INSIGHTS INTO THE GENETIC AND ENVIRONMENTAL BASES OF MYCOTOXIN CONTAMINATION IN KENYAN MAIZE

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Samuel Kilonzo Mutiga

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INSIGHTS INTO THE GENETIC AND ENVIRONMENTAL BASES
OF MYCOTOXIN CONTAMINATION IN KENYAN MAIZE

Samuel Kilonzo Mutiga, Ph.D.
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Mycotoxins are toxic fungal secondary metabolites that contaminate an estimated 25% of foods globally. Aflatoxin and fumonisin are major mycotoxins that contaminate maize in tropical countries. Kenya's frequent aflatoxicosis outbreaks and the associated human fatality rates have received global attention. The objective of this dissertation was to investigate the extent and the drivers for mycotoxin contamination in Kenyan maize. Between May 2009 and March 2010, surveys were conducted in three provinces (Rift Valley, Western and Nyanza) of western Kenya, the country's grain basket and a region where mycotoxin outbreak had not been recognized. Aflatoxin contamination above the regulatory limit of 10 ppb was observed in 15% of the flour samples that had been collected from the patrons of local mills. Drought and monocropping were identified as drivers for increased aflatoxin contamination. A longitudinal survey in farmers' storage sheds and at local mills in Western Province revealed vulnerability of the most popular varieties to mycotoxins. Surveys were conducted in 10 districts of Eastern Province during an aflatoxin outbreak in 2010. Aflatoxin contamination above 10 ppb was observed in 39% of the flour samples from patrons (n=1500) of local mills, while 37% were above the 1 ppm regulatory limit for fumonisin. Reduced aflatoxin accumulation was associated with intercropping, larger farms and high grain yield. Visual grain sorting reduced fumonisin but not aflatoxin levels. Analysis of aflatoxin in naturally-infected diverse maize germplasm grown in eastern Kenya showed higher contamination under low nitrogen (N) than under optimal N. Early-maturing maize had reduced aflatoxin accumulation under low N, possibly because of faster utilization of the limiting and diminishing soil N. A mature kernel screening assay of diverse maize inbred lines showed that colonization and aflatoxin accumulation were influenced by the ear environment (where maize were grown). High sulfur content in grain was associated with reduced kernel colonization and aflatoxin accumulation. Management strategies should include: 1. surveillance across all maize-producing regions, 2. breeding for early maturing maize, and 3. spectral grain sorting. Experiments should be conducted to identify the intercrop species and the mechanism for aflatoxin control, and to elucidate the maize ionome - mycotoxin relationship.

BIOGRAPHICAL SKETCH

Samuel was born and brought up in eastern Kenya, where his parents were peasant farmers. While at high school, he had a great interest in Chemistry and Biology, and had a desire to pursue a career leading to the application of the knowledge. During his childhood, Samuel did not witness his parents buy certified seed or apply pesticides in their farming practices. Samuel has experienced the effect of food shortage and vividly recalls incidences of drought and famines that forced their family members to live on famine relief maize, locally termed as "*kathĩĩka*", and moldy maize. Samuel did not know that moldy maize contained the mycotoxins which have been the major focus for his PhD research.

Samuel's career in agriculture begun when he joined Moi University (Kenya) for a B.Sc. degree in Horticulture in 2000. Despite his desire to be a Chemistry major, compelling advice from Dr. M. E. Omunyin, the then Head of Horticulture department, made him keep within. Samuel was undecided of his career until about his graduation with the B.Sc. in 2004, when he was compelled to be a crop protectionist by the larger grain borer, *Prostephanus truncatus*, which ground his nine 90-kg bags of maize grain that he had stored at his cousin's store into unpalatable flour. Samuel earned a Master of Philosophy in Plant Protection from Moi University in 2008, under mentorship of Dr. L. S. Gohole and Prof. E. O. Auma.

Samuel's desire to advance his career in crop protection got a boost after his acceptance at Cornell University's department of Plant Pathology & Plant-Microbe Biology in 2008. His research interests matched and were further shaped by Dr. Rebecca Nelson, who put enormous effort in developing research proposals to support Samuel's research. Rebecca collaborated with Drs. Michael Milgroom, Charles Nicholson and Vivian Hoffmann in a research proposal on understanding aflatoxin accumulation in Kenyan maize, which was funded by the Cornell Center for Sustainable Future (CCSF, now ACSF) in 2008. During her 6-month (Jan-June 2009) sabbatical at Biosciences eastern and central Africa (BecA) – International livestock Research Institute (ILRI), Nairobi, Dr. Nelson discussed the aflatoxin project proposal with the host scientists and charted Samuel's role in the project and his placement at the institution. As a member of R.Nelson lab, Samuel conducted his Ph.D research at Cornell University and at BecA–ILRI, Nairobi. At BecA, Samuel was co-supervised by Dr. Jagger Harvey, and had an opportunity to participate in grant proposal that was funded by the Australian Agency for International Development (AusAID). Besides attendance of several workshops relating to mycotoxin management in Eastern Africa, Samuel participated in the establishment of a mycotoxin platform at BecA-ILRI, Nairobi.

Samuel's interests are in management of plant diseases and pests using conventional and molecular breeding. He plans to spend the rest of his future career in doing research that could boost crop productivity, and food safety and security.

I dedicate my thesis to my cousin, Paul Muchee Kairiba

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I would like to acknowledge the support that my thesis committee members have given to me since I joined Cornell University. Dr. Rebecca Nelson worked hard to ensure that I got admitted to Cornell University, and I joined her lab. Dr. Nelson has been a great mentor and supportive in many ways during my research. I cannot forget the many times she introduced me, and made arrangements for me to meet Kenyan researchers who were to be of importance in my work. Dr. Nelson taught me the importance of database in handling large datasets. Besides the research advisory role, Dr. Nelson always alerted me about the need to take care of my health. I would also like to acknowledge Dr. Michael Milgroom for playing a key to role to ensure that I got admitted to Cornell University when he was the director of graduate studies in the PPPMB. Besides his support for my research in Kenya, Dr. Milgroom has been a source of my inspiration to pursue the understanding of the biology of plant pathogens. I would like to acknowledge Dr. Margaret Smith. I am honored to have Dr. Smith in my thesis committee as a mentor in Plant Breeding and Genetics (PB&G). My great desire to practice the plant breeding methods in managing plant diseases rhyme with the effort that Dr. Smith has put in the research extension and as a professor at Cornell University. I am very grateful to all my committee members for the advice, logistical support and handling of the paperwork that facilitated my 3-year leave of absence from Cornell University while I was doing my research at BecA-ILRI, Nairobi.

I would like to appreciate the accommodation at BecA. I thank Drs. Segenet Kelemu (former BecA director) and Jagger Harvey, BecA-ILRI, Nairobi, for having accepted me to work in the BecA laboratories. The thousands of maize samples that I collected and stored in five freezers during my placement shocked most researchers, but the hub accommodated me. The research assistance provided by Gordon Otieno, Vincent Were, Damaris Njeri, and Samuel Angwenyi is appreciated.

I would like to acknowledge RNelson lab. members who contributed to my work. Drs. Santiago Mideros and Chialin Chung introduced me to the laboratory standard operating procedures. Santiago maintained frequent check of my research progress even when I was in Kenya. Dr. Judy Kolkman ensured that I got my laboratory supplies in time. Laura Morales has been of great help in not only discussing my data, but also participating in exploratory analysis. Nelson Chepkwony provided technical support for my experiments in the lab at Cornell University.

My work would not have been successful without consultations and collaboration with different researchers. Dr. Vivian Hoffmann (University of Maryland) participated in planning and the execution of

Kenyan surveys. Vivian was involved in in-depth discussions about the study designs, participated in the development questionnaire and in sample collection. Dr. James Gethi (KARI, Kenya, now CIMMYT, Harare, Zimbabwe) has been a mentor and was always willing to share ideas about breeding methods and germplasm for reduction of aflatoxin accumulation in Kenyan maize.

Lastly, my PhD program could not have been successful without the funding that I received from different sources. My admission into Cornell University was made possible by a teaching and research assistantship that I was offered by the department of Plant Pathology and Plant-Microbe Biology from 2008-2009. The Kenyan surveys were funded by the Cornell Center for Sustainable Future (CCSF, now ACSF) from 2009-2010. Dr. Christopher Barrett, a faculty fellow in the ACSF, played a big role in facilitating additional 3-year financial support for the Kenyan surveys through Stimulating Agricultural and Rural Transformation (StART) initiative of the Cornell International Institute of Food, and Agriculture Development (CIIFAD). Additional funding was obtained from the Global Hunger Alleviation award that I received from The Presbyterian Church of Ithaca. I received a 2-year graduate fellowship from the Australian Agency for International Development (AusAID) through BecA's Capacity and Action for Aflatoxin Reduction in Eastern Africa (CAAREA). The CAAREA funding was supplemented with The Norman Borlaug Leadership Enhancement in Agriculture Program (LEAP) fellowship. My stipend and laboratory consumables were financed by The McKnight Foundation funds to RNelson's lab at different times between 2010 and 2014.

PREFACE

This dissertation presents work that is aimed at understanding the drivers for accumulation of mycotoxins in maize. Although some work was done in the USA, the project is more relevant to Kenya or the East African food system where mycotoxin accumulation threatens people's lives. The dissertation contains an introductory chapter, and four research paper chapters. The introductory chapter provides the literature review of the two mycotoxins studied in later chapters, aflatoxin and fumonisin, and also lists the global objectives of the dissertation. I took a leave of absence from Cornell University between November 2010 and July 2013, and remained at BecA in order to be near the research sites. I did the work presented in chapters 2-5 when I was on placement as a graduate research fellow at BecA-ILRI, Nairobi. The research paper chapters are presented in the format of the journal *Phytopathology*, one of leading journals in Plant Pathology.

Chapter 2 presents findings of survey work that was done in Kenyan maize grain basket, which consists of Rift Valley, Western and Nyanza Provinces. The paper presents the most comprehensive studies to-date on the prevalence of and the factors associated with accumulation of aflatoxin and fumonisin in the region. The work was carried out between 2009 and 2010.

Chapter 3 has been published in its current form in *Phytopathology*. It presents work involving analysis of the prevalence of aflatoxin and fumonisin, and a statistical analysis of drivers for aflatoxin accumulation in maize. I did this work between 2010 and 2012, and the project was hosted by BecA-ILRI, Nairobi.

Chapter 4 is based on work in which R.Nelson lab collaborated with the International Maize and Wheat Improvement Center (CIMMYT) in a project named Improved Maize for African Soils (IMAS). The paper presents findings of aflatoxin accumulation in a diverse maize germplasm that was being used by CIMMYT to study maize tolerance to low soil N in eastern Kenya. We took the advantage of the germplasm and the design to study the role of low nitrogen stress on aflatoxin accumulation in maize in the aflatoxin hotspot. Maize samples were provided by the lead scientist, Dr. Biswanath Das, to BecA-ILRI, Nairobi, for analysis of aflatoxin and other traits of interest. This work was carried out between 2012 and 2013.

Chapter 5 is a paper from research that was conducted at R.Nelson Lab., Cornell University. This study was carried out between 2010 and 2012. I did most of the assays between January and November 2010, but the rest of the samples were analyzed by Nelson Chepkwony, a biology major Cornell University undergraduate student who was working in the lab. Nelson Chepkwony is included in the manuscript as one of the co-authors.

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CHAPTER 1

LITERATURE REVIEW: INSIGHTS INTO THE GENETIC AND ENVIRONMENTAL BASES OF MYCOTOXIN CONTAMINATION IN KENYAN MAIZE

Introduction

Mycotoxins are toxic low-molecular-weight compounds produced as secondary metabolites by filamentous fungi (148). These metabolites are dissimilar in chemical structures or modes of action, but are grouped together because the members can cause disease and death in human beings and other vertebrates (19). Mycotoxicosis is the disease caused by the toxic effects of mycotoxin exposure through diet, inhalation, and skin (19). Severity of mycotoxicosis depends on the toxicity, the extent of exposure, age and nutritional status of the individual and possible synergistic effects of other chemicals or conditions to which the individual is exposed (201). Mycotoxin contamination of food and feed has been known since the 17th century, and the most recognized human mycotoxicosis are through consumption of contaminated rye, rice, maize, cassava and peanuts (201,19,215). Mycotoxicoses are categorized as acute or chronic. Acute toxicity is caused by a toxin exposure above the maximum tolerable limit, has a rapid onset and could be fatal, while chronic toxicity is due to low-dose exposure over a long time period, which can result in cancers and other irreversible effects (19).

Mycotoxin contamination occurs on crops or foodstuffs that are colonized by mycotoxigenic fungi. Mycotoxigenic fungal species exist in certain crop plants as endophytes, saprophytes and/or pathogens (195,197). Interestingly, some fungal species can occupy different niches within the same crop species. For example, *Aspergillus flavus* can be a saprophyte and a weak pathogen that cause ear and kernel rot in maize (198). Similarly, certain *Fusarium* species including *F. verticillioides, F. proliferatum* and *F. graminearum* can be endophytes, cause ear and/or stalk rot, and can be mycotoxigenic in maize (228). While it is not well known why fungal species produce mycotoxins, certain plant pathogenic fungi that produce mycotoxins are more virulent than the atoxigenic strains. For example, *Fusarium* species that produce trichothecenes and zearalenones have increased disease on wheat (254,216). Environmental conditions for production of individual mycotoxins differ greatly in fungal species, and

mycotoxins have been reported to contaminate food and feed globally (279). It has been estimated that mycotoxins contaminate 25% of foodstuffs, and co-contamination is not uncommon (48). Conditions for mycotoxin production are more prevalent in the sub-tropics and tropics. Examples of general temperature ranges for production of some common toxins are: low to moderate temperatures (8-25°C) for deoxynivalenol (DON), fumonisin, and ergot, and high temperatures (28–37°C) for aflatoxins and ochratoxins (5,201,273).

Because of their damaging effects, mycotoxins are regulated by the United Nations World Food Programme (WFP) and by individual countries across the world (25,215). Strict mycotoxin regulation exist only in developed countries such as in the United States, Canada and some European nations (242,215). Mycotoxin-related economic losses include qualitative and quantitative crop yield decline; mycotoxin prevention costs (e.g., surveillance and research); and treatment of human and livestock mycotoxicosis (25,279). The costs associated with mycotoxin regulation, control and prevention are high. For example, in the US, the annual agricultural economic losses due to aflatoxin contamination of food and feed are estimated at $270 million (48). The high costs for effective management of mycotoxins are not within the reach of most developing countries (266). Because of inability to meet the costs for effective mycotoxin surveillance and regulation, most African countries have unknown levels of mycotoxin contamination in foodstuffs, and their populations are faced with high levels of exposure (266).

Aflatoxins and fumonisins are the major food safety threats in the maize- and peanut-dominated sub-Saharan African countries' diets (265,98). Aflatoxin contamination is endemic for maize in Kenya, Malawi, Uganda, and Tanzania (11,134,113,127,61), and for peanuts in Kenya, Zambia, Malawi and Zimbabwe (237,177). Fumonisin contamination in maize has been associated with growth retardation and esophageal cancer in South Africa, Kenya, and Tanzania (253,126,233). Kenya, Malawi and Zimbabwe are among the leading African maize-consuming countries, and their populations are faced with high risk of exposure to aflatoxin and fumonisin (265,266).

