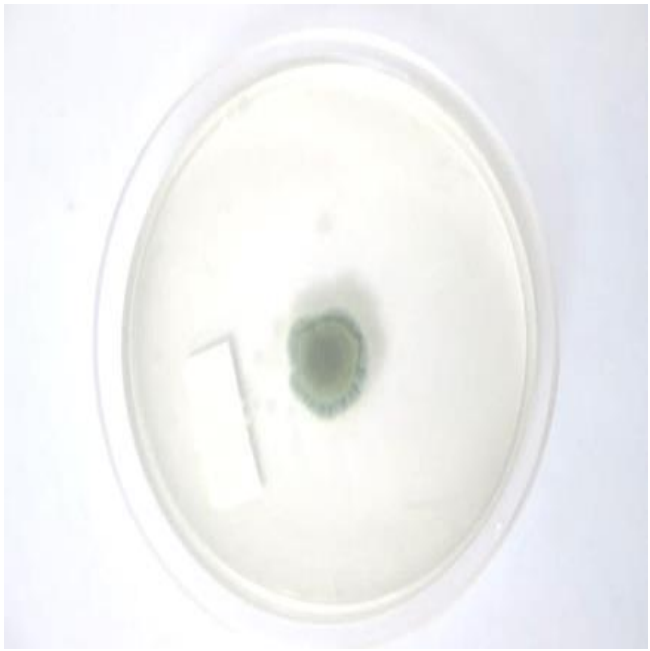

Figure 15: *Aspergillus fumigatus* blue grey surface on CZ.

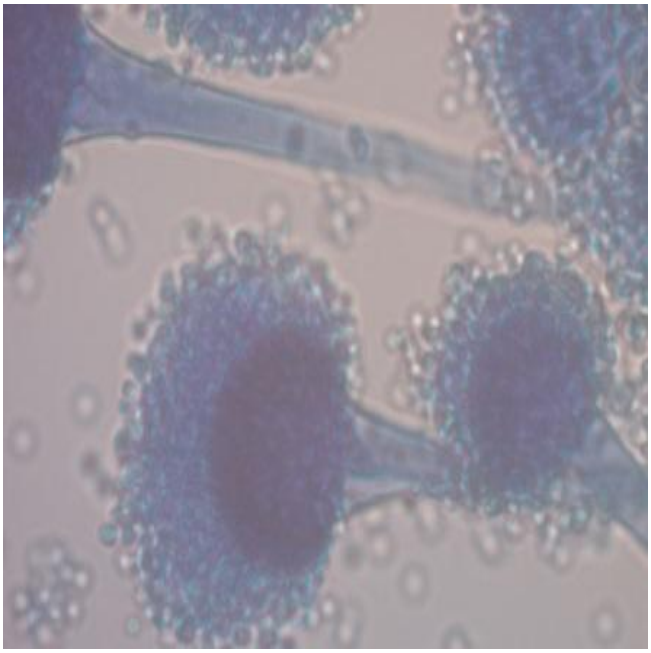

Figure 16: *Aspergillus fumigatus* subglobose vesicle (Mg=1000x).

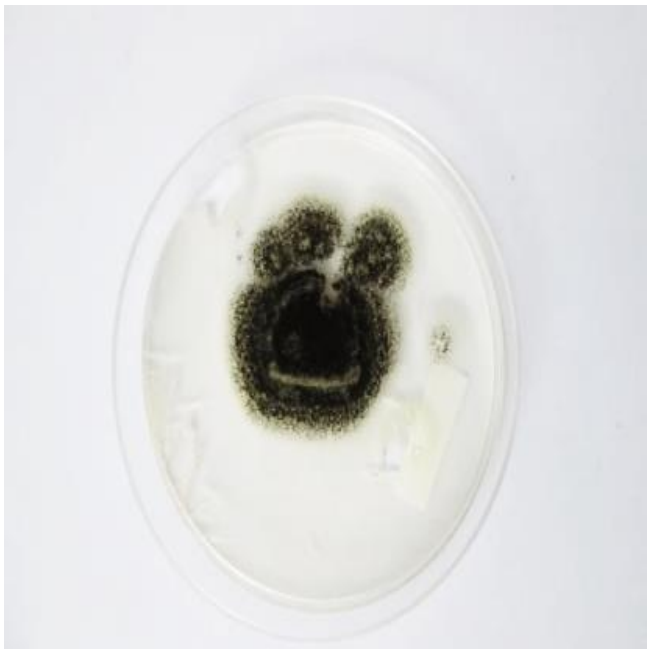

Figure 17: *Aspergillus niger* black surface on CZ.

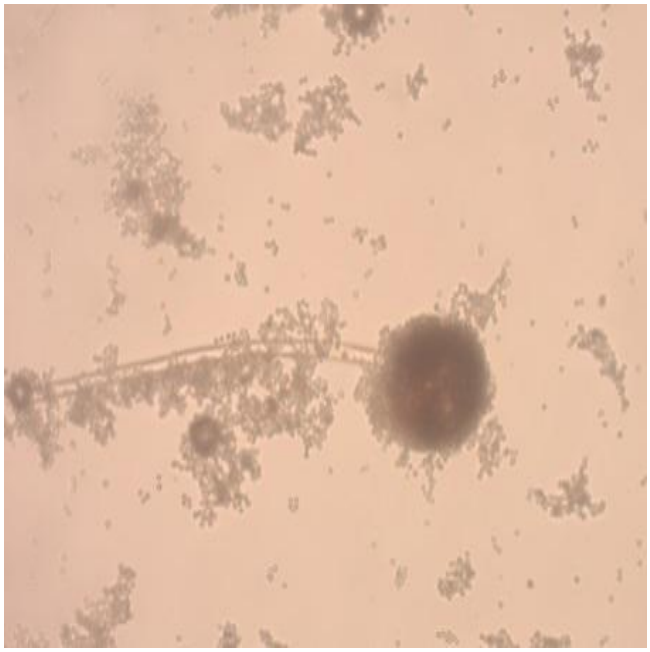

Figure 18: A brownish, relatively long and smooth conidiophore of *A. niger* (Mg=400×).

Figure 19: *Fusarium verticillioides* surface on PDA after 7 days of incubation.

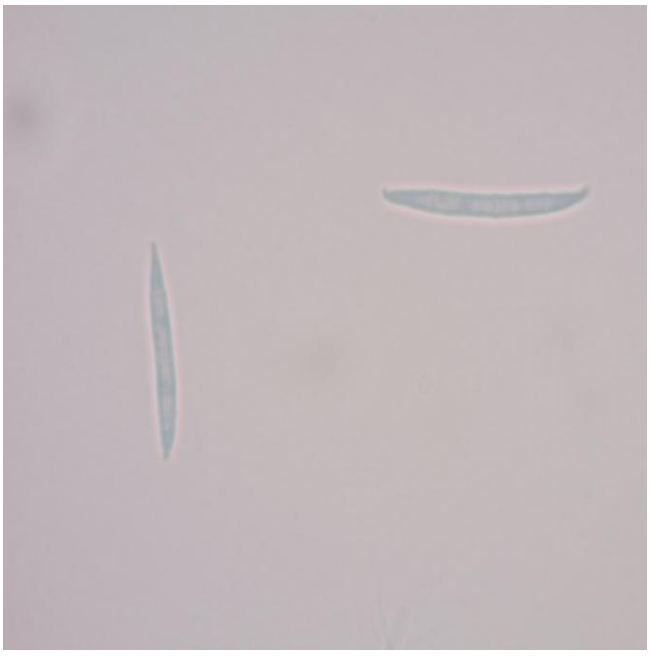

Figure 20: *Fusarium verticillioides* macroconidia (Mg=1000x).

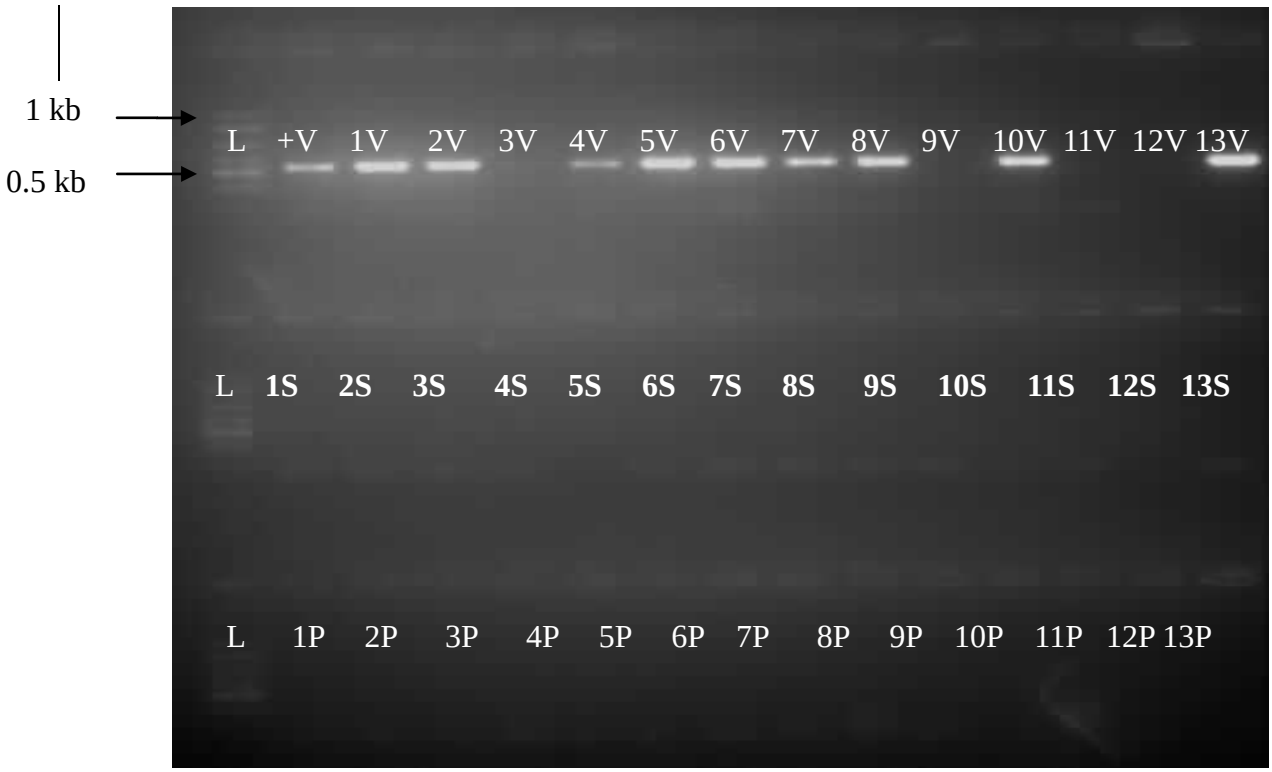

Figure 21: Gel electrophoresis of PCR amplified Translation elongation factor-1 alpha gene (611 bp) on 13 isolates of *Fusarium* section *Liseola*. Isolates signified as V, *F. verticillioides*; S, *F. subglutinans*; and P, *F. proliferatum*. Lane L: 1kb base pair ladder; +, positive control for *F. verticillioides*. Electrophoresis was performed on 1.2 % agarose gel.