Because of the outbreak of acute aflatoxicosis that claimed 125 lives in eastern Kenya in 2004, there has been significant worldwide attention to the aflatoxin problem in the region (184). Although Kenya has adopted the regulatory limit of 10 ppb (10 µg/kg) in human food, implementation mechanisms are lacking (61). Aflatoxin contamination of maize and its

consequences are underreported because of lack of surveillance. Because sampling is difficult and assays are expensive and require sophisticated facilities, the extent of contamination and the factors that influence the frequent massive maize contamination have not been well understood. Existence of highly toxigenic strains of the aflatoxin-producing fungi has been reported as one of the possible causes of the observed high level of contamination in eastern Kenya (211). Recent projects have focused on maize resistance breeding and biocontrol of the aflatoxin-producing fungi.

The regulatory limit of 1 ppm (1,000 µg/kg) for fumonisin in human food in Kenya was adopted from that of the WFP. As with aflatoxin, there is inadequate surveillance and control mechanisms to effect the regulation (152). Several recent incidences of Fusarium ear rot and fumonisin contamination have been noted in Kenyan maize, especially in Kenya's grain basket region of Western and Rift Valley provinces (121,4). The fumonisin-producing fungus, *F. verticillioides*, has been reported as one of the most prevalent maize-colonizing fungal species in the major maize-producing regions of Kenya (176,121,3). An association between incidences of esophageal cancer and the toxin was recently reported in Kenya's North Rift (253). High levels of contamination of maize with fumonisin has been associated with growth retardation and esophageal cancer in Tanzania and South Africa (164,126). The many reported incidences and high levels of fumonisin contamination could imply a hidden major health problem in the region.

Aflatoxin Contamination of Maize and Health Implications

Aflatoxins are difuranocoumarin derivatives produced by the polyketide pathway of many strains of *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. bombycis*, *A. ochraceus* and *A. pseudotamari* (19). Aflatoxins were first isolated and characterized after an incidence of massive poisoning and death of hundreds of thousands of turkeys that had consumed moldy peanut meal in Europe. The toxin name *Aspergillus flavus toxin* was derived based on the causal organism for the Turkey X disease (270). There are 14 different types of aflatoxin that exist in nature (31). The four most important are B1, B2, G1, and G2. *A. parasiticus* produces the four aflatoxins, but *A. flavus* mainly produces the B types (198). Nomenclature of the toxins in based on their color of fluorescence under ultra-violet (UV) light (i.e. B types, blue; and G types, green) and relative mobility under thin-layer chromatography (84,78). Aflatoxin B1 and B2 are metabolized and are released as M1 and M2 in mammalian milk and poultry eggs, respectively (122,239).

The absorbance maximum for the four aflatoxin types are detected under UV at 265 nm and 360–365 nm (104). Pure aflatoxins occur as pale-yellow crystals of high melting point (237-289 °C) (79). Heat stability of the four common aflatoxins follow the order B2>B1>G1>G2, and the molecular weights are in the order G2>G1>B2>B1 (79). The thermostability of aflatoxins hinders degradation during most food processing and cooking methods (104,75). They are slightly soluble in water, insoluble in non-polar solvents, and soluble in moderately polar organic solvents (e.g., chloroform and methanol) and especially in dimethyl sulfoxide (104). Aflatoxins are unstable to UV light in the presence of oxygen, to pH extremes (< 3, > 10) and to oxidizing agents such as ozone (153,104,79). The aflatoxin lactone ring is susceptible to alkaline hydrolysis, and is degraded by reaction with ammonia or sodium hypochlorite (128).

Aflatoxin contamination of foodstuff occurs due to successful colonization of the crop species by the aflatoxigenic fungi. *A. parasiticus* colonizes and contaminates below-ground parts of some crop species such as peanuts, while *A. flavus* is a dominant fungal species and a contaminant of some above-ground parts of cereal crops (e.g. maize) and cotton seed (84). All aflatoxins are poisonous when ingested in contaminated food or feed, and the resultant disease is termed as aflatoxicosis (24). Aflatoxicosis effects vary greatly depending on the aflatoxin type and amount ingested (acute or chronic exposure). Aflatoxin B1 is the most toxic and carcinogenic natural substance known globally, and has been grouped as a class 1 carcinogen by the International Agency for Cancer Research (IARC) (104,234).

Acute exposure to aflatoxin B1 has been reported to cause death in humans and livestock (270). For example, in Kenya, 125 people died in 2004 after consumption of highly contaminated maize (184). Aflatoxin B1 is genotoxic, and causes significant heritable deoxyribonucleic acid (DNA) mutations that promote cell proliferation and liver cancer (also termed hepatocellular carcinoma or hepatocarcinoma) (207). While the globally recognized cause of liver cancer is hepatitis B, exposure of hepatitis B patients to aflatoxin B1 increases chances of developing the cancer by 50-fold (244,167). Exposure to aflatoxin B1 was associated with reduced immunity and increased morbidity (225). Other health problems associated with exposure to aflatoxin B1 are increased HIV incidence, poor nutrient absorption, and growth retardation in fetus and children (265,266).

Maize is the third most important crop in the world, and a staple to the majority in Africa (95). Unfortunately, it is also an excellent substrate for *A. flavus*, the major aflatoxin B1 producer

(98). Maize-dependent populations of sub-Saharan Africa are thus at high risk of exposure to aflatoxin B1 and its associated health problems (265). Kenya has the worst reported aflatoxicosis fatalities, and is among the countries with the highest aflatoxin contamination in maize globally (265,252). There is an increasing evidence that Kenyan dairy products are contaminated with aflatoxin M1 above the limits allowed for human consumption (117,116). The high prevalence of the carcinogenic aflatoxin B1 in maize-based foodstuffs, and their metabolite M1 implies a great health burden to the African countries which are already faced with malnutrition and other diseases (265,266).

Biology of *Aspergillus flavus* and Aflatoxin Production

Aspergillus flavus belongs to the order eurotiales and class ascomycetes. Members of the order eurotiales are capable of growing at high temperatures (169). The fungus reproduces asexually in nature, but a parasexual reproduction mechanism has also been recognized. Because of the difficulty that had been encountered in identifying the sexual reproduction mechanism in nature, the fungus had been classified as a deuteromycete (214). A recent discovery of the teleomorphic stage, based on laboratory experiments, led to renaming the fungus *Petromyces flavus*, and although the stage has been confirmed in nature, the name *A. flavus* is still in general use (101,102).

A. flavus populations are known to be highly distinct genetically within a small geographic location, and a great variability has been observed when isolates from the tropics and sub-tropics were characterized by using DNA markers, vegetative compatibility groups (VCGs), morphological features and toxigenicity (18,58,209,191). Interestingly, the highly diverse populations have been shown to be correlated with adaptation to the wide range of environmental conditions in which the fungus exists, and the diverse genotypes have generally been isolated in similar conditions (45,209,210).

A. flavus is a ubiquitous soil-borne fungus that is predominant in the warm tropical and sub-tropical climates (221). The fungus can grow over a wide range of temperatures (12 to 48 °C), but its optimum for growth is 37°C temperature and 85% relative humidity (131). The conditions for the growth of this fungus suggests that it could survive under a wide range of environmental conditions and colonize diverse hosts. *A. flavus* sclerotia survive under extreme environmental conditions in soil and plant debris, and invade susceptible plants when conditions are favorable

(102). Sclerotia that germinate on a susceptible host (or on substrate) form mycelia that extends and colonizes the plant or substrate (131,197). Depending on temperature and nutrient status of the substrate, mycelia produces conidia and sclerotia within 2 to 8 days (131). The conidia disperse to other host plants mainly through wind, but other factors include rain splashes, insects, birds and other herbivores (229,198).

Aspergillus flavus colonizes cereal crops (e.g. maize, rice, and sorghum), cassava, cotton and peanuts (196). The fungus is a weak maize pathogen that depends on pre-existing plant stress factors to successfully colonize the maize ear and to cause ear rot (217). Its adaptation to grow in dry media makes *A. flavus* colonize maize under heat stress and drought conditions (221,198). *A. flavus* conidia infect maize via the silk channel through any of the following mechanisms: direct germination on the silk, capillary action of conidia-carrying water droplets, and wind-blown inoculum onto open husks. Other mechanisms of maize ear or kernel infection include direct soil contact with propagules in the soil due to plant lodging, and via wounding by insect-pests or birds (100,1).

Maize is more vulnerable to pre-harvest infection and colonization by *A. flavus* under stress conditions such as drought, disease and insect pressure, and poor soil nutrient levels (196). Successful germination of conidia through the silk, rachis and via the pedicel at the milk stage of maize kernel development leads to colonization of the maize ear by *A. flavus (135)*. Colonization of the kernels is mediated by the rachis tissue, and prior to spread of the rot to individual kernels (240). Infection through other mechanisms could occur at any stage in kernel development, depending on prevalence of the inoculum and transmission mechanism (131,100). Post-harvest infection occurs on improperly dried maize kernels (e.g. kernels dried on the ground), and the fungal propagules from the two stages of infection can multiply in damp and improperly ventilated grain storage conditions (97,19). Infection of mature kernels is mainly through wounded pericarp, but the pedicel route has also been observed (281).

Studies aimed at elucidation of how *A. flavus* infects maize have not yet determined the pathogenicity mechanisms (83). The fungus shows saprophytic and pathogenic characteristics which makes the classical method for classifying plant pathogens based on their interaction with the host (as either biotroph, necrotroph, or hemibiotroph) a puzzle in its classification (83). A microscopic analysis showed that the fungus can break down the maize kernel cell wall during its

growth to facilitate penetration (240), implying necrotrophic pathogenicity (159). However, the colonization of kernel tissue from resistant maize lines exhibited increased levels of salicylic acid and unchanged levels of jasmonic acid (144), suggesting a biotrophic pathogenicity (88). Proteomics-based techniques have identified several virulence proteins produced by *A. flavus*, most of which are hydrolytic enzymes (154). Examples of these enzymes are amylases, cellulases, chitinases, cutinases (e.g., phyto-cutinase), lipases, pectinases, proteases such as alkaline protease, and xylanases (92,57,36,53,39,33,139,200). These enzymes might play an important role in the fungal saprophytic lifestyle which involves catabolism of decaying plant materials (154,83).

Colonization of maize is internal to the kernel, and often leads to aflatoxin contamination if conditions are favorable (135). Aflatoxin is produced at high temperature (15-37°C), with an optimum at range of 28 - 30°C (186). Mechanisms that trigger the fungus to produce aflatoxin are unclear, but at least 20 genes are involved in the biosynthetic pathway (230,216). The biological role of aflatoxin is not well understood, but the most compelling hypothesis has been to protect the fungus against oxidative stress (125). Aflatoxin is neither a pathogenicity nor a virulence factor, and the literature on the correlation between maize ear rot and aflatoxin level is contradictory (159,269). Aflatoxin level differs greatly in kernels of the same ear due to variation in extent of kernel colonization, and within a field due to variation in plant stress factors and inoculum distribution (133). Although scientists have synonymously used Aspergillus ear rot to imply aflatoxin contamination, the inconsistency in correlation between the two traits has hindered progress in surveillance and management of the problem, especially for small-scale farmers and consumers who rely on visual assessment to sort and improve grain quality.

While recent research has linked aflatoxin production with protection of the fungus against oxidative stress (125), approximately half of *A. flavus* strains in a population are atoxigenic (130,20). Interestingly, some members of the atoxigenic strains can competitively exclude the toxigenic populations in the field, and have been used as biocontrol agents (211). The aflatoxigenic species differ in qualitative and quantitative toxigenicity potential (187,210). Two *A. flavus* types of strains are recognized based on their aflatoxigenicity, and are mycologically characterized by sclerotia formation in *V8* agar medium as small (S-strains, produce copious amounts of toxin and many small sclerotia) and large (L-strain, produces few large sclerotia and less toxin) (236). The drought-prone Eastern Province of Kenya is an aflatoxin hotspot and home to the recently

described highly toxigenic *A. flavus* S-strains, which possess genetic sequences different from those recognized in other parts of the world (210).

Fumonisin Contamination of Maize and Health Implications

Fumonisins are polar polyketide-derived mycotoxins produced by certain members of the genus *Fusarium*. Their structure consists of chains of about 20 carbons with acidic ester, acetylamino and sometimes other substituents. There are at least 28 different forms of fumonisins; designated as A-, B-, C-, and P-series, as identified based on carcinogenicity assays (145). The B-series is the most naturally abundant fumonisins and includes fumonisin B1, B2, B3, and B4 (147,220). Fumonisins dissolve in other polar compounds like water, methanol and acetonitrile, but not in non-polar solvents. Fumonisins can withstand temperature up to 150^0C, and hence remain unchanged under most cooking processes, except when maize is boiled under alkaline conditions (e.g., through the nixtamalization process of making tortillas in which maize is boiled in a calcium hydroxide solution) (68,67). Fumonisin B1 (FB1) is the most abundant (occurs in 70-80% of samples contaminated with fumonisin) and toxic, and is mainly produced by *F. verticillioides* (224). Other Fusarium species that produce FB1 are *F. proliferatum* and *F. nygamai* (145). FB1 was first characterized and its effects on humans first reported during an outbreak of mycotoxicosis in the maize-dependent community of Transkei, South Africa and in China, but has then been found to contaminate maize globally (220,148).

Fumonisin production has mainly been associated with plant stress factors such as drought, insect damage, and plant disease pressure (164). Exposure to fumonisin is mainly by ingestion of contaminated maize. The toxin is poorly absorbed after ingestion at 6%, and does not significantly permeate human skin and hence has no significant systemic health risk after dermal exposure (215,32). Ingestion of FB1 has been correlated with esophageal cancer and birth defects in humans (165,266). In animals such as horse and equine, ingestion of fumonisin is known to cause neurotoxicity and hepatotoxicity in a fatal syndrome called leukoencephalomalacia (63). Fumonisin also causes neural tube defects in horses by inhibition of a major enzyme (ceramide synthase) involved in biosynthesis of sphingolipids (compounds that are found on the brain and that provide protection to the cell surface against harmful environmental factors by forming a mechanically stable and chemically resistant outer leaflet of the plasma membrane lipid bilayer, and also involved in cell recognition and signaling) (245,249).

Owing to a lack of metabolic conversions and low bioavailability of fumonisin upon ingestion, exposure to the toxin can only be monitored by either analysis of the contaminated foodstuff or by use of biomarkers (249). An inference of exposure based on analysis of the consumed contaminated foodstuff may be inaccurate because of the potential subsequent biochemical activities upon ingestion and the skewed distribution of mycotoxins in a sample (249). The downstream events of disruption of sphingolipid metabolism include an increase in the ratio of two sphingoid precursors, sphinganine and sphingosine, and the altered ratio has been observed in tissues, serum, and urine from animal models, and was proposed as a biomarker for fumonisin exposure. However, in most sample animals, the correlation between the biomarker and fumonisin exposure has been inconsistent because the sphingoid precursor levels are influenced by other factors besides fumonisin (249,63).

Ineffective mycotoxin regulation in maize-dependent countries could lead to a serious health problems due to exposure of the maize-consuming population to fumonisin (243). High consumption of fumonisin contaminated maize has been correlated with incidences of esophageal cancer in China, Italy, Iran, Kenya, Zimbabwe, United States and Brazil (243,266). In animal studies, combined ingestion of aflatoxin and fumonisin were shown to increase chances of liver cancer. In *in-vitro* studies, Carlson et al. (46) showed that fumonisin B1 (FB1) promotes aflatoxin B1-initiated liver tumors in trout. Pre-treatment of rats with aflatoxin followed by a provision of a diet which was contaminated with both aflatoxin and fumonisin increased hepatocytoxicity, presumably by rendering the liver more susceptible to the toxic effects of FB1 (46,86). This implies that co-occurrence of aflatoxin and fumonisin in maize could be a major health problem, if the above models are applicable to human beings.

Biology of *Fusarium verticillioides* and Fumonisin Production

F. verticillioides (teleomorph *Gibberella moniliformis*) is a filamentous fungus that belongs to the order Hypocreales and an Ascomycete that exhibits both anamorph and teleomorphic reproductive stages in nature (180). Strains of different mating types can be found on the same plant, suggesting that sexual recombination could occur within an individual plant (120).The fungus is of great economic importance because it is a maize pathogen that produces fumonisins (180). The fungus shows great diversity as assessed using genetic molecular and biochemical markers, and vegetative compatibility groups (VCGs) (120,65,189,247), and are found distributed under diverse climatic

conditions, including temperate, alpine, tropical and subtropical and deserts (180,181,69). The wide distribution of the diverse strains could be accounted for by the ability of these fungi to grow on a wide range of substrates, efficient dispersal mechanisms and sexual recombination, which enhances selection for adaptation of the strains to new environments (181). The genetic diversity of the fungus is not correlated with fumonisin production (219,189).

F. verticillioides is ubiquitous in soil and its spores are found in air (181,147). It can colonize and grow under different hosts or media under a wide range of temperatures (8–37°C), with an optimum of 22 - 30°C (109,168,205). It produces fumonisin at an optimum temperature range of 20–28°C (109,146,180). It is capable of establishing both endophytic and parasitic relationship with maize, and it grows within maize roots systemically through the stalk to the ears and kernels (171,174,205). The endophytic association is asymptomatic, while the parasitic relationship causes important diseases such as seedling blight and stalk and ear rot (275,13,171,190). The fungus also lives a saprophytic life in rotten maize kernels, plant debris and other organic substrates (171). *F. verticillioides* often produces fumonisin during maize colonization, but the relationship between fumonisin production and fungal pathogenicity or virulence is inconsistently presented in the literature (65,247,59). In an evaluation of virulence of a progeny derived from a cross between fumonisin- and non-fumonisin-producing strains on maize seedlings, Desjardins et al. (65) concluded that fumonisin plays a role in virulence, but fumonisin production is not necessary or sufficient for the seedling blight disease. Similarly, Desjardins et al. (64) analyzed severity of ear rot in maize that had been inoculated with either atoxigenic or toxigenic strains of *F. verticillioides*, reporting that the non-fumonisin producing strains caused ear rot. They concluded that fumonisin was not required for *F. verticillioides* to either cause ear infection or ear rot (64).

The fungus can be transmitted either vertically or horizontally (173,171). The fungal propagules include micro- or macro-conidia that remain in the soil and can infect maize seed approximately 48 hours after germination, growing systemically with or without symptoms within the seedling (175). The asymptomatic growth does not cause harm to the host, and is characterized by intercellular fungal germination, while the symptomatic association is parasitic and can kill the seedlings in 2 to 3 weeks. Plants that recover from the infection grow asymptomatically, but can develop stalk rot disease in later stages of their development

(173,171,190). The long-term endophytic fungal growth is characterized by relatively low fumonisin production (72). The endophytic biotrophic association between the fungus and maize leads to approximately 10% vertical transmission through infected seed to kernels (119,172,173). Horizontal transmission of *F. verticillioides*, in which spores are spread by wind, in debris, insect vectors, and/or by water splashes, is also common and accounts for the highest percent incidences of maize ear rot (172,173).

The major entry of Fusarium ear rot causing fungi is the silk channel, but several other routes of entry are also recognized (173,171,190). The silk channel infection court involves an airborne conidia that infects and grows through the silks (172,173). The other routes of penetration of maize include entry via damaged ears or wounded kernels (172,173). Environmental conditions, water availability, and the genetics of the plant and the pathogen may all be important factors in ear rot development (66,141,71,143). Infection can be effectively managed with fungicide application, but fungicides are not commonly used in small-scale maize farming systems (171,188). Furthermore, control, prevention, and detection of the endophytic infections are difficult because of poor correlation between kernel appearance and extent of fungal colonization (163,190,72).

F. verticillioides produces several mycotoxins that are harmful to humans and domesticated animals. Toxigenic and atoxigenic *F. verticillioides* strains exist, and both are capable of cause ear and kernel rot in maize (64). Fumonisins can be detected in symptomatic and asymptomatic maize kernels, but there is a generally good correlation between the starburst symptoms of kernel rot and fumonisin levels because of the superficial nature of colonization within the pericarp (72). Post-harvest colonization of maize kernels by *F. verticillioides* is minimal or does not occur if maize grain is dried to below 24% moisture (or below water activity, $a_w \approx 1.0$) (259,226). A significant correlation between fumonisin accumulation and kernel developmental stage was previously reported (259). The highest fumonisin contamination was observed at the dent stage compared to earlier kernel development stages, suggesting that fumonisin biosynthesis increases with increase in grain starch content (203). FB1 biosynthesis increases dramatically with kernel maturity, peaking at the same time as the endosperm starch content (259,28).

Chemical and Physical Control of Aflatoxins and Fumonisins in Maize

Certain chemical methods are known to reduce the mycotoxins through some form of reactions, dissolution, conversion, binding and/or neutralization, and include nixtamalization, binding to clays, and decontamination (68,67). Nixtamalization is a traditional Latin American process used in making of tortillas that involves cooking maize in excess water and lime (calcium hydroxide) at temperatures near boiling, followed by steeping (68,67). The process was reported to remove mycotoxins from maize whole-grain at rates of 76% (FB1), 94% (AFB1) and 90% (other aflatoxins) (47,68,73). Although nixtamalization was reported to significantly degrade the two mycotoxins, the some studies have shown that the toxins are either regenerated by digestive processes or the resultant molecules are more toxic than the mycotoxins (208,155). Studies have reported improved degradation of the two toxins and reduced formation of toxic residues when nixtamalization was combined with extrusion (using lime and hydrogen peroxide) and addition of sodium bicarbonate (137,73).

Treatment of maize with ammonia and ozone are two other chemical methods for mycotoxin decontamination. Treatment of maize grain with ammonia is called ammoniation, and was reported to reduce aflatoxin from thousands of parts per billion to below 20 ppb (14). Application of ammonia on cottonseed feedstock was also observed to have reduced AFB1 exposure and subsequent AFM1 release in dairy cattle (15). Ammoniation is a widely used method for lowering the level of aflatoxin contamination in cottonseed and maize in the United States and in peanuts in countries such as France, South Africa and Mexico (78). Ozone treatment of cereals that were infected with mycotoxigenic fungi was reported to hinder the growth of the fungi and to inhibit mycotoxin production (93,6). Ozone was found to detoxify AFB1 and total aflatoxin in peanuts at rates of 65.8% and 65.9%, respectively (49). Ozone treatment of maize flour reduced AFB1 by 80%, and other aflatoxins by 85% (140). Unfortunately, there has been no evidence of FB1 decontaminated by ammoniation process, and its degradation by ozone forms 3-keto-fumonisin B1, a compound that is equally toxic (183,153).

Certain mycotoxins are known to bind to certain types of clay that are used to avoid caking of animal feeds. A good example is the phyllosilicate clay also known as NovaSil that has been reported to be aflatoxin-selective and binds tightly to the toxin. NovaSil has been under investigation as an enterosorption strategy for mycotoxin control with some significant progress

(202,32). It has a high affinity and binding capacity in the gastrointestinal tract for aflatoxins and significantly reduces the bioavailability of the toxins without interfering with the utilization of vitamins and other micronutrients (202,85). Inclusion of the clay in animal feed has been shown to prevent aflatoxicosis in a variety of animals (150,85). This strategy is undergoing biosafety evaluation as a potential remedy for acute aflatoxicosis in humans, and has shown a promising reduction in aflatoxin exposure as measured by biomarkers (202,166). Recent *in vitro* studies (based on hydra assays) showed that a refined NovaSil can effectively absorb aflatoxin and fumonisin at pH levels that are relevant to the human gastrointestinal tract, resulting in decreased bioavailability (32). Furthermore, the efficacy of the clay is not influenced by cooking methods (74).

Certain physical methods have been reported to have reduced aflatoxin and fumonisin levels in maize (21). Examples of the physical methods are de-hulling, sieving, flotation and density segregation, spectral and visual grain sorting. Mechanisms through which individual physical methods reduce mycotoxins differ, and the efficacy is dependent on the correlation between the target kernel damage and the mycotoxin(s). It should also be noted that the application of these methods may differ greatly among individuals, communities, available resources and even the economic status of the maize consumers. The already recognized mycotoxin heterogeneity in maize samples could highly influence the performance and interpretation of physical methods of reducing maize contamination (261). The most researched physical methods of mycotoxin control are dehulling and the sorting.

Dehulling is the removal of the maize seed coat by abrasion prior to cooking or other cooking processes (21). Siwela et al. (238) analyzed aflatoxin on twenty paired samples that were either dehulled or not de-hulled, and reported a 92% decrease in aflatoxin levels in dehulled compared with undehulled maize meal (238). Similarly, Mutungi et al. (178) reported that dehulling decreased aflatoxin levels by 47% in Kenyan maize containing 10.7–270 ng/g aflatoxin. In a study in which dehulling efficacy of three types of mills were compared, dehulling reduced fumonisin level by up to 65% (76).

Grain sorting is the deliberate removal of kernels that are visually assessed to be of poor quality or have some discoloration that is characteristic of damage by mycotoxigenic fungi. Sorting of maize based on apparent damage or moldiness is common with most maize consumers. For

example, Kimanya et al. (127) found that over 90% of maize consumers in Tanzania sorted maize prior to storage and/or consumption. While there is a dearth of information on the effect of sorting on aflatoxin and fumonisin, it is known that maize ear and/or kernel rot shows a better correlation with fumonisin or moldiness than with aflatoxin (44,231,56). Sorting maize based on blue–green-yellow fluorescence (BGYF) under UV light (also known as black light test) has been a practice to reduce aflatoxin contamination in maize (21). Due to the fact that observed fluorescence is caused by the presence of kojic acid, a metabolite produced by several fungi, the method has been found to have high false detection rates and is currently obsolete (21). Current efforts are aimed at developing spectral sorting devices, and tremendous progress has been reported with development of machines that can detect and remove both aflatoxin and fumonisin from maize (199).

Farm Management Practices for Aflatoxin and Fumonisin Control in Maize

Factors that reduce crop vigor or induce plant stress during growth and development favor infection of maize by both *A. flavus* and *F. verticillioides,* and could lead to accumulation of aflatoxin and fumonisin (42). Studies have shown that pre-harvest management of the plant stressors, and proper peri- and post-harvest handling are key to control of mycotoxins (41). Pre-harvest management practices that could reduce mycotoxin contamination include timely planting and harvesting, and management of biotic (e.g. mycotoxigenic fungal genotypes, diseases, pests, birds etc.) and abiotic (soil fertility, drought, etc.) factors. A recent review by Hell and Mutegi (98) highlighted the approaches and challenges to aflatoxin management in sub-Saharan Africa. Among the key aspects that complicate pre-harvest management of aflatoxin is the nature of the maize value chain, which is characterized by high percentage of crop production by resource-poor small-scale farmers, rudimentary maize processing, high level of self-provisioning, lack of surveillance and ineffective regulations (98).

Cultivation of well-adapted, drought tolerant, insect and disease resistant maize cultivars (or varieties) is essential in managing biotic stress (164,41,267,271,98). Incidence of maize infestation by thrips (*Frankliniella williamsi,* Hood) was recently correlated with fumonisin B1 levels, and it was proposed that their feeding and movement could play a role in spreading *F. verticillioides* spores (193). Similarly, studies have shown significantly lower aflatoxin contamination in Bt-maize compared to non-Bt-maize (268,94). Use of maize with multiple disease resistance would help in reducing stress induced by other maize pathogens. More effort

is needed in identification and introgression of appropriate resistance into the maize varieties across different environments in the developing world (182,7). Irrigation and use of fungicides, herbicides and insecticides could be useful in management of drought, diseases, weeds and pests, but the approaches are not accessible to small-scale farmers in most developing countries (98). Use of insect resistant Bt-maize is hindered by the fact that transgenic maize is regulated in most countries in Africa (136,98).

Malnourished plants are more susceptible to infection by mycotoxigenic fungi (137,278). For example, cultivation of maize under nitrogen (N)-depleted soils has been associated with mycotoxin contamination in maize (110,248,246,42). In a recent three-year study conducted at North West Italy, Blandino et al. (27) observed an increase in aflatoxin and fumonisin contamination in maize when the crop was grown under inadequate or N-depleted soils, but application of too much of the fertilizer was also associated with an increase in fumonisin contamination. Mechanisms through which increased soil nitrogen would reduce aflatoxin or fumonisin are not well-known, but it is generally understood that plant stress leads to vulnerability to fungal colonization and mycotoxin accumulation (41,142). Although application of synthetic fertilizers would reduce plant nitrogen deficiency, resource-poor farmers often cannot afford to buy them. Another affordable option for improving soil nutrition is to intercrop maize with leguminous crops, which would fix nitrogen (96,80).

Aflatoxin and fumonisin contamination could be reduced through certain peri- and post-harvest practices. Farm practices that have been reported to reduce contamination of maize by mycotoxigenic fungi include timely harvesting, proper maize ear handling at harvest and shelling, and proper drying to avoid further infection of kernel by mycotoxigenic fungi (41,114,252,10). Shelling methods that lead to kernel breakage predispose maize to fungal infection and colonization (114,112). Mycotoxin production is reduced if maize is stored at below 15% grain moisture for aflatoxin and below 18% grain moisture for fumonisin (114,268). Additional post-harvest practices that could reduce mycotoxin contamination are storage under adequate ventilation, application of weevil protectant pesticides, and grain sorting to remove the moldiness and other forms of grain damage (41,54). Improper storage can allow *A. flavus* and *F. verticillioides* to proliferate during grain storage (12,118,192,54). Metal silos have been improvised and farmers are being encouraged to use them for long-term storage of grain in most maize-producing sub-Saharan countries (29).

Biocontrol of toxigenic strains of *A. flavus* based on competitive exclusion with atoxigenic strains has been reported to be effective in reducing aflatoxin contamination in several crops, including maize (129,24,272). There have been recent efforts to identify atoxigenic strains for use in control of the deadly *Aspergillus* species in Kenya (209,211). Given that *Aspergillus* spp. are potential human pathogens that could cause aspergillosis, among other biosafety issues, there has been concern over whether certain countries would permit application of atoxigenic strains in agricultural fields (123). Biological control of *F. verticillioides* during the endophytic growth phase using an endophytic bacterium, *Bacillus subtilis*, and during grain storage using *Trichoderma* spp. have been reported (13).