4.4.3 Incidence of *Aspergillus* and *Fusarium* species

The respective distribution of six *Aspergillus* and *Fusarium* species in push-pull and monocrop systems were as follows: *A. flavus* 39.8% and 60.2%; *A. fumigatus* 55.2% and 44.8%; *A. niger* 35.6% and 64.4%; *A. parasiticus* 71.4% and 28.6%; *A. terreus* 53.3% and 46.7%; *A. tamarii* 20% and 80%; *F. verticillioides,* 50% and 50%; and *F. graminearum,* 66.7% and 33.3% respectively (Table 5). A high incidence of total and individual fungi was observed during long than short rain seasons respectively, except *A. parasiticus* which was abundant in short (78.6%) than long rain season (21.4%). Majority of aflatoxigenic fungi were positive for aflatoxins (81.5%) with only 8.3% and 23.7% of *A. parasiticus* and *A. flavus*, respectively being atoxigenic (Table 6).

Table 5: Population of *Aspergillus* and *Fusarium spp.* in different cropping systems and seasons

Fungi species	Cropping system		Season	
	Push-pull	Maize monocrop	Short Rain	Long Rain
A. flavus	39 (39.8)	59 (60.2)	31 (31.6)	67 (68.4)
A. fumigatus	37 (55.2)	30 (44.8)	12 (17.9)	55 (82.1)
A. niger	16 (35.6)	29 (64.4)	9 (24.3)	28 (75.7)
A. Parasiticus	20 (71.4)	8 (28.6)	22 (78.6)	6 (21.4)
A. terreus	16 (53.3)	14 (46.7)	2 (6.7)	28 (93.3)
A. tamarii	1 (20.0)	4 (80.0)	1 (20.0)	4 (80.0)
F. verticillioides	9 (50.0)	9 (50.0)	2 (11.1)	16 (88.9)

n, number of isolates; (%), raw percentages calculated based on counts within district, cropping system and season.

Table 6: Percentage of selected section *Flavi* isolates tested for aflatoxigenicity

Species	No. of isolates	Toxigenic	Atoxigenic
A. flavus	15	73.3%	23.7%
A. parasiticus	12	91.7%	8.3%
Total	27	81.5%	18.5%

No, Number; %, percent

4.4.4 The population density of *Aspergillus* and *Fusarium* species in soil

There was low population density of fungi was observed in push-pull (2,266.1 $CFUg^{-1}$) than in monocrop plots (2,499.9 $CFUg^{-1}$). *Aspergillus parasiticus* was the only species which had high population in push-pull (333.3 $CFUg^{-1}$) than in the monocrop system (133.3 $CFUg^{-1}$), with relatively small insignificant difference ($p<0.067$). During long rain season, a significantly high population of *A. flavus*, *A. fumigatus*, *A. terreus* ($p<0.001$) and *A. parasiticus* ($p<0.05$) were also observed.

Table 7: Population (CFU g^{-1}) of *Aspergillus* and *Fusarium species* in different cropping systems and seasons

Fungi	Cropping system			Season		
	PPT (mean $CFUg^{-1}$)	MM (mean $CFUg^{-1}$)	P-values	LR (mean $CFUg^{-1}$)	SR (mean $CFUg^{-1}$)	P-values
A. flavus	650.0	983.3	0.405	1,116.7	516.7	0.0012
A. fumigatus	616.7	500.0	0.330	916.7	200.0	0.001
A .niger	266.7	483.3	0.090	433.3	316.7	0.550
A. parasiticus	333.3	133.3	0.067	100.0	366.7	0.054
A. terreus	266.7	233.3	0.464	466.7	33.3	0.0001
A. tamarii	16.7	66.7	0.311	66.7	16.7	0.311
F. verticillioides	116.0	100.0	0.761	200.0	16.7	0.0045
Total	2,282.8	2,516.6	0.856	3316.8	1483.5	0.001

PP, Push-pull; MM, Maize monocrop; $CFUg^{-1}$, Colony forming unit per gram of soil; LR, Long rain season; SR, Short rain season; Significance level ($p=0.05$).

4.5 Discussion

There was higher (averagely 80%) incidence of *Aspergillus* than other fungi in all districts, cropping systems and seasons observed in this study. This corroborates findings of other studies in different agro-ecological areas in Kenya that reported relatively higher incidence of *Aspergillus* relative to other fungi (Okoth *et al.*, 2012; Karanja, 2013). However, insignificant difference in incidence of *Aspergillus* between push-pull and maize monocrop systems contradicted several findings which showed significant increases in *Aspergillus* population with minimum tillage and organic matter ammendments (Nesci *et al.,* 2006; Zablotowicz *et al.,* 2007; Dubova *et al.,* 2016). Thus, more *Aspergillus* expected on a conserved system like push-push which improves organic matter content in the soil and reduces the amount of tillage was not observed. This observation could be explained on the basis that historically, and depending on the cropping season and amounts of rainfall, most farms in western Kenya more often have maize intercropped with food legumes such as common bean (*Phaseolus vulgaris* L.) and peanuts (*Arachis hypogaea* L.) (Mudavadi *et al.,* 2007; Mutegi, 2010). Such edible legumes provide beneficial ecological services of soil improvement through addition of organic matter and nitrogen fixation that could increase *Aspergillus* incidence in the soil.

Soil is the main reservoir for both *A. flavus* and *A. parasiticus* with relatively higher frequency of the former (Klich, 2007). However, the frequency of *A. parasiticus* is comparatively higher and more endemic in soils where peanut or sugarcane is grown relative to that under maize (Diener *et al.,* 1987; Garber and Cotty, 2014). In the current studies, there was higher frequency of *A. parasiticus* in soil samples from push-pull compared to maize monocrop systems. Similarly the frequency of incidence was higher during the short rainy season relative to the long rainy season. Although not measured in the current study, soil temperature has been reported to influence incidence of these fungi, with lower temperatures favoring *A. parasiticus* relative to *A. flavus* (Horn, 2005). Pitt and Miscamble, (1995) and Horn, (2005) showed that the optimal temperature of 22 ^{o}C is suitable for growth of *A. parasiticus* while 30-37 ^{o}C for *A. flavus*. This cool soil temperature is encouraged by cultural practices such as cover cropping, reduced tillage (Sławiński *et al.,* 2012), and wet season (Horn *et al.,* 1995) resulting into increased soil moisture.

Therefore, high frequency of *A. parasiticus* in soil samples from push-pull system was possible since *Desmodium* provides cover cropping for a longer period due to its perennial nature compared to annual edible intercrop legumes often used in western Kenya. Push-pull is also practiced on a reduced tillage in both land preparation and weeding. For the long rain season, *A. parasiticus* was less frequent since the study area is characterized by long dry spells which increases soil temperature than during the short rainy season (Mugalavai *et al.,* 2008).

Several studies on aflatoxin production have reported fewer incidences of non-aflatoxin (atoxigenic) producers amongst *A. parasiticus* isolates (Tran Dinh *et al.,* 1999; Barros *et al.,* 2006), except in few cases (Okoth *et al.,* 2012; Salano *et al.,* 2016). The current study supports these findings as 8.3% of *A. parasiticus* isolates compared to 23.7% of *A. flavus* were positive for aflatoxin production. With more aflatoxigenic fungi, the merit of conserved systems in increasing soil agricultural sustainability might also expose crops to aflatoxin contamination by increasing their *A. flavus* propagules in soils (Zablotowicz *et al.,* 2007). However, contamination of maize is not entirely dependent on the population of *A. flavus* in the soil (Horn *et al.,* 1994) since maize intercropping which encourages more *A. flavus* has shown low aflatoxin contamination compared to sole cropping system (Mutiga *et al.,* 2015). Therefore, as revealed in these studies, intercrops are able to reduce *Aspergillus* infections and contamination through other factors such as increased soil nitrogen and limiting insect damage (Bruns, 2003).

The frequency of *A. parasiticus* or ratios of *A. flavus/A. parasiticus* in this study suggest the potential levels of contamination in maize. Studies show that *A. parasiticus* is comparatively a poor colonizer of aerial plants like maize (Horn, 2003) and have low spore density in air (Horn *et al.,* 1995) than *A. flavus* (Hedayati *et al.,* 2007). In deed, studies by Angel *et al.* (1982) and Lillehoj *et al.* (1980) observed almost complete infection of maize ears with *A. flavus* despite high incidence of both *A. parasiticus* and *A. flavus* presence in soil. Therefore, increased frequency of occurrence of *A. parasiticus* in push-pull relative to maize monocrop warrants further investigation.

Soil as the main reservoir for both *A. flavus* and *A. parasiticus* has relatively higher frequency of the former *Aspergillus* species than the latter (Klich, 2007). However, the frequency of *A. parasiticus* is comparatively higher and more endemic in soils where peanut or sugarcane is grown relative to that under maize (Garber and Cotty, 2014). Although not measured in the current study, soil temperature has been reported to influence incidence of these fungi, with lower temperatures favoring *A. parasiticus* relative to *A. flavus* (Horn, 2005). Optimally, *A. parasiticus* grow at temperature of 22 ^{o}C while *A. flavus* at 30-37 ^{o}C (Horn, 2005). This cool soil temperature is encouraged by cultural practices such as cover cropping, reduced tillage (Sławiński *et al.,* 2012), and wet season (Horn *et al.,* 1995). In the push-pull system, *Desmodium* provides soil cover for a longer period due to its perennial nature compared to annual edible intercrop legumes common (bean and peanuts) in western Kenya. The push-pull system also manifest limited tillage practices during land preparation and weeding for conservation, and from cover cropping of *Desmodium,* respectively. This explain probable low soil temperature in PP thus higher population of 71.4% was observed on *A. parasiticus* in soil samples from push-pull compared to 28.6 % in maize monocrop systems. The long dry spells which increases soil temperature in long rainy season than the short rainy season (Mugalavai *et al.,* 2008) also account for low (21.4%) population of *A. parasiticus* during the long rainy season relative to 78.6% during the long rainy season in our observation.

The results of the current study show that *A. terreus*, *A. niger* and *A. fumigatus* had high prevalence in soils after *A. flavus*. This observation corroborates reports of most studies on distribution of microflora in the soil (Horn *et al.,* 1995; Horn, 2005; Sharma and Raju, 2013). However, studies by Salano *et al.* (2016) reported a high *A. niger* than *A. flavus* in eastern province of Kenya. These species have less impact on quality of most grains, although they play a role in mineralization of other plant nutrients as well as production of other mycotoxins and human infections. For instance, *A. niger* is effective in solubilization of phosphate (Reena *et al.,* 2013) besides current report on production of fumonisins and ochratoxins A (Mogensen *et al.,* 2010; Palencia *et al.,* 2010). *A. terreus* also produces toxin known as territrem (El-Sayed Abdalla *et al.,* 1998), while *A. fumigatus* is the cause of invasive aspergillosis (Hedayati *et al.,* 2007).