Maize Breeding for Resistance to Aflatoxin and Fumonisin

Breeding for resistance to pre-harvest infection and aflatoxin and fumonisin accumulation processes in maize has been ongoing, and holds potential. Although complete resistance to mycotoxins could save the resource-poor farmers' cost of inputs for management of plant stress and other mycotoxin-related aspects, the resistance trait is complex and cannot stand alone without good crop management (252,98). Maize resistance to aflatoxin and fumonisin accumulation are quantitative traits that are controlled by multiple small-effect genetic loci and require a lot of effort and cost to dissect (25,267,157). Because of the costs associated with mycotoxin quantification, and since the mycotoxigenic fungi cause ear rots, most breeders and pathologists thought that tackling ear rots would tackle the mycotoxin problem as well. Breeding for resistance to aflatoxin and fumonisin in maize has always involved phenotyping for the ear rots (2,157,158). While fumonisin has been correlated with Fusarium ear rot, Aspergillus ear rot is not always correlated with aflatoxin (223,2). Use of ear rots as proxy for the two mycotoxins has been helpful in tackling the ear rot problem and fumonisin, but not aflatoxin (156,206,40).

Because of the complex nature of mycotoxin resistance, breeding efforts have also involved identification and selection for other correlated traits such as drought tolerance, insect resistance and maturity (90). Most breeding programs have mainly been focused on pre-harvest aflatoxin and fumonisin accumulation than with post-harvest contamination problem (2,157,158). However, a few studies have involved mature maize kernel assays that could possibly mimic post-harvest colonization of maize by *A. flavus* (38,35,162). Mechanisms of resistance to infection by the two mycotoxigenic fungi differ, and there has been more progress

in understanding *F. verticillioides* –maize (or Fusarium ear rot, FER) pathosystem compared to the *A. flavus*-maize (or Aspergillus ear rot, AER) pathosystem.

The multiple aflatoxin resistance loci and their effects were described in a meta-analysis by Mideros et al. (162). The potential mechanisms through which such quantitative resistance loci operate in the complex system are described by Poland et al. (204). The mechanisms of action of the genes vary greatly, and are associated with different aspects that the host plant possess or encounters in nature. Host-related resistance mechanisms include morphological/structural, physiological and chemical composition aspects, while the environmental aspects includes the role that biotic and abiotic factors play in the pathosystem (161,162). The proposed resistance mechanisms for each of the pathosystems are described separately herein.

Mechanisms of Maize Resistance to Aspergillus Ear Rot (AER) and Aflatoxin.

Resistance to aflatoxin accumulation in maize has been reported by multiple researchers, and is strongly influenced by the environment where maize is grown, and the interaction between maize genetic factors and environment (GxE) and farm management aspects. Because of the high proportion of the phenotypic variance that is explained by the environment and GxE, some researchers concluded that resistance does not exist (62). Resistance is characterized by low heritability, which has been reported in different studies to range non-existent to H^2=0.68 (162). A search for resistance has involved analysis of different tissues that were hypothesized to play a role in conferring resistance including silk, husks, rachis, glume, developing and mature kernels (195,240,16,277,160). Multiple quantitative trait loci (QTL) associated with aflatoxin resistance have been identified, but most of them are of small effect and have not been useful in maize breeding. Some maize lines have been registered with a tag of aflatoxin resistance, but the resistance has not been introgressed into commercial maize varieties in most parts of the world because of its low heritability, strong GxE, and the lack of major QTL (157,257,82,83,162). Some maize lines with most promising aflatoxin resistance have been included in further conventional and molecular selection breeding programs in different parts of the world (267,157,40).

Certain morphological features (e.g. ear secondary traits) including husk coverage and tightness, silk channel length, and nod (ear droop) have been associated with infection of maize by *A. flavus* (17,16). In an artificially-inoculated trial, Balconi et al. (16) observed reduced pre-harvest aflatoxin in maize hybrids with longer silk channel and tighter husk coverage. A negative correlation between the two traits implied that hybrids with better husk coverage at pollination stage would exclude *A. flavus* propagules until maturity, reducing chances of infection and subsequent aflatoxin accumulation. Although ear droopiness (ear nod) has been hypothesized as a method through which maize ears would have less water accumulation in the husks and reduced fungal growth and aflatoxin, there is a dearth of empirical evidence of the mechanism (194,3).

Stylar canal (an opening in the developing kernel adjacent to the silk where the two anterior and one posterior carpels fuse) was hypothesized to be a determinant of kernel colonization by fungal pathogens such as *A. flavus* (103). Although maize genotypes with open stylar canal were found to be more susceptible to infection by *F. verticillioides* (72), chances of such an infection court are limited because they depend on colonization of the silk immediately after pollination and before silk abscission (213,195).

Aflatoxin resistance breeding effort has included selection for drought tolerance and insect resistance (42,91). Transgenic maize with resistance to insect damage has been shown to accumulate less aflatoxin compared to conventional maize (263,268,271). While early maturity genotypes have been used to avoid drought, maturity is not well-correlated with aflatoxin resistance in maize (22,99). Proteomic comparisons of maize kernel proteins between resistant and susceptible maize identified stress-related and antifungal proteins that are associated with resistance to *A. flavus* colonization and aflatoxin accumulation (51). Among the stress-related proteins that are highly expressed in maize kernels that are infected by *A. flavus* are those that are known to be induced by drought (52).

Maize kernel structural and textural characteristics have been implicated in resistance to colonization by *A. flavus*. Compactness of the endosperm is determined by arrangement of starch granules and seed storage zein proteins. Maize grain is classified based on texture as flint (has hard starch and compact endosperm) to dent (has high soft starch and less compact endosperm) (81). In a field evaluation of diverse maize inbred lines and their test-crosses, Betran et al. (23)

reported reduced aflatoxin accumulation in flint compared to dent maize genotypes. Flint maize has been registered with an aflatoxin resistance tag in other breeding programs (151).

Maize kernel chemical composition has been associated with susceptibility of maize to aflatoxin accumulation. Quality Protein Maize (QPM), a maize germplasm that contains approximately 70% more lysine and tryptophan than other maize varieties, and γ-zeins (essential for maintenance of protein body density and starch grain interaction), was found to be less susceptible to aflatoxin compared to non-QPM maize (26,23,276,232). It was hypothesized that the thickness of the seed coat in the QPM maize acted as a barrier to colonization by *A. flavus* (232). However, the relationship between aflatoxin resistance and pericarp thickness and wax is inconsistent in literature (34,92,87). Maize grown under nitrogen-rich soils has higher grain protein content and is less susceptible to aflatoxin accumulation (110,248,27), a phenomenon that is presumed to be due to reduced crop stress under improved soil fertility.

Brown et al. (34) showed that resistance to colonization of mature kernels by *A. flavus* exists in a living embryo. This finding suggests that a host living embryo possesses a defense mechanism against fungal pathogens, and/or the host plant stores some mechanism for protecting the seed against pathogens within the kernel. Proteomics of mature maize kernels identified aflatoxin resistance associated proteins (RAPs) and some putative molecular markers for aflatoxin resistance breeding in maize (52,37,138,50). Additional resistance has been identified based on QTL mapping (82,264). There has been a great interest in application of molecular markers in marker assisted selection breeding for aflatoxin resistance (267,157,256). Progress in breeding efforts is, however, not efficient because of the strong GxE, which causes poor association between markers and the phenotype (83,162).

Mechanisms of Maize Resistance to Fusarium Ear Rot (FER) and Fumonisin

Fusarium ear rot is caused by multiple *Fusarium* spp. including *F. verticillioides*, *F. proliferatum* and *F. subglutinans* (70). While FER is can be caused by multiple or either of the *Fusarium spp*., the environmental conditions determine which one of the species ends up predominating the kernels (164,70). FER symptoms have been positively correlated with FB1 which is mainly produced by *F. verticillioides* (223,222). The correlation between FER and fumonisin has been reported to vary greatly among maize genotypes, *Fusarium* strains and environments (64,223,2,222). Breeding for resistance to fumonisin accumulation in maize has been

synonymous to FER resistance, but the variation in correlations implies that under certain conditions, use of FER as a proxy for contamination could be misleading.

Dissection of the resistance to fumonisin accumulation in maize has focused on plant physical barriers (or morphological features) of fungal entry, developmental traits (or stages), and kernel structural and chemical composition (56,55). The genetic bases for resistance have been described in different ways, but there is a consensus that the trait is polygenic (157,30,227). Toxigenic strains of *F. verticillioides* differ greatly in toxin production potential, and the interaction of fungal strains with maize genotypes vary across environments (12). The variation in toxigenicity potential could be possible cause of the GxE in fumonisin resistance evaluation across locations, seasons and years.

The known physical or morphological barriers of maize infection by *F. verticillioides* include husk coverage and tightness, silk channel length, and stylar canal openness (43,72). In a diallel study, Butron et al. (43) reported significant differences in fumonisin levels in maize hybrids that differed in husk tightness. Greater husk coverage and tightness were associated with lower chances of entry of thrips through the tip of the silk channel, and reduced FER (258). A longer silk channel leads to a delay in the movement of conidia on the surface of the silk by capillarity (72). Once the developing *Fusarium* hyphae overcomes silk resistance (or other morphological barriers to silk colonization), it could face the closed stylar canal as the next resistance barrier (72,251). Maize genotypes with closed stylar canals were found to be more resistant to colonization by *F. verticillioides* compared to susceptible ones that had open stylar canals (72).

The stage of maize kernel development has been associated with resistance of maize to infection by *F. verticillioides* (203,274). An artificially inoculated analysis of fumonisin production by *F. verticillioides* at different stages of kernel development (blister, milk, dough, and dent) showed significant differences in FB1 among the development stages, with the least at blister and highest at dent stages (259,203). A chemical analysis showed a significant influence of concentration of different types of carbohydrate and pH, and the dent stage was shown to have a high level of branched starch (amylopectin) and acidic pH (259,203). Bluhm and Woloshuk (28) showed that amylopectin induces FB1 production and this was confirmed by the increased expression of major fumonisin biosynthesis genes at the dent stage. Although the starch levels at

dent stage have been associated with high fumonisin production by *F. verticillioides*, ear rot was more correlated with FB1 than the amylopectin and pH (203,274).

Owing to the recognized relationship between maize maturity and drought tolerance, maturing time has been investigated for its association with fumonisin resistance in breeding programs (223). Early maturing maize exhibits drought tolerance because such varieties can do well under less rainfall compared to late maturing genotypes, and could possibly have less susceptibility to fumonisin (164,41). Early maturity could also be associated with a higher rate of ear dry-down to moisture levels below the water activity of *F. verticillioides* (226,149). Breeders have thus resorted to integrated breeding programs that include traits that are potentially correlated with fumonisin resistance in maize (e.g., insect resistance and drought tolerance) (164,60,158).

Fatty acid levels in varying maize hybrids were correlated with fumonisin production (60). Higher fumonisin levels were reported in maize hybrids with higher linoleic acid content compared to those with low levels of the acid (60). Other chemical compounds that have been found associated with reduced kernel infection of maize kernels by *F. vertillioides* include pericarp propanoids and wax (227), but these traits have not been of any meaningful importance in the breeding programs (164,158).

Mycotoxin Surveillance and Regulation

Because mycotoxins are frequent food contaminants, there has been need to monitor their occurrence, prevalence and incidences in order to prevent associated losses in food and feed. Aflatoxin and fumonisin are plant-stress-related mycotoxins that occur over wide climatic conditions globally, especially in the tropics and sub-tropics (41,54). Given the known health implications, there is inevitable need for surveillance across environments to inform whether either or both of the mycotoxins contaminate food and/or feed, the extent of contamination, and the factors associated with the occurrence or levels of the toxins. Once the occurrence has been established, there is need to investigate the level of exposure of the people. Insights on these factors could help the policy makers to apply the appropriate control strategies to protect maize consumers.

Mycotoxin monitoring is essential but its implementation strategy is not universal. Mycotoxin monitoring practice varies from non-existent in developing countries, to stringent

monitoring in developed countries (25,250). Mycotoxin surveillance involves sampling of maize (or other foodstuffs) over time and space. The surveillance data could possibly inform the policy makers on the level of intake of the affected food stuff, and the limits that could possibly be established to reduce exposure to the damaging toxins (215). Mycotoxin regulation is aimed at reducing exposure by barring movement, trade, and consumption of contaminated foodstuff across geographical locations (77). Different countries have their own food and feed mycotoxin regulations, but the United Nations' World Food Programme (WFP) in collaboration with World Health Organization (WHO) and Food and Agriculture Organization (FAO) has some set the maximum tolerable limits (MTL) for different mycotoxins in different foodstuffs or agricultural produce (77,250). Regulations are set based on indices such as results of *in-vitro* model experiments, and the expected daily intake of the target food item (242,215). Aflatoxin level in cereals is regulated at ranges from 5–30 ppb in different countries, but the WFP limit is 10 ppb (10 mg/kg) (250,132). Fumonisin is regulated at ranges from 2–4 ppm, while the WFP limit is 1 ppm (1,000 mg/kg) or (132). Limits allowed in feed vary, and are higher than those set for human food (77,132).

Surveillance of mycotoxins in maize is costly and challenging due to sampling problems caused by the huge variability in contamination among maize kernels (262,261). Surveillance has been successful in developed countries because of their financial and technological capability. People in the developed world have access to a wider variety of food commodities, and are not greatly affected by the stringent mycotoxin limits on specific crop produce. A majority of the developed countries have managed low levels of fumonisin and aflatoxin in food and feed through regulation that is implemented through different processing levels in the maize value chains (250).

In contrast to the situation in the developed world, most developing countries lack financial, technological capacity and policy resources to effect mycotoxin regulation (265). For example, mycotoxin surveillance in sub-Saharan Africa has been supported through international donor funding or is conducted through research institutions that have international grants. Unfortunately, donor funding chances are on a sporadic and patchy basis. Maize value chains lack sophisticated processing steps, and the majority of consumers eat what they produce on their farms (115). Stringent mycotoxin regulation in maize is likely to impact costs of living of the maize-dependent populations in the developing world. The challenges associated with

surveillance of the toxins in tropical and sub-tropical developing countries have left the maize-consuming populations with high risk of exposure to these carcinogens, but the extent of the exposure is not well known in most African countries (266).

A great within a sample variability in mycotoxin levels has been observed in maize. Pre-harvest aflatoxin levels in different kernels of the same ear from artificially inoculated maize ranged from 0–80,000 ppb (133,241). Similarly, fumonisin levels have been found to vary by up to 30 times within the same maize sample (262). Critical issues arise with regard to collection of representative samples from bulks of maize kernels with varying contamination levels (e.g., sampling from heaps of grain such as those stored in plastic and metallic silos, and polypropylene and gunny bags) (260,261). Although there is no specific sampling method that gives the most accurate representation of grain contamination, international sampling standards have been established (84,261). Given the skewed nature of mycotoxin distribution, and the need for regular mycotoxin surveillance, the sampling strategy and cost of analysis remain major challenges. These challenges are well evident in a country like Kenya where maize is staple to majority of the people, mycotoxin occurrence and mycotoxicosis are common, yet the mycotoxin management capacity and policy is inadequate.

The Kenyan Maize Value Chain

Maize is staple food for approximately 96% of Kenyans, accounting for 65% of total staple food caloric intake and 36% of total food caloric intake (9). Because of the cultural importance attached to maize, a country-wide shortage in the production makes Kenyans declare famine during that year or season. Approximately 80% of Kenyan land is either arid or semi-arid, and experiences frequent droughts and crop failure (115). Maize is cultivated on about 1.4–1.6 million hectares, and the national average production for the last ten years is about 1.8 tons/ha (89). Within the last decade, Kenyan maize consumption average has ranged from 3–4 million tons/year, while the average production ranged from 1.4-2.6 million tons/year (89,218). Although Kenya does not produce sufficient maize to support its consumption, there is potential to increase the grain yield if the known production constraints are reduced (89). The produced grain either circulates and is consumed within the country, or is purchased by the government and is stored in the grain reserve for future famine relief (185).

Kenyan maize cultivation is mainly rainfed (89). Maize is historically known to do well in agroecological zones (AEZs) of the following ranges of annual rainfall: sub-humid to semi-arid (600-1,100 mm), semi-humid (800-1,400 mm) and sub-humid (1,000-1,600 mm) (108,107,280). The major maize-producing zones are located in the west of Rift Valley, in seven districts (Bungoma, Uasin Gishu, Tran-Nzoia, Kericho, Nandi, Lugari, and Nakuru) which constitute the Kenyan maize grain basket (218). Maize is also cultivated in wetter and even in drier AEZs (106,107). The semi-subsistence farmers in the semi-arid AEZ (e.g., in lower Eastern and parts of Nyanza provinces) endeavor to produce the crop under drought conditions from season-to-season (235). Because of the known relationship between aflatoxin and drought (91), cultivation of maize under the water-stress conditions has been presumed to be one of the factors driving the frequent aflatoxin contamination and aflatoxicosis in lower Eastern Kenya.

Maize is cultivated on small-scale (average farm size is less than two hectares) and large-scale (average farm size is over twenty hectares) (235). Most of the maize (75%) is produced by about 3.5 million small-scale farmers for semi-subsistence use, while the rest (25%) is produced by commercial large-scale farming which is mainly practiced in Rift Valley province by about 1,000 farmers (185,89). Small-scale farming is practiced in almost every part of the country where there is either some rainfall or irrigation water (280). The predominance of semi-subsistence farming is due to the cultural value attached to the crop, and the need to utilize the little available land for production of food and for cash income. Because of bottlenecks in the maize value chain, the unit production and income from maize farming is lower for small-scale than for the large-scale farmers.

Generally, maize yields vary greatly between small- and large-scale farmers, and among the small-scale producers (255). Besides locational differences (large-scale farming is located in areas with better climatic factors for maize production), access to certified seed, differences in farm management, and the ability to manage crop stress factors account for the difference in the yield (255,280). Cultivated maize consists of commercial hybrids, synthetic varieties and open-pollinated varieties (89). The varieties are planted as monocrop (mainly by commercial growers) and as intercrops with other crop species (by semi-subsistence small-scale growers). While large-scale farmers can afford the basic production inputs such as certified seed, fertilizer and pesticides, small-scale farmers may not have the financial capacity to access these inputs (255). A majority of the semi-subsistence small-scale farmers save seed of the hybrid varieties from

previous seasons, or grow local open-pollinated varieties (8). Provision of credit facilities to farmers is also restricted to commercial maize growers (185,255). These factors lead to the production of most of Kenyan maize under sub-optimal conditions.

Provision of certified seed is a mandate of several interlinked governmental and private organizations (115). The cultivated maize seed is conventionally bred by the Kenya Agricultural Research Institute (KARI), the International Maize and Wheat Improvement Center (CIMMYT) and a few private breeders (115). KARI is mandated with the public breeding, and releases varieties which pass through the Kenya Plant Health Inspectorate Service (KEPHIS) for certification (115). Seed is thereafter multiplied, packaged, distributed and marketed by several seed dealers (89). The seed dealers also market fertilizers and pesticides through local outlets and agents across the country (89,115). Varietal adoption is facilitated through government extension education agencies in the ministry of agriculture, and a few non-governmental organizations (NGOs) (115). The Kenya Maize Development Programme (KMDP) and the Tegemeo Institute (Egerton, Njoro) are examples of NGOs that play a role in research and provision of information to maize farmers (89,115).

Timing of specific agronomic practices is important in rainfed agriculture, especially in regard to management of mycotoxins. In Kenya, the majority of farmers rely on their historical knowledge of seasons and the maize development stages to establish the planting, maturity and harvesting dates. The lack of timely weather forecast information to farmers has been associated with unpreparedness at critical times in the maize crop production, and has led to pre-and post-harvest losses of maize produce (124). Recent efforts by organizations such as integrated regional information networks (IRIN) and global framework for climate services (GFCS) could help in timely provision of weather forecasting data and hence impact farmers' approaches crop production practices (105). Timely planting and execution of important pre-harvest agronomic practices could reduce crop stress, while proper timing of harvesting and post-harvest practices could reduce occurrence of ear and kernel rot and mycotoxins.

The peri- and post-harvest practices are determined by the intended use of the produce. Maize meant for fresh market consumption is harvested by the dough stage of ear development, while that which is consumed as mature dry whole grain or flour must be allowed to reach physiological maturity (185,115). The distinct stages are important for small-scale semi-subsistence or commercial farmers, as a majority of large-scale farmers produce mature dry

whole grain. Although fresh maize consumption is common across all maize-consuming communities, its demand has recently increased at the small-scale open-air roadside markets (179). Fresh maize is harvested at milk or dough stage and the ears are sold for immediate roasting, steaming or boiling (179). Maize for consumption of mature whole grain status is allowed to attain physiological maturity, it is field dried and harvested using different methods, depending on the scale of production (179). Mature maize is ether left to dry on erect plants or the whole plant is cut and stacked in the field for a few days prior to harvesting (removal of ear from the stalk and dehusking) (212,89).

Post-harvest bottlenecks within the maize value chain include improper harvesting methods, inadequate and improper drying methods (e.g., sun-drying of the grain on the ground), shelling by manual whipping (which may cause massive kernel breakage), and improper grain storage (115). After field drying, large-scale farmers carry out mechanized harvesting and shelling, and treat the grain with synthetic weevil protectants prior to packaging into 90-kg polypropylene bags and storage in modern stores (111). On the contrary, the small-scale farmers have no mechanized shelling, but package maize ears into gunny bags and shell by manual strikes (212). The small-scale farmers do may not afford to buy the synthetic pesticides, but apply locally known grain preservatives (or botanicals) prior to packaging their maize into the 90-kg polypropylene bags, plastic or metallic containers, and storage in cribs (111). Small-scale farmers store their produce and sell and consume it in small amounts. Large-scale farmers sell their maize at relatively good prices to either the government through the National Cereals and Produce Board (NCPB), or to the larger grain millers, or to foreign companies. Large-scale farmers are also able to hoard the grain for sell at higher prices (185).

The government of Kenya does practice a direct control of maize markets, but could cause intervention by setting prices at which the NCPB buys the grain (115). Maize produced by small-scale farmers is either consumed at home or is sold through middlemen to local markets or to millers (170). Farmers who exhaust their grain before the next harvest often buy from the local grain traders (or from nearby village-based mills called *posho* mills) at inflated prices. Maize prices are determined by factors such as expected future crop failure, consumer demand, and demand for famine relief within the country or abroad (170). The middlemen include bicycle traders or local grain dealers who buy and hoard maize or could sell the grain to large-scale traders who hoard the grain or sell directly to NCPB, and/or to large-scale millers (115,170). Generally, the small-scale

producers face the following market bottlenecks: lack of knowledge of prices and hence exploitation by market cartels; lack of efficient means of transportation; experience more grain quality loss (e.g., kernel breakage, moldiness and mycotoxin contamination); and the majority of them lack skills to account for the profit or losses associated with their maize farming activities (115).

The Kenyan maize value chain lacks sophisticated and regulated maize processing methods which could help in quality evaluation and monitoring (115). Dry mature grain is processed and/or consumed in different forms, including two favorite Kenyan meals, *githeri* (boiled mix of whole maize grain and common beans), and *ugali* (stiff porridge made from milled maize), while fresh maize is consumed roasted or steamed. A majority of maize (60%) is milled at posho mills at approximately 10% the cost of the grain (115). The sifted flour from the maize processed at large-scale mills is sold at wholesale stores or supermarkets to retail stores (179,115). The by-products of large-scale milling process are sold as livestock and poultry feed at retail agro-vet stores (115).

Thesis Objectives and Rationale

This dissertation addresses the mycotoxin problem in maize in tropical sub-Saharan Africa using the Kenyan maize value chain as a model. The first three research chapters (chapters 2-4) consist of work that was done in Kenya, while work for the last chapter (chapter 5) was done at Cornell University, United States of America. The first two research chapters are from survey studies for aflatoxin and fumonisin contamination. The third research chapter is derived from an investigation of aflatoxin accumulation in naturally-inoculated experimental maize hybrids that were grown in field trials at aflatoxin hotspot areas. The last research chapter consists of laboratory-based mature kernel assays using seed of publicly available maize inbred lines that were grown at different locations in the United States of America.

Chapter 2 (*Cross-sectional and longitudinal assessment of mycotoxin contamination of maize in western Kenya*) investigated contamination of maize by aflatoxin and fumonisin in a Kenyan area where most maize is produced (the grain basket). The chapter presents two studies that were carried out between 2009 and 2010. The first study investigated whether there was a problem in this area where no aflatoxin contamination or aflatoxicosis outbreaks had been recognized, and to identify factors (if any) associated with the problem in the wider grain basket region, Nyanza, Rift Valley and Western provinces. The second study investigated aflatoxin and fumonisin accumulation over

time at four points in the maize value chain (at harvest, two and four months after harvest, and at the mill) in major maize varieties in Western province.

Chapter 3 (*Extent and drivers of mycotoxin contamination: Inferences from a survey of Kenyan maize mills*) investigated the nature and extent of maize contamination in Eastern Kenya, the aflatoxin hotspot area, during an aflatoxin contamination outbreak in 2010. Prevalence of the two mycotoxins, aflatoxin and fumonisin, is presented and the drivers for aflatoxin accumulation were analyzed. In addition, the efficacy of visual sorting of grain (based on apparent moldiness and breakage) as a means of reducing the level of maize contamination by the two toxins was investigated. Sorting is a common practice in the maize value chain in sub-Saharan Africa. Chapters 2 and 3 mainly investigated the mycotoxin problem at the posho mills because these mills are the last point before consumption of maize by most Kenyans, and they could be good points for mycotoxin surveillance in the Kenyan maize value chain.

Chapter 4 (*Insights on susceptibility of maize to aflatoxin: influences of plant stress, agronomic traits and Aspergillus flavus populations*) investigated how soil fertility influences susceptibility of maize to aflatoxin, and maize genetics and pathogen-related (*A. flavus*) factors that influence severity of aflatoxin accumulation under nitrogen-depleted soils. The chapter focuses on identifying aflatoxin resistance using materials that were grown under N-depleted conditions (similar to maize production conditions for majority of farmers), and tests the association between aflatoxin resistance or susceptibility and maize agronomic traits. Maize genotypes were grouped based on maturity, and the level of aflatoxin contamination was assessed within and among the maturity categories. Investigations were also done on the relationship between aflatoxin and *A. flavus* biomass (assessed using real-time PCR) or *A. flavus* toxigenicity in maize grown under N-depleted soils.

Chapter 5 (Insights into the role of ear environment in post-harvest susceptibility of maize to toxigenic *Aspergillus flavus*) presents work on the effect of environment where the parent maize plant is grown on susceptibility of maize to a toxigenic *A. flavus* strain (based on mature kernel assays) using diverse maize inbred lines (NAM founders) that were grown at seven environments in the USA. The chapter also presents correlations between the grain ionomic content and maize colonization by aflatoxigenic *A. flavus*, or aflatoxin level. The analysis was based on a

laboratory-based assay of mature dry kernels of the intermated B73xMo17 (IBM) population that was grown at Clayton, NC in 2005.

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CHAPTER 2

CROSS-SECTIONAL AND LONGITUDINAL ASSESSMENT OF MYCOTOXIN CONTAMINATION OF MAIZE IN WESTERN KENYA[1]

Abstract

We report a two-part survey of mycotoxins in maize in western Kenya. In a cross-sectional survey conducted in 2009 across three agroecological zones within three provinces, milled maize samples were collected from 985 patrons of 26 hammer mills. Aflatoxin contamination above the regulatory limit of 10 ppb was observed in 15% of the samples overall, while 65% of the samples from a drought-prone area were over the limit. In a longitudinal survey conducted in Western Province, we investigated aflatoxin and fumonisin accumulation in four popular maize varieties from harvest through a four-month storage period. We collected whole-grain samples from farmers' storage sheds and milled samples from patrons of local mills. Aflatoxin arithmetic means were identical for the sample sets from the sheds and the mills. Per the highly skewed distribution of toxins in grains, mycotoxin frequency was higher at mills, while the variances were much higher at the sheds. Mycotoxins did not increase during storage. The most popular maize varieties were apparently vulnerable to mycotoxins and weevils. Mycotoxin surveillance is important not just in known aflatoxin hotspots, but in all maize-producing regions. Given the highly skewed toxin distribution and the feasibility of sorting, fumonisin exposure could be reduced through sorting.

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Introduction

There is increasing concern about mycotoxin contamination in tropical food systems. Significant attention has been focused on certain recognized "hotspots" where mycotoxin outbreaks or high levels of endemic contamination tend to occur. For example, the recurrent outbreaks of fatal aflatoxicosis in Eastern Kenya have received considerable attention (41,16,34). However, little information is available on the occurrence and the extent of mycotoxin contamination in most areas of Africa, including the western region of Kenya, the country's grain basket.

Maize is produced in Kenya under agro-climatic conditions that favor fungal colonization and mycotoxin accumulation (36,43). Approximately 75% of Kenyan maize is produced by resource-poor farmers under sub-optimal conditions that could predispose the crop to mycotoxin contamination, and the maize value chain lacks quality control mechanisms (34). The factors that influence vulnerability of maize to mycotoxins along the African maize value chains are poorly understood. Mycotoxin surveillance at key points along the maize value chain is important to facilitate management strategies that would reduce health risks.

The best-known mycotoxins in maize include aflatoxin, produced by *Aspergillus flavus* and *A. parasiticus*, and fumonisin, produced by *Fusarium verticillioides* (68,11). Acute exposure to aflatoxin is fatal, while chronic exposure has been associated with stunting in children and with increased rates of liver cancer (66,37). Consumption of fumonisin-contaminated maize has been associated with stunting of children and with increased rates of esophageal cancer (37). Co-exposure to the two mycotoxins has been shown to increase the frequency of liver cancer in animal studies (12). The World Food Program (WFP) has set the regulatory limits for aflatoxin at 10 ppb (10 µg/kg) and for fumonisin at 1 ppm (1 mg/kg) (58). Because of the high cost of mycotoxin analysis, few developing countries effectively monitor mycotoxin levels in their food systems. Kenya has adopted the WFP limits, but the country lacks capacity for systematic mycotoxin monitoring and the government has not fully operationalized the regulations (63,24,62).