Previously, study had shown low frequency of *Fusarium* section *Liseola* and *F. graminearum* isolates in soils from maize fields (Okoth and Siameto, 2010). Similar observation of low incidence of *Fusarium* section *Liseola* with no incidence of *F. graminearum* was made in this study. The most plausible explanation for this occurence could be due to their inherent scarcity (Okoth and Siameto, 2010) or effects of organic matter in the soil (Alakonya *et al.*, 2008) from intercropping systems common in western Kenya. But importantly, low soil *Fusarium* incidence herald more infection from aerial spores and external sources.

The cultural identification in *Fusarium* section *Liseola* is demanding and limiting (Summerell *et al.*, 2003), thus molecular methods are used for confirmation. In molecular identification of *F. verticillioides* using translation elongation factor 1-alpha (TEF) gene, 140 isolates culturally identified as *F. verticillioides*, 133 and 4 isolates were confirmed as *F. verticillioides* and *F. proliferatum*, respectively (Rahjoo *et al.*, 2008). Therefore, further identification of species in *Fusarium* section *Liseola* using TEF genes is more accurate and reliable. In this study, 13 isolates that were initially identified by cultural characteristics as *F. verticillioides*, 9 isolates were positive for *F. verticillioides* using TEF gene. However, *F. proliferatum* and *F. subgluinans* were not present amongst the isolates.

In conclusion, seasons had significant influence on distribution of *Aspergillus* and *Fusarium* fungi in soil while cropping system did not. The high *Aspergillus* fungi in the soil in this study show that soil fungal community within the field is a potential risk for aspergillus ear rot infection and aflatoxin contamination, while the low frequency of *F. verticillioides* and *F. graminearum* in the soil samples suggest external inoculum as important for both gibberella and fusarium ear rot infection in the field.

4.6 References

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CHAPTER FIVE

GENERAL DISCUSSION

5.1 Incidence of ear rot infection and fungal soil inoculum

Inocula sources of *Aspergillus* and *Fusarium* including soil, crop debris and insects are important in epidemiology of maize ear rot diseases. However, for ear rot disease development, several interactions involving other factors such as growth stage of maize (anthesis), infection route, dispersal method of conidiophores and environmental factors such as temperature and humidity are crucial (Wicklow, 1994; Munkvold *et al.*, 1997; Argyris *et al.*, 2001). In the present study, the distribution of *Aspergillus* was high in soils, but aspergillus ear rot incidence was low. This observation is in agreement with views by Abbas *et al.* (2009) that categorized *A. flavus* as non-aggressive during pre-harvest stage despite being the main causative agent of aspergillus ear rot. However, in the event of insect damage or maize phenotype with open tips where the kernels are exposed or compromised, occurrence of aspergillus ear rot is often observed. Thus from earlier times (Riley, 1882) the appearance of greenish-yellow spores typical of *A. flavus* on ear or kernel was observed on ears compromised by insect damaged in the field. The presence of high *A. flavus* propagules in the soil thus implies prospects of high aflatoxin production in the crop (Shearer *et al.*, 1992) rather than aspergillus ear rot infection.

In the current study, a high gibberella ear rots incidence and absence of *F. graminearum* isolated from soil samples is likely to suggest a distant inoculum source. This could be explained by a common practice of cutting maize stalks (stooks) to dry, and heaping them either at points on the maize fields or within homesteads in western and rift valley. The stooks are the main inoculum source for gibberella ear rot infection because they have more maize debris. This is in line with findings of Andries *et al.* (2000) and Dill-Macky and Jones (2000) who reported that maize debris were a major source (83%) of peritheca, the main body that produces ascospores which initiate infection by *F. graminearum*. Since ascospores are more dispersed (Shah *et al.*, 2000; Xu, 2003) than macroconidia (Horberg, 2002), high gibberela ear rot might have been from sources near the field.

5.2 Seasonal variation of soil fungi and ear rot incidence

The current study found significantly higher incidence and severity of fusarium and aspergillus ear rots, as well as populations of their corresponding fungi in the long rainy season. However, during the same period, the incidence and severity of gibberella ear rot were low, with no propagules in the soil samples. This could be explained in terms of the rainfall patterns and dry spells in the study region. The cessation of rainfall often results in dry spells which expel soil moisture resulting in high soil temperatures which favor germination, growth and sporulation of causal agents of aspergillus and fusarium ear rots (Horn *et al.,* 1995; Vincelli *et al.,* 1995). In western Kenya, this occurs after the long rainy seasons in which the dry spells are often more prolonged than that following the short rainy seasons (Mugalavai *et al.,* 2008). As such, in order to deal with this current challenge of crop infestation, farming practices that improve and/or conserve soil moisture such as cover cropping have a potential to reduce distribution of fungal spores.

5.3 Aflatoxigenic fungal population and incidence and levels of aflatoxins on ear samples

There was high density of aflatoxigenic *A. flavus* and *A. parasiticus* in soil samples from both cropping systems. Studies suggest that a high population of *A. flavus* in soil indicates a concomitant increase in the chances of aflatoxin outbreaks (Jaime-Garcia and Cotty, 2004), as was reported in Iowa aflatoxin epidemic in 1988, that resulted from high soil *A. flavus* propagules (about 1,231 CFU g^{-1}) (Sheare *et al.,* 1992). This could be the reason for the higher incidence and levels of aflatoxin in symptomatic ears samples relative to the values obtained from the asymptomatic ears in the current study. However, soil density of *Aspergillus* species is not the only predictor of infection frequency (Horn *et al.,* 1994). Study has shown that intercrop and conserved tillage systems increase organic matter content of the soil which favors *A. flavus* increase in soil (Zablotowicz *et al.,* 2007); however, less aflatoxin contamination on maize from intercrop systems has been reported (Mutiga *et al.,* 2015).

This could be explained by other factors and practices such as addition of nitrogen, insect control and cover cropping (Bruns, 2003) which also play a role in reduction of aspergillus ear rot infection and aflatoxin contamination.

In this respect, push-pull adds nitrogen to the soil through desmodium and also protects the maize ears from insect damage in addition to lowering soil temperature through cover cropping (Khan *et al.,* 2011).

5.4 Incidence of gibberella ear rots and zearalenone and deoxynivalenol

The current study reported gibberella ear rot as the most frequent mycotoxigenic ear rot. Similarly, high incidence levels were reported for its related mycotoxins, zearalenone (ZEA) and deoxynivalenol (DON). These findings indicate that gibberella ear rot and incidence of ZEA and DON might be related since more symptomatic samples had more samples with high levels of these mycotoxins. A less exposure to DON has been previous reported in Kenya in maize samples and feedstuff (Muthomi *et al.,* 2008), local brews (busaa) and dairy feeds (Makau *et al.,* 2016). However, the high incidence and levels of DON in this study and those detected in some household maize (23,586 μg/Kg) in Tanzania (Degraeve *et al.,* 2016) suggest more human and animal exposure. Together with high incidence (100%) and level of ZEA on all maize samples studied, human and animal exposure was revealed. Therefore, awareness on management of DON and ZEA among maize stakeholders is necessary.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The current study revealed potential of push-pull cropping system in reduction of ear rot incidence and severity. The incidence and high levels of mycotoxins in symptomatic ear samples was also confirmed as the primary source of mycotoxins in the maize value chain. Thus, pre-harvest control of ear fungal infection represents possibility to reduce ear rot fungal inoculum and control progression of mycotoxin at post-harvest stage. Since, aspergillus ear rots were poorly developed at harvesting time (had low incidence), and one "clean" or asymptomatic maize sample was found with some levels of aflatoxins, this study confirm *Aspergillus* species as non-aggressive at pre-harvest (Abbas *et al.,* 2009). However, their population in the soil and percentage of aflatoxigenic species show potential risk in terms of mycotoxin contamination.

The population of *A. parasiticus* was insignificant in both cropping systems. However, a higher frequency of *A. parasiticus* was observed in push-pull relative to the maize monocrop system. The change in *A. flavus/A. parasiticus* ratio in soil ecology could affect aspergillus ear rot development and aflatoxin production. For penicilium ear rot, both incidence and severity were the lowest in comparison to other ear rots. This shows that penicillium ear rot atypical pre-harvest ear rot. However, fusarium and gibberella ear rots were well developed at pre-harvest. This means the latter ear rots, gibberella and fusarium, can be controlled from the fields and their mycotoxins, DON and ZEA reduced as well.

Apart from AFB and FB, there were presence and high levels of ZEA and DON on maize samples in these findings showing additional potential of mycotoxin exposure in the maize value chain. However, incidence and level of these two mycotoxins in symptomatic ears were high. Previous studies suggested that these mycotoxins were major a problem in wheat (Muthomi *et al.,* 2008). However, our study and another in Tanzania confirm incidence of these mycotoxins at high levels in maize. Thus, more awareness and sensitization of these emerging mycotoxins should be carried out along traditional mycotoxins such as aflatoxins and fumonisins.

The molecular studies revealed that identification of *Fusarium* section *Liseola* is precise and not limiting. These were in agreement with findings by Rahjoo *et al.* (2008*)* that reported lower number of *F. verticillioides* from previous number and additional species, *F. proliferatum* from same population identified as *F. verticillioides*.

6.2 Recommendation

These studies reveal potential impact of push-pull system on ear rots and mycotoxins, although the level of aflatoxigenic fungi was high in soil samples from both push-pull and maize monocrop systems. Apart from traditional mycotoxins (fumonisin and aflatoxins) in sub-Saharan Africa, emerging mycotoxins (deoxynivaleneol and zearalenone) were found at high levels and occurrence in maize ears. Therefore, the following recommendations were made:

1. Integration of push-pull cropping system with current methods for management of grain quality and quantity amongst smallholder farmers.
2. Awareness and sensitization of farmers and consumers on emerging mycotoxins in Africa such as zearalenone and deoxynivalenol.
3. The effects of desmodium legume on ear rot fungal species.

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APPENDICES

Appendix 1: Quantity of mycotoxins from 76 samples of asymptomatic and symptomatic ear

Ear type	AF µg/Kg	FB µg/Kg	DON µg/Kg	ZEA µg/Kg
Asymptomatic	0	5,960	0	0
Asymptomatic	0	0	0	0
Asymptomatic	0	0	0	56.2
Asymptomatic	0	0	0	25.9
Asymptomatic	0	5,650	3,760	34.8
Asymptomatic	0	0	1,362	31.4
Asymptomatic	0	1,420	1,360	21.8
Asymptomatic	0	0	4,360	39.9
Asymptomatic	0	6,350	240	22.1
Asymptomatic	0	3,850	0	21.8
Asymptomatic	0	5,110	0	14.7
Asymptomatic	0	0	560	26.6
Asymptomatic	0	4,780	1,640	30.6
Asymptomatic	0	2,740	650	25.0
Asymptomatic	11.7	2,710	0	24.2
Asymptomatic	0	0	270	15.1
Asymptomatic	0	2,720	0	20.9
Asymptomatic	0	0	0	16.8
Asymptomatic	0	0	0	25.6
Asymptomatic	0	0	0	27.2
Asymptomatic	0	0	970	31.5
Asymptomatic	0	0	1,860	21.6
Asymptomatic	0	0	390	23.7
Asymptomatic	0	6,460	420	29.7
Asymptomatic	0	0	0	29.0
Asymptomatic	0	0	0	27.0