Maize is the main staple food in Kenya, contributing 65% of staple food calories and 36% of the total caloric intake (1). Small-scale farmers store maize under varying and often sub-optimal conditions for up to four months before home use or sale (30,23). Families often consume some

of the maize they grow, sell some to raise cash, and buy locally-marketed maize as need arises (1). An estimated 60% of maize is processed by consumers at local, small-scale mills known as *posho* mills (34).

As a basis for developing effective mycotoxin mitigation strategies, there is need to assess the extent of contamination and the factors associated with the problem. The final level of mycotoxin contamination of a maize sample depends on the severity of fungal colonization, growth and toxin production at each stage along the value chain (24). Posho mills are the last processing points for approximately 60% of Kenyan maize, and are strategic sites for surveillance of mycotoxin contamination levels in maize destined for immediate consumption (34). In the current study, we investigated the prevalence and level of mycotoxin contamination during harvest, at farmers' stores and at the mill in western Kenya.

Western Kenya (Nyanza, Rift Valley and Western provinces; hereafter W. Kenya, to distinguish the region from the former administrative province) produces over 75% of the maize consumed in Kenya. Consumers in most parts of the country depend of the maize that is produced in W. Kenya (50). Mycotoxicosis outbreaks have not been recognized in the region, though the lack of fatalities and mycotoxin surveillance could mean that problems have gone unnoticed. The few mycotoxin-related studies conducted in the region had shown that fumonisin could be more prevalent than aflatoxin, but the studies were limited in sample size and geospatial spread. Kedera et al. (36) collected maize samples (n=197) from the storage sheds across different districts in W. Kenya, reporting fumonisin in 47% of samples (n=93), with 5% contamination above the regulatory limit. Alakonya et al. (2009) assessed the effect of delayed harvesting in four maize varieties at 24 farms in Western Kenya, and reported aflatoxin up to 20 ppb in three samples (3). Mutegi and Ngugi (40) reported a high prevalence of aflatoxin contamination in groundnuts (*Arachis hypogaea* L.; also known as peanut) in W. Kenya, but peanut production and consumption are not as widespread as maize. Analysis of human blood serum samples taken across the country in 2007 revealed lower levels of human exposure to aflatoxin in W. Kenya than elsewhere in the country (71).

The factors that increase the vulnerability of maize to mycotoxin contamination include pre-harvest biotic and abiotic stress, genetic factors of the pathogen and maize that favor colonization and toxigenesis, and improper post-harvest management (65,26). Drought, insect pressure and

soil degradation/infertility are among the environmental factors of relevance (10). The toxigenicity of the fungal populations is another variable that is poorly understood; *A. flavus* genotypes of varying toxin production potential have been reported in Kenya (43,48). Maize genotypes differ in susceptibility to mycotoxin accumulation, and maize breeding is one of the potential mycotoxin management strategies (67,24). Complete resistance of maize to aflatoxin and fumonisin does not exist and the two complex traits are controlled by multiple genes (67,19). Mycotoxin accumulation is influenced by maize and fungal genotypes (G_M and G_F), environment (E), farm management factors (M), and the interactions among these factors (G_M x G_F x E x M) (9,19). Grain characteristics such as kernel texture are among the genetically-determined traits of maize that could influence susceptibility to pre- and post-harvest mycotoxin accumulation (8).

The objectives of this study were to assess prevalence of aflatoxin in a major maize-growing area where an outbreak had not been recognized, to analyze drivers for aflatoxin contamination in maize, and to assess aflatoxin and fumonisin contamination in selected maize varieties at different points on the maize value chain. Sampling strategies were designed to gain insights at different stages of the maize value chain; at harvest, after two and then four months of storage, and finally at the mill. The levels of mycotoxins detected in samples from the mills were an indicator of the level human exposure at the time of conducting this study. To understand whether contemporary varieties differ in their vulnerability to mycotoxin accumulation under farmers' conditions and at key points in the value chain, we tested whether major varieties grown in W. Kenya differed in vulnerability to mycotoxin accumulation. We considered maize grain texture in selection of the four varieties, based on prior reports that this kernel trait could influence mycotoxin levels (15,8).

Materials and Methods

Study sites. Cross-sectional and longitudinal surveys were conducted in western Kenya in 2009 and 2010 to assess mycotoxin contamination in maize. The cross-sectional survey involved sampling of flour at posho mills in Nyanza, Western and Rift Valley provinces of W. Kenya between May and July 2009 (Table 1, Fig. 1). Sites were identified to sample three maize-producing agro-ecological zones (AEZs) - humid, sub-humid and semi-humid - using a geographical information system (GIS) overlay of publicly available data on Kenya's provincial

administrative boundaries and AEZs. Six towns that served as headquarters of administrative districts within three provinces were selected as sampling hubs (Fig. 1). Villages that represented different AEZs and that were located within a 20-km radius from the sampling hub were selected as target sampling sites. After stratifying by AEZ, 26 villages were randomly selected from this sampling frame. One mill within each selected village was randomly selected for inclusion, conditional on the consent of its owner (Table 1). Sampled sites were grouped based on existing geo-climatic data and AEZs into 11 geo-climatic locations; samples collected from all mills within the same AEZ in the same district were considered to have originated from the same geo-climatic location (Tables 1 & 2).

The longitudinal survey involved sampling of maize grain from farmers' grain storage sheds and maize flour from posho mills within seven villages located in a humid AEZ of Bungoma district, Western province, August 2009 - March 2010. The mills included in the longitudinal study were different from those in the cross-sectional survey. The longitudinal study sites were identified with guidance from authorities in the Kenyan Ministry of Agriculture to fulfill the following criteria: relative climatic uniformity, agro-climatic conditions representative of the major maize growing area of W. Kenya, maize as the predominant crop, small-scale maize production, cultivation of multiple maize varieties that represented the diversity of maize in the region, maize harvest coinciding with the start of our survey, and few or no non-governmental maize development agencies operating in the area (such organizations were expected to influence farmers' maize production activities, and instructions from such organizations might have either interfered with our survey or influenced its results). The seven villages were identified as sampling sites based on pre-harvest assessment visits (reconnaissance) to 13 villages within a 10-kilometer radius from the Bungoma town center. For each of the 13 villages, 30 farmers were interviewed about the maize varieties they grew, time to maturity of each variety and the expected harvest date. Further information about the characteristics of each of the varieties was obtained from the Tegemeo Institute working paper (55), from brochures of seed traders, and by inspection of kernel traits. For evaluation of varietal effect on mycotoxin accumulation, we selected four maize varieties based on their popularity in the study area and their differences in kernel texture.

Study design and sampling strategy. For the cross-sectional posho mill survey**,** centrally-trained research assistants interviewed and collected paired samples (n=985) of whole-grain and

milled maize from individuals who processed maize at the 26 posho mills. From each posho mill, 30-45 samples were collected in a 3-5-day period. For each sample, a questionnaire was administered on how the respondent acquired the maize. For those with home-grown maize, we collected data on pre- and post-harvest management of the crop.

For the storage shed survey, we selected farmer participants (*n*=186) who grew at least one of the four selected varieties within the seven villages. A further subset of farmers (*n*=40) was randomly selected from the participating group to assess whether the commonly-used grain weevil deterrents (e.g., commercial synthetic pesticides, botanicals, or ash, hereafter "preservatives") influenced mycotoxin accumulation. For each of the home-grown maize varieties, the participants in the grain preservatives study were provided with a pair of woven polypropylene storage bags (the typical maize storage bags in East Africa; 30), which could hold 10 kg of maize. Participants were asked to put their treated and non-treated home-grown maize grain in the appropriately labeled bags, and to store the sample bags in their sheds during the sampling period. Interviews were conducted and samples collected at three intervals: at harvest, and at two and four months after harvest. Interviews at harvest provided information on pre-harvest management practices, while later interviews provided data on post-harvest conditions.

Interviews at harvest and ear rot assessment were conducted within the first two days of harvest (and prior to maize shelling). Ear rot was visually assessed on randomly selected maize ears of each variety that filled a polypropylene bag of 10-kg grain capacity. Ears were grouped into categories of percent of rotten kernels as follows: 1= no rot; 2 = below 25% but with some rot; 3 = 26-50%; 4 = 51-75% and 5 = greater than 75%. Data were recorded as number of ears that fell in each of the score categories per bag, and the average varietal ear rot scores were calculated.

Upon shelling and packaging into the 90-kg polypropylene bags, whole-grain maize samples were collected from up to three bags that the participants stored, and also from the 10-kg bags for the participants in the grain preservative experiment. Maize was sampled using two devices: a double-tube spear for the grain that was stored in the 90-kg bag, and a closed spear for the 10-kg bag (49). Grain was sampled as follows: the sampling device was used to obtain grain from different depths from the mouth of the bag, and nine sub-samples of approximately 100 grams each were randomly drawn. During each of the grain sampling dates, grain moisture and

weevil incidence were assessed. Grain moisture was measured using an SC 4A digital sensor (Zhejiang Top Instrument Co., Ltd., China) at nine points (three at the top, three in the middle, and three at the bottom) for every three 90-kg polypropylene bags. Weevil incidence was scored on each of the nine sub-samples using the following scale: 0 = no weevils; 1 = 1-2 weevils; 2 = 3-5 weevils; 3 = 6-10 weevils; 4 = >10 weevils. After scoring weevils, the sub-samples were pooled, mixed, and a final 100-gram whole-grain sample was drawn for mycotoxin analysis.

In addition, we collected 100-gram flour samples from individuals who processed maize that was destined for immediate consumption at a major posho mill within each of the villages where there was a concurrent storage shed survey. Research assistants collected the flour samples and survey responses from 30 adults at each mill in a 3-day period during each of the three sampling periods for the storage study. The flour sample was sub-sampled from well-mixed flour bulks, which ranged among individuals from 2-10 kg. Individuals were interviewed on maize variety and pre- and post-harvest management practices.

Sample handling and mycotoxin analysis. Aflatoxin was analyzed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit, the performance of which had been previously validated (see chapter 3). Aflatoxin was extracted from 5-gram sub-samples of maize flour using 70% methanol and was analyzed following the ELISA kit's manufacturer's protocol (Helica Biosystems Inc., Fullerton, CA). The solid-phase direct competitive aflatoxin ELISA kit (Helica Cat. No. 941BAFL01-96) consisted of a 96-well micro-plate coated with an antibody that had been optimized to cross-react with the four aflatoxin types at the following rates: B1 (100%), B2 (77%), G1 (64%) and G2 (25%). The lower and upper limits of quantification of the kit were 1 and 20 ppb (µg/kg), respectively, per the supplier's information. Samples with aflatoxin levels above the upper quantification limit were diluted and re-tested.

Fumonisin was extracted from 10-gram sub-samples of maize flour using 90% methanol and analyzed with a commercially available ELISA kit per manufacturer's protocol (Helica Biosystems, Inc., Fullerton, CA). The solid-phase direct competitive fumonisin ELISA kit (Helica Cat. No. 951FUM01C-96) consisted of a 96-well micro-plate coated with an antibody that was optimized to cross-react with three fumonisin subtypes (B1, B2, and B3). The lower and upper limits of quantification of the kit were 100 and 6,000 ppb (or 0.1 and 6 mg/kg), respectively, per supplier's information. Samples with toxin values below the limit of

quantification were considered as containing no detectable toxin. Optical densities of the reactions for the two mycotoxins were quantified using a micro-plate reader (BioTek Instruments, Inc., Winooski, VT) with an absorbance filter of 450 nm. Test values were interpreted with reference to standards that were included in each experiment.

Statistical analysis. Statistical analysis was done in JMP Pro ver. 10 (SAS Institute Inc., 2012, Cary, NC.). For the cross-sectional study, aflatoxin prevalence was analyzed by computing the percentage of samples with various aflatoxin contamination levels for each of the geo-climatic locations. Correlations were computed between publicly available rainfall estimates [based on satellite imaging and global telecommunication system (70)] and the percentages of samples with aflatoxin or with aflatoxin above the regulatory limit of 10 ppb across the geo-climatic locations. Factors potentially influencing aflatoxin levels were analyzed using samples from Nyanza Province. Aflatoxin data from the other regions were too skewed to permit a similar analysis. Aflatoxin data were transformed to logarithm (concentration in ppb+1) and analyzed as a dependent variable in a model in which the 26 factors included in the survey questionnaire served as independent variables. District and village nested within a district were modeled as random effects. Results of the analysis were presented as geometric means (GM) that were derived by back-transformation of the least squares means of the transformed aflatoxin data.

For the longitudinal study, the means, variances and percentages of samples with contamination at different levels were computed for whole grain collected at farmers' storage sheds and for the flour collected at the mills. Mycotoxin data of the whole grain collected from the storage sheds was analyzed separately from the data of contamination of flour samples from the mills; these were not considered to be directly comparable due to the differences in sampling methods. To assess the factors associated with the presence or absence of mycotoxins in the longitudinal study, a nominal logistic regression analysis was used on binary coded mycotoxin data. Aflatoxin and fumonisin were binary coded as follows: samples with toxin level above the detection limits (1 ppb, aflatoxin; 100 ppb, fumonisin) were considered positives and coded 1, while those with lower values were considered as negatives and coded 0. The questionnaire-based variables used in the regression analysis were sequentially dropped from the logistic model until the best model, based on Akaike's Information Criterion (AIC), was obtained.

Pair-wise comparisons between mycotoxin levels and covariates were performed using *t*-tests and correlation tests. Spearman's correlation test was used for comparisons that involved

small sample sizes, and for data for which the type and distribution did not meet the assumptions for Pearson's correlation test. Paired *t*-tests were used to compare mycotoxin levels in maize that was either treated or non-treated with preservatives. A chi-square test was performed for difference in the levels of weevil infestation between preservative-treated or non-treated maize, for the difference in the proportions of samples with weevil infestation and mycotoxin contamination among the sampling periods, and for the differences in weevil and mycotoxin contamination among maize varieties.

Results

Aflatoxin prevalence in western Kenya, 2009. Among the 985 maize samples collected from posho mills in 11 geo-climatic locations, 49% had detectable levels of aflatoxin, and 15% were contaminated above the regulatory limit (> 10 ppb) (Table 2). The highest percentage of aflatoxin-contaminated maize was observed in two drought-prone districts (Homa Bay, 66%; Rachuonyo, 93%) of Nyanza province (Table 1 & 2). Similarly, Nyanza province had a relatively high proportion (8-65%) of samples with aflatoxin contamination above the regulatory limit (Table 2). The association between aflatoxin and the questionnaire variables was therefore analyzed using aflatoxin data for the samples from Nyanza (n=118). The frequency of significant aflatoxin contamination among samples from Rift Valley and Western provinces was insufficient to support the analysis of drivers.

Aflatoxin levels were significantly influenced by AEZ (P=0.038). The level of aflatoxin contamination increased with decrease in moisture across the AEZs as follows: humid (geometric mean, GM=1±0.3 ppb), sub-humid (GM=2.4±0.4 ppb) and semi-humid (GM=9.2±0.4 ppb). The level of contamination differed between humid and semi-humid zones (P<0.05). Similarly, the amount of rainfall during the growing season of the sampled maize was negatively correlated with the percent of samples with aflatoxin above the regulatory limit (r= - 0.79, P=0.004) (Fig 2).

In Nyanza province, aflatoxin levels were influenced by the way in which individuals obtained the maize (P=0.016). Separation of means for the mixed model with samples from Nyanza (n = 118) showed that home-grown maize had significantly (P=0.016) lower levels of contamination (GM=2.4±0.3 ppb; n=56) compared to purchased maize (GM= 4.6±0.3 ppb;

n=62). A univariate analysis of factors that influenced aflatoxin levels in home-grown maize samples (*n*=52) showed that intercropping significantly reduced aflatoxin (*P*=0.023). Maize grown in monoculture was three times more contaminated (GM=4.8±0.5 ppb; *n*=8) than maize grown in an intercrop (GM=1.6±0.3 ppb; *n*=44).

A descriptive analysis of land size and cropping system showed that the sampled maize was cultivated on larger farms at Trans Nzoia, Uasin Gishu and Bungoma districts (approximately 1 ha) compared to Rachuonyo, Homa Bay, and Kisii districts, where farm size ranged 0.4-0.5 ha. An area with relatively large farms under maize production in Kenya (the semi-humid region of Trans Nzoia) had the highest percentage (63%) of home-grown samples (Table 2). The humid area of Bungoma district had the lowest percentage (27%) of home-grown maize; but this region was intensively sampled, representing 23% of the overall sample size. When samples from the humid area of Bungoma district were excluded, the percentage of samples from home-grown maize in the cross-sectional survey was 48%, up from 32% (Table 3).

Farmers' criteria for varietal choice and the determinants of mycotoxin accumulation in the selected varieties in storage. An reconnaissance study of varietal frequency in 13 villages showed that the four varieties included in the longitudinal study were cultivated by 47% (*n*=122) of the respondent farmers, with another 20 minor varieties also grown in the region. A pre-harvest survey of 186 farmers in seven villages revealed the following varietal cultivation percentages: H614 (44%), H513 (36%), WH505 (13%) and Local 8 (7%). Some farmers grew more than one maize variety; 26 farmers (14%) grew two varieties, and two farmers (1%) grew three varieties (Table 4). Factors influencing farmers' varietal choice(s) included seed availability, drought tolerance, earliness of maturity, grain yield and flour quality (Table 4).

Grain moisture was significantly influenced by the sampling period (*P*<0.05). The grain moisture (12.8%) observed right after harvest was significantly (*P*<0.0001) higher than that observed in the subsequent sampling periods, 11.4% for the second and 11.3% for the fourth months of storage. There were no significant maize varietal differences in grain moisture during storage (*P*=0.119), and mycotoxin levels were not correlated with grain moisture (Table 5).

The grain preservative experiment showed that all maize samples were free of weevils at the first sampling right after harvest, but over 20% were infested at the second and fourth months of storage (Fig. 3). A chi-square test showed that the proportion of maize samples with different

levels of weevil infestation did not differ statistically χ^2 = 2.602 (*P*=0.271, *n*=196, *df*=2) at the second versus fourth months of storage, but the varieties differed significantly in infestation χ^2 =19.5 (*P*=0.0005, *n*=196, *df*=4). Variety Local 8 was not infested, while the other three varieties had varying levels of weevil infestation (Fig. 3). The percentage of preservative-treated samples (22%) that was infested by weevils was marginally less than that of untreated maize (24%), but overall was not significantly different (χ^2=0.056, *P*=0.972, *n*=196, *df*=2).

Analysis of paired sets of treated and non-treated maize samples showed that the proportion of fumonisin-contaminated maize was lower (*P*=0.046, *n*=84) when farmers applied preservatives (24%) compared to maize stored without preservatives (44%). In contrast, the proportion of samples with aflatoxin contamination was not different with (10%) or without (12%) treatment with the preservatives (*P*=0.77, *n*=119). Interestingly, mycotoxin levels were not correlated with weevil incidence or ear rot (Table 5). Moreover, aflatoxin and fumonisin levels were uncorrelated (*r*=-0.004).

Among the samples (*n*=488) of the four maize varieties that were studied over the three sampling periods, the overall aflatoxin mean was 2.3 ppb, while the standard deviation was 745% of the mean. The percentage of samples with detectable aflatoxin was 4% (Fig. 4). The likelihood of aflatoxin contamination differed significantly (*P*=0.004) among the varieties (Table 6). Samples of Local 8 and WH505 accounted for 19% of the overall sample size and did not have detectable aflatoxin. For both H614 and H513, the percentages of samples with aflatoxin contamination were slightly below 10%, while contamination above the regulatory limit was 7% for H614 and 2% for H513 (Fig. 5). The likelihood of aflatoxin contamination did not differ between H513 and H614 (odds ratio, *OR*=0.774, *P*=0.514). Sampling period did not influence the likelihood of aflatoxin contamination in maize (Table 6).

Of the 488 samples collected at the storage sheds, 233 (48%) were analyzed for fumonisin (Fig. 4). The overall percentage of samples with detectable fumonisin was 41% (Fig. 5). The overall fumonisin contamination mean was 0.533 ppm, while the standard deviation was 198% of mean. The percentages of samples with detectable fumonisin ranged 25-54% (Fig. 5), with fumonisin contamination above the regulatory limit varying among the varieties (31% for H614, 17% for Local 8, 11% each for WH505, and H513). The likelihood of fumonisin contamination differed significantly (*P*=0.005) among the maize varieties (Table 6). The likelihood of fumonisin

contamination was higher in H614 than in H513 (*OR*=0.27, *P*=0.001) and WH505 (*OR*=0.34, *P*=0.012). The likelihood of fumonisin contamination did not differ significantly among the rest of the varieties (*P*>0.05). Sampling period did not influence the likelihood of fumonisin contamination in maize (Table 6).

Mycotoxin contamination based on longitudinal survey at the posho mills. Flour samples collected at the mill were from maize that was either home-grown (57%) or purchased (43%). The percentage of samples that represented purchased maize was lowest at harvest time and increased over time (36, 42 and 51% at the three posho-mill sampling periods). Of the 574 samples collected at the mill, nearly half had detectable aflatoxin (41%), while 4% of the samples exceeded the regulatory limit for aflatoxin (Fig. 6). The overall mean aflatoxin level at the mill was 2.3 ppb, and the standard deviation was 166% of the mean. Analysis of a subset of the samples for fumonisin (*n*=125) showed that 87% had detectable fumonisin, while half of the samples exceeded the regulatory limit for fumonisin (Fig. 6). The overall mean fumonisin concentration was 1.9 ppm, and the standard deviation was 156% of the mean.

Of the 574 samples collected at the mill, 178 (31%) were reported to be of the four maize varieties that had been collected at the storage sheds. The overall aflatoxin concentration in the samples that were reported to be from the four popular varieties was 2.0 ppb, and the standard deviation was 144% of the mean. The percentage of samples of Local 8 and WH505 that were analyzed for aflatoxin at the mill accounted for 5% of the overall samples collected at the mill. Among the four popular varieties, 30-60% of the samples had detectable levels of aflatoxin contamination (Fig. 7). The proportion of samples of these varieties with detectable aflatoxin did not differ among the sampling periods (*P*>0.05). None of the four popular varieties had samples above the regulatory limit for aflatoxin, except for H614, which had 1% (1 sample) above 10 ppb.

Across all samples collected from the mills, the likelihood of aflatoxin contamination differed significantly (*P*=0.0007) among the sampling periods. The likelihood of aflatoxin contamination was lowest in samples that were collected right after harvest, and highest 2 months after harvest. Samples collected two months after harvest were 1.6x more likely to have aflatoxin than those collected right at harvest (Fig. 8). The likelihood of aflatoxin contamination at harvest did not differ significantly (*P*>0.05) with that observed four months later. The percentages of with aflatoxin contamination above the regulatory limit were across the sampling periods were as

follows: 2% right after harvest, 6% two months later, and 4% in the fourth month. The proportion of samples with aflatoxin did not differ significantly (P>0.05) between the home-grown and purchased maize at any of the sampling periods (Table 7).

Of the 125 samples that were analyzed for fumonisin at the mills, 49 (39%) were reported to be of the four maize varieties that had been collected at the storage sheds. The overall fumonisin concentration in the samples that were reported to be from the four popular varieties was 2.177 ppm, and the standard deviation was 160% of the mean. The percentages of fumonisin-contaminated samples among the selected varieties ranged from 60-85% (Fig. 7). The percentage of samples with fumonisin contamination above the regulatory limit was 69% for H614, 50% for H513, and 20% for WH505. Samples of Local 8 from the mill were not analyzed for fumonisin, while those of WH505 accounted for 1% of the overall samples collected at the mill (Fig. 7). Varieties did not differ significantly in the likelihood of fumonisin contamination (P>0.409). Sampling periods differed in the likelihood of fumonisin contamination (P=0.01). The likelihood of fumonisin contamination at harvest was 22x higher than in the second sampling period (P=0.02). The likelihood of contamination was not significantly different (OR>999.99, P=0.24) in flour samples collected at the mill at harvest and four months later. The likelihood of fumonisin contamination in the samples that were collected in the third was significantly (OR>999.99, P=0.02) higher than in the second sampling period.

Across all samples that were analyzed for fumonisin from the mills, the likelihood of fumonisin contamination differed significantly (P=0.01) among the sampling periods. The percentage of samples with detectable fumonisin at different sampling periods were 90% at harvest, 82% at 2 months later and 96% at the 4^{th} month after harvest (Fig. 8). The percentage of samples with fumonisin above the regulatory limit was highest at harvest (75%), and decreased to 45% within the two months later, followed by a further decrease to 32% in the subsequent two months (Fig. 8). The proportion of samples with detectable fumonisin did not differ between the purchased and the home-grown maize over the three sampling periods (Table 7).

Discussion

Outbreaks of fatal aflatoxicosis caused by consumption of contaminated maize have frequently been reported in eastern Kenya (6,16,34). It is less clear whether mycotoxins are a chronic or periodic problem in regions of Kenya where fatalities have not been reported. In particular, W. Kenya is the country's grain basket and thus of importance to maize consumers across the country, but no comprehensive mycotoxin survey had been conducted to establish the prevalence of aflatoxin or fumonisin contamination in maize. The few published studies on the occurrence of mycotoxins in maize provided some insight, but were based on small geographical areas and/or small sample size and/or relied on data from one point in the maize value chain (36,2,4,40). We extended the understanding through a deeper sampling across locations and at multiple points along the maize value chain.

We used a cross-sectional survey to assess the prevalence of aflatoxin in maize destined for immediate consumption over three agroecological zones in each of the three provinces. We detected significant influences of climate (rainfall) and management (intercropping v. monoculture) on aflatoxin contamination in maize. We further used an in-depth longitudinal survey within an area with less climatic variation (a humid AEZ in Bungoma, Western Province) to assess the extent of maize contamination at harvest and during storage, and to make inferences regarding the susceptibility of major maize varieties to aflatoxin and fumonisin. We provide more information about susceptibility of three of the maize varieties that Alakonya et al. (3) had previously investigated. We report mycotoxin contamination at four key time-points in the maize value chain (at harvest, two and four months during maize storage, and at the mill), and relate the contamination with maize storage practices (use of pesticides and other preservatives in storage).

While we observed an overall low frequency and level of aflatoxin contamination in western Kenya, certain geo-climatic locations exhibited high levels of contamination. The level of contamination observed in Nyanza province was similar to the reported prevalence in the relatively well-studied regions of eastern Kenya where frequent fatal outbreaks of aflatoxicosis have occurred (6,16). A recent study that involved aflatoxin analysis in human blood samples that were collected from across the country showed that Nyanza population had the lowest levels of aflatoxin exposure of all provinces (71). The low level of aflatoxin observed in the blood samples within Nyanza contradicts the current finding of high levels of maize contamination, which suggests high levels of exposure. The difference across the two studies could be due to temporal

variation in mycotoxin levels, which could be influenced by environment and changes in diet. Such temporal variation is of policy significance because it points to a potential public health problem in the grain basket region where mycotoxin outbreaks have not been recognized, and the need for more frequent monitoring even in locations where contamination levels are not typically high. Results of this study imply that aflatoxin contamination is common but has remained unnoticed because of a lack of surveillance, and that attention to the problem should not only focus on the known aflatoxin hotspots of eastern Kenya.

We observed a significant association between climatic conditions (rainfall and agroecological zones) and aflatoxin contamination. Trans Nzoia and Uasin Gishu typically experience more rainfall than the other districts studied, and were found to have less aflatoxin contamination. The highest proportion of samples above the regulatory limit was observed in the drier semi-humid AEZ and the drought-prone districts. These findings showed the expected relationship between moisture stress and aflatoxin levels, and are in agreement with the previous reports that drought makes maize vulnerable to colonization by aflatoxin-producing fungi (31,32,21,39).

Based on the cross-sectional survey data, the level of aflatoxin contamination was influenced by how individuals obtained their maize. Higher contamination was observed in purchased maize than in home-grown maize. Hoffmann and Gatobu (27) found that Kenyans place substantially higher value on maize they have grown themselves relative to maize that is available for purchase at the local markets perhaps because they expect it to be of higher quality and safety. In the current survey, farmers reported that local maize traders cushion themselves against grain losses caused by moldiness by blending maize of different qualities. Blending of grain could increase aflatoxin contamination in marketed maize compared to home-grown maize.

Among the home-grown maize samples, intercropped maize was observed to have lower aflatoxin contamination compared to monoculture. These findings are consistent with those of the recent survey in eastern Kenya (see chapter 3), but contradict previous reports on the effect of intercropping on aflatoxin accumulation (22,54). Aflatoxin accumulation is correlated with crop stress (14). Intercropping is intended to reduce competition between neighboring plants and to introduce complementary functions that increase system performance. For example, intercropping of maize with a leguminous crop enhances nitrogen fixation in the soil and provides a consequent boost in crop vigor, a phenomenon that might reduce susceptibility to

opportunistic fungal pathogens. Some cover crops used in intercropping might reduce plant stress by reducing the rate of evapotranspiration, suppressing weeds, and controlling some diseases (56,53). There is need for experimental studies to establish the specific intercrops and mechanisms through which intercropping reduces aflatoxin contamination.

We observed low aflatoxin contamination in the two districts with unimodal rainfall pattern. Kenyan smallholder maize growers typically store their maize grain until the subsequent harvest. Maize growers in the unimodal rainfall areas have one crop per year, while farmers in the bimodal pattern harvest two maize crops per year (28). Farmers store maize for a longer period in the unimodal compared to those in bimodal rainfall pattern. In the current study, samples from districts within the unimodal rainfall pattern were collected approximately seven months after harvest, while those from the rest of the districts were collected at approximately three months after harvest. If duration of storage were a major determinant of aflatoxin accumulation, samples collected from the two districts with the unimodal rainfall pattern would have been the most contaminated with aflatoxin. Because of short time intervals between seasons, we speculate that farmers in the bimodal pattern may lack time and space to adequately dry the produce and could have elevated chances of mycotoxin accumulation.