Asymptomatic	0	0	0	32.8
Asymptomatic	0	0	0	24.8
Asymptomatic	0	2,370	880	405.8
Asymptomatic	0	0	470	194.7
Asymptomatic	0	0	460	198.9
Symptomatic	0	0	0	565.5
Symptomatic	0	0	0	124.3
Symptomatic	0	0	820	18.7
Symptomatic	28.9	4,430	18,260	608.0
Symptomatic	0	6,820	4,830	60.5
Symptomatic	0	7,640	1,180	184.4
Symptomatic	2.1	0	520	50.9
Symptomatic	0	2,510	0	42.2
Symptomatic	0	0	520	612.9
Symptomatic	26.6	0	0	438.5
Symptomatic	0	3,170	16,790	529.5
Symptomatic	0	6,090	1,610	233.6
Symptomatic	0	0	2,260	111.4
Symptomatic	0	7,440	540	129.4
Symptomatic	0	6,970	10,690	510.1
Symptomatic	16.3	7,500	3,130	57.0
Symptomatic	14.1	7,910	3,970	32.0
Symptomatic	28.7	7,530	8,230	216.9
Symptomatic	0	7,410	1,640	676.2
Symptomatic	0	0	12,480	104.8
Symptomatic	0	4,370	560	36.7
Symptomatic	12.5	7,630	330	49.7
Symptomatic	0	6,810	1,990	554.7
Symptomatic	0	6,610	0	457.9
Symptomatic	0	8, 280	0	256.9

Symptomatic	18.5	0	0	506.8
Symptomatic	28.6	4,770	2,830	681.6
Symptomatic	0	3,810	700	186.5
Symptomatic	0.4	6,870	830	81.0
Symptomatic	11.5	0	690	88.3
Symptomatic	0	6,640	3,180	34.4
Symptomatic	0	7,360	-	107.5
Symptomatic	0	7,130	-	670.7
Symptomatic	27.1	7,920	-	125.3
Symptomatic	28.4	5,910	-	39.7
Symptomatic	0	0	-	168.7
Symptomatic	23.3	5,830	-	172.6
Symptomatic	0.4	6,920	-	301.3
Symptomatic	0	8,060	-	606.0
Symptomatic	0	0	-	54.9
Symptomatic	0	0	-	310.1
Symptomatic	0	0	-	125.5
Symptomatic	0	0	-	409.1
Symptomatic	-	-	-	75.7
Symptomatic	-	-	-	688.2
Symptomatic	-	-	-	511.8
Symptomatic	-	-	-	396.8
Symptomatic	-	-	-	163.7

Appendix 2: Media compositions of Spezieller Nährstoffarmer Agar (SNA)

Chemical	**Quantity**
KH_2PO_4	1 g
KNO_3	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
Glucose	0.2 g
Sucrose	0.2 g
Agar	20 g

Appendix 3: The volume of single reaction of Mastermix

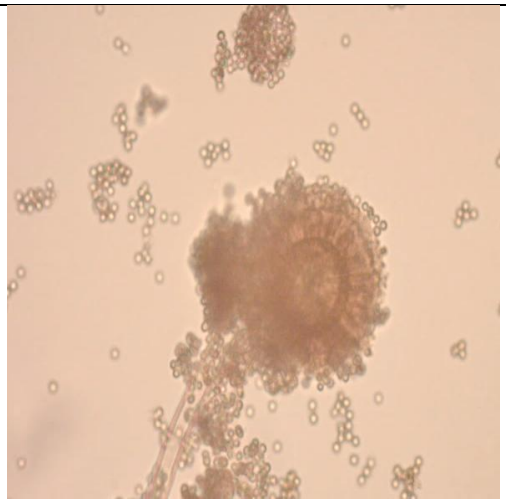
Reagent	Quantity (µl)
PCR ddH_2O	11.1
5X Buffer	5.0
25mM dNTPs mix	0.2
25mM $MgCl_2$	1.5
Primer F	1.0
Primer R	1.0
Taq pol	0.2
Mastermix total volume	20
Template	5
Total volume	25

Appendix 4: Maximun levels of mycotoxins in unprocessed maize for human consumption

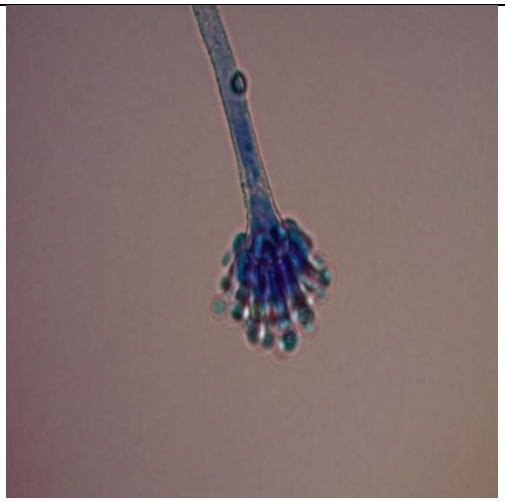
Mycotoxins	EU limit (µg/Kg)	KEBS limit (µg/Kg)	Used in study (µg/Kg)
Total aflatoxin	10	10	10
Total Fumonisins	4,000	-	4,000
Deoxynivalenol	1,750	-	1,750
Zearalenone	350	-	350

EU, European Union; KEBS, Kenya Bureau of Standards.

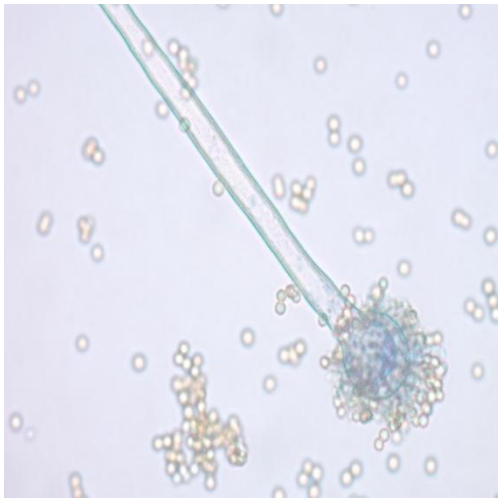
Appendix 5: Main micromorphological features used for identification of *Aspergillus*



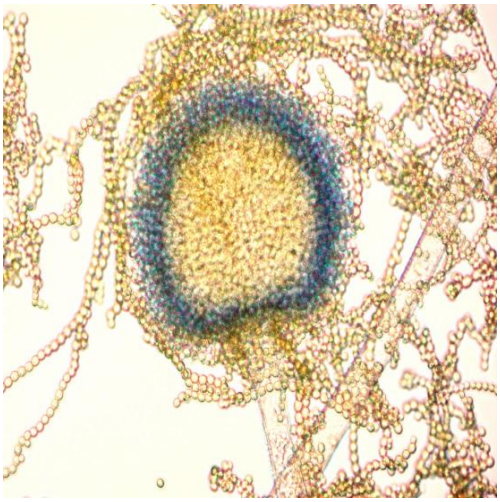
Biserated head



uniserated head

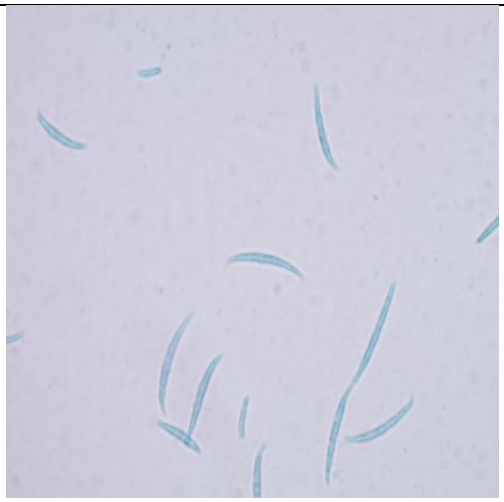


Conidia scattered

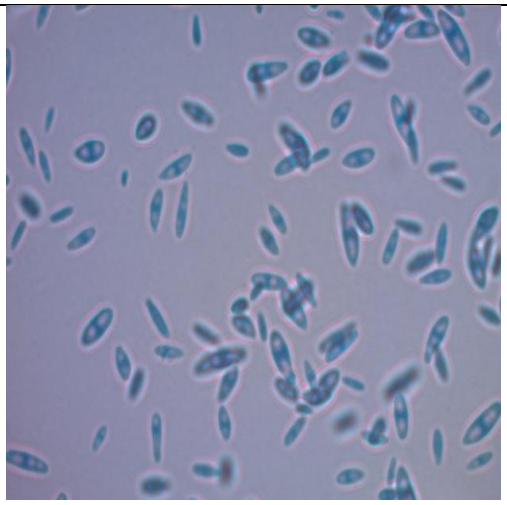


Conidia arranged in chains

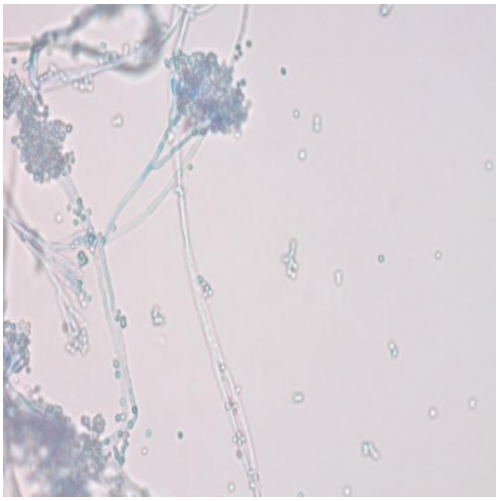
Appendix 6: Micromorphological features used for identification of *Fusarium*, *Alternaria* and *Penicilium*



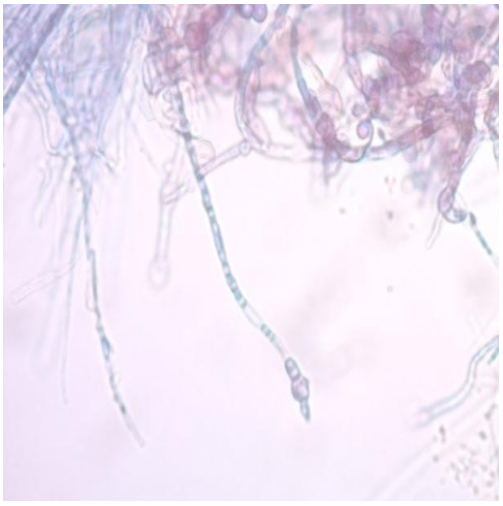
Fusarium macroconidia



Fusarium microconidia



Penicillium



Alternaria