Except for the Rachuonyo and the Homa Bay districts, which are prone to drought, the other districts surveyed received relatively high rainfall and had a lower percentage (3% to 7%) of samples with aflatoxin above the regulatory limit. Most farmers in the Bungoma, Trans Nzoia and Uasin Gishu districts have larger farms, and are likely to have higher income from maize production, as well as better pre- and post-harvest management relative to farmers with smaller farms (<1 ha) in the drought-prone areas of Nyanza Province (42). Recent studies in eastern Kenya showed that farmers with larger maize farms and grain yield had relatively lower likelihood of aflatoxin maize contamination (see chapter 3).

We observed a greater extent of maize contamination with detectable and above the regulatory limit for fumonisin than aflatoxin, and neither the occurrence nor the levels of contamination by the two mycotoxins were correlated. The greater extent of fumonisin contamination is in agreement with previous studies (36,4), and requires urgent investigations of possible health implications of fumonisin in the region. The observed high percentage (50%) of samples that had fumonisin above the regulatory limit of 1 ppm suggests exposure to unacceptable levels of the toxin. Recent studies reported an increase in esophageal cancer in

western Kenya, a disease that has been correlated with consumption of fumonisin contaminated foods (59,45). According to recent global statistics on age-standardized cancer death rates, Kenya was ranked the 8th for esophageal cancer and 76th for liver cancer (13,38). The Kenyan rates of cancer per 100,000 person-years were as follows: esophageal cancer (incidence, 17.6; mortality, 16.5), and liver cancer (incidence, 5.4; mortality, 5.0) (18). Previous animal co-exposure studies have shown that a combined ingestion of aflatoxin and fumonisin can increase chances of liver cancer (12,20). Although Kenya was ranked among the countries with moderate death rates due to liver cancer, the observed co-occurrence of aflatoxin and fumonisin implies a co-exposure and a likelihood of an increase in incidences of the disease. Co-occurrence of these mycotoxins could also lead to increased human morbidity and stunted growth in children (66,52).

The rates of contamination observed at farmers' storage sheds and at mills were quite different, and the results must be considered in light of the different sampling strategies used at each of the sites. The samples collected at the mills were from larger grain samples that were homogenized through milling prior to sampling, whereas the samples collected at the storage sheds were from a fixed amount of bulked whole grain that was not milled prior to the sampling process. The observed higher mycotoxin frequency at the mills compared to the storage sheds was possibly because a smaller sample was analyzed from the sheds compared to the mills. The distribution of mycotoxins is known to be highly skewed among grains, especially for aflatoxin (51,64,61). The higher variances observed at the storage sheds compared to the mills for both mycotoxins are consistent with the expectations based on the sampling design. The observed similar aflatoxin means for the sheds and mills implies that the aflatoxin levels in large samples collected at each site averaged out and the magnitudes of contamination reflected what was expected for the sampled site. Because of small samples of the popular varieties that were collected at the posho mills, data from the mills were used to give insights on the level of mycotoxin contamination in maize that was being consumed at the time of the survey. Additionally, the data complemented inferences about the performance of the popular varieties.

To prevent storage losses, farmers apply different types of preservatives, including botanicals (locally-known plant derivatives), synthetic pesticides, ash, etc. Application of the preservatives was associated with a marginal decrease in weevil incidence, and a decrease in fumonisin contamination, but weevil incidence was not correlated with mycotoxin levels. Weevil damage of maize in storage has been previously correlated with mycotoxin accumulation in other

tropical regions. Kaaya et al. (33) observed a significant association between aflatoxin and weevil damage on stored maize in Uganda. Similarly, Ono et al. (44) observed a positive correlation between fumonisin accumulation in maize and weevil damage (33, 35). The lack of correlation between fumonisin and weevil incidence in the current study implies the possibility of direct control of fumonisin-producing fungi by the preservatives applied. Unlike *A. flavus*, which is known to exhibit internal colonization, growth of *F. verticillioides* is superficial and its growth might have been inhibited by the preservatives (16). Direct experimental evidence will be needed to test this hypothesis.

We did not detect a significant difference in aflatoxin and fumonisin contamination among the sampling periods during storage. While reports of fumonisin accumulation during storage are inconsistent (7,44,5), several studies have reported aflatoxin accumulation in stored maize, especially in the tropics (22,35,54). In their 12-month experiment in which two groups of maize samples, pre-dried to 11 and 14% and monitored for fungal colonization and fumonisin levels, Ono et al. (44) reported no significant increase in fumonisin. They attributed this finding to low water activity that might have inhibited growth of fumonisin-producing fungi. In the current study, grain moisture during storage ranged 11.3 to 12.8%, and was below the maximum 13% that is recommended for grain storage in the tropics, and below the 15% which is favorable for growth and toxin production by *A. flavus* and *F. verticillioides* (49,33). The low grain moisture and the observed low weevil incidence during storage suggests that maize was stored under conditions that were not optimal for aflatoxin and fumonisin accumulation. Although variation in toxigenicity of fungal populations could influence the frequency and amount of mycotoxins, we did not carry out toxigenicity experiments (46,48).

Across the sampling periods at the mill, the proportion of aflatoxin-contaminated samples was highest in flour samples that were collected two months after harvest, while the percentage of samples with fumonisin above the regulatory limit was highest at harvest and decreased over time. Because the observed trends were maintained even when only home-grown maize samples were considered, we speculate that they were caused by grain sorting. In a study in eastern Kenya, we had observed a higher reduction of fumonisin than aflatoxin levels when Kenyan maize consumers sorted their maize prior to milling (see chapter 3). A low correlation between visible moldiness and aflatoxin contamination has been previously reported, and visual sorting was not as efficient in reducing aflatoxin as it was with fumonisin (17,57). The observed increase

in aflatoxin in the second sampling period, followed by a decrease implies that the Aspergillus kernel rot was not visually detectable until the third sampling period when the sorting method was able to lower the proportion of contaminated samples. The observed reduction in fumonisin contamination over time was presumably because farmers sorted and removed moldy maize gradually.

We observed significant varietal differences in mycotoxin levels at grain storage sheds and the mills. H614 and WH505 showed consistent contamination by both aflatoxin and fumonisin at the two types of sampling sites. Local 8 and WH505 were contaminated by fumonisin but not aflatoxin at the storage sheds. However, Local 8 and WH505 were highly contaminated by the two mycotoxins at the mills. H614 and H513 were the most popular among the farmers because of high grain yield. However, these two most popular varieties were vulnerable to weevil damage and mycotoxin accumulation. The observed vulnerability of the two most popular varieties implies that mycotoxin and insect resistance traits were not adequately considered during breeding for increased grain yield. Previous work showed that flint maize genotypes were more resistant to pre-harvest aflatoxin accumulation than dent genotypes (8). If texture had a major effect on mycotoxin accumulation, H614 would have been the most resistant variety. Insect damage has previously been associated with mycotoxin accumulation (25,69). However, we did not find a correlation between mycotoxins and weevils. The observation that the four major varieties were highly contaminated with fumonisin in storage and at the mill implies that fumonisin is a health threat to maize consumers. The findings that Local 8 and WH505 did not have detectable aflatoxin in storage would have led us to conclude that they were resistant, but the complementary findings at the mill showed otherwise. There is need for breeding efforts to combine mycotoxin and insect resistances in local varieties.

In conclusion, the cross-sectional posho mill survey provided evidence of aflatoxin occurrence and its association with drought and cropping systems in a wide geographical area of Kenya's maize grain basket. To the best of our knowledge, this was the first aflatoxin prevalence study at a large sample size and wide geographical coverage of the region. More importantly, this study was tailored to discern aflatoxin contamination at the posho mills, the last point in the maize value chain prior to consumption. The parallel studies conducted at storage sheds and posho mills confirmed the previously reported higher prevalence of fumonisin than aflatoxin in Western Province, and gave insights on varietal differences in mycotoxin accumulation at

farmers' stores. Posho mills are potentially important venues for several mycotoxin mitigation approaches, including surveillance, creation of awareness, introduction of mycotoxin binding agents (60), and spectral grain sorting (47). Mycotoxin surveillance and mycotoxin-related work should include all maize-producing regions of Kenya, and other similar tropical agro-climatic locations.

Acknowledgements

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Tables and Figures.

Table 1. Geo-climatic locations, rainfall patterns, and sites (mills) where cross-sectional survey maize samples were collected in three provinces of western Kenya, 2009. Data on Agroecological zones (AEZs) and expected rainfall was adapted from the Farm Management Handbook(s) of Kenya (28-30).

Province	District	Sampling Hub	Agroecological zone (AEZ)	Altitude (masl)	Rainfall Pattern [1]	Maize Growing season (Days)	Expected rainfall range (mm)	Rainfall (mm) in 2008/2009 [2]	Number of mills (*n*) [3]
Nyanza	Rachuonyo	Kosele	Semi-Humid	1,140 - 1,350	Bi-modal	135-154	350-500	578	2
Nyanza	Rachuonyo	Kosele	Sub-Humid	1,300 - 1,500	Bi-modal	135-154	700-950	624	1
Nyanza	Homa Bay	Homa Bay	Humid	1,300 - 1,500	Bi-modal	135-154	700-950	617	2
Nyanza	Homa Bay	Homa Bay	Sub-Humid	1,140 - 1,450	Bi-modal	135-154	500-800	679	1
Nyanza	Kisii	Kisii	Humid	1,800 - 2,165	Bi-modal	135-154	400-700	462	3
Western	Bungoma	Bungoma	Humid	1,350 - 1,500	Bi-modal	195-214	700-800	879	6
Western	Bungoma	Bungoma	Sub-Humid	1,350 - 1,550	Bi-modal	195-214	650-700	857	2
Rift Valley	Trans-Nzoia	Kitale	Semi-Humid	1,650 - 1,850	Uni-modal	195-214	950-1,020	852	2
Rift Valley	Trans-Nzoia	Kitale	Sub-Humid	1,700 - 1,950	Uni-modal	195-214	1,050-1,250	840	3
Rift Valley	Uasin Gishu	Eldoret	Semi-Humid	1,900 - 2,400	Uni-modal	195-214	900-1,300	827	2
Rift Valley	Uasin Gishu	Eldoret	Sub-Humid	2,300 - 2,400	Uni-modal	195-214	1,150-1,220	969	2
Total									26

[1]Two-crop seasons per year (bimodal) and one-crop season per year (unimodal).
[2]Rainfall data for the year 2008/2009 was retrieved at World Climate repository http://www.worldclim.org.
[3]Sampling sites within an AEZ in each administrative district.

Table 2. Aflatoxin prevalence across the geo-climatic locations within districts in western Kenya. Flour and grain samples were collected in 2009 from persons who brought maize for milling at local mills and analyzed for aflatoxin. Lower limit of aflatoxin quantification by direct competitive ELISA is 1 ppb (µg/kg). Kenyan aflatoxin regulatory limit is 10 ppb (µg/kg).

District	AEZ	Number of samples	Complete questionnaires	Home-grown maize [x]	Top quartile range [y]	% samples with aflatoxin (ppb) in categories [y]		
		(n_1)	(n_2)	(%)	ppb	< 1	> 1[z]	>10
Rachuonyo	Semi-Humid	71	44	45	16 - 710	7	93	65
Rachuonyo	Sub-Humid	33	19	59	2 - 51	58	42	9
Homa Bay	Sub-Humid	44	10	57	15 - 19	34	66	32
Homa Bay	Humid	69	20	56	13 - 51	29	71	29
Kisii	Humid	125	48	40	2 - 18	55	45	8
Bungoma	Sub-Humid	83	71	43	2 - 24	55	45	2
Bungoma	Humid	226	172	27	2 - 52	57	43	3
Trans-Nzoia	Semi-Humid	65	52	63	1 - 200	51	49	7
Trans-Nzoia	Sub-Humid	127	99	29	1 - 32	62	38	3
Uasin Gishu	Semi-Humid	75	53	54	0 - 79	80	20	5
Uasin Gishu	Sub-Humid	67	58	38	1 - 31	69	31	4
Total		985	646	32	2 - 710	51	49	15

[x & y] Based on number of completed questionnaires (n_2) and number of samples (n_1) from each geo-climatic zone, respectively.
[z] includes samples with aflatoxin >10 ppb (µg/kg).

Table 3. Mode of maize acquisition and percent of samples with aflatoxin levels above the regulatory limit of 10 ppb (µg/kg). Percentages were computed based on aflatoxin levels of samples that matched with well-completed questionnaires (*n*=646) in a cross-sectional survey in western Kenya, 2009.

Maize source	***n* (%)**	***n*>10 ppb (% > 10 ppb)**
Local market	396 (61)	40 (10)
Home-grown	210 (32)	10 (5)
Gifts	33 (5)	9 (27)
Food aid	7 (1)	1 (14)

Table 4. Varietal types, kernel texture and reasons for farmers' preference of the four major varieties in Bungoma district. Data are based on a pre-harvest survey that was conducted with randomly selected farmers (*n*=186) in maize growing villages (*n*=7) in a humid agroecological zone between July and August, 2009.

	Variety			
Factor	H614	H513	WH505	LOCAL 8
Varietal type	Hybrid	Hybrid	Hybrid	Open pollinated
Kernel type	Flint	Semi-Flint	Dent	Dent
Number of farmers (*n*)	93	77	28	14
Percent of farmers (%)	44	36	13	7
Percent of farmers in a category				
Seed obtained by:				
Bought	42	35	13	6
Saved	0	11	22	67
Other[1]	58	54	65	27
Reason for preference:				
Cost of seed	2	9	19	7
Seed availability	39	14	5	7
Drought tolerance	0	11	10	21
Earliness	2	0	0	21
Yield	47	32	38	14
Pest tolerance	10	7	5	7
Flour quality	0	25	24	21

[1] farmers did not disclose, missing entry, or those that did not match the questionnaire coding e.g., barter trade

Table 5. Pair-wise correlations between mycotoxins and covariates measured at harvest and during four-month storage of maize at Bungoma, Western province, Kenya. Except for ear rot and ear weight, which were only analyzed at harvest, factors were measured at harvest and twice after harvest at two-month intervals. Numbers inside the parenthesis represent the sample size. For grain moisture and ear rot v. mycotoxins, the numbers outside the parenthesis are Pearson's correlation coefficient, *r*. For weevil v. mycotoxins, the numbers outside the parenthesis are Spearman's rank correlation coefficient, Rho.

		Aflatoxin(ppb)			Fumonisin(ppb)		
	Sampling time	At Harvest	2 Months after Harvest	4 Months after Harvest	At Harvest	2 Months after Harvest	4 Months after Harvest
Grain Moisture	At Harvest	0.05 (149)	-0.10 (140)	0.14 (118)	-0.12 (54)	0.25 (50)	0.12 (50)
	2 Months after Harvest	0.04 (108)	-0.06 (113)	0.14 (91)	0.14 (38)	-0.23 (54)	0.10 (47)
	4 Months after Harvest	0.02 (98)	-0.08 (101)	-0.07 (104)	0.02 (42)	-0.09 (48)	0.14 (51)
Weevil							
	At Harvest						
	2 Months after Harvest		-0.04 (31)	-0.05 (27)		-0.12 (26)	-0.10 (23)
	4 Months after Harvest			-0.08 (29)			-0.11 (24)
Ear rot	At Harvest	-0.05 (44)	0.05 (41)	-0.16 (40)	0.09 (44)	0.14 (41)	0.18 (40)

*, Correlation significant at α=0.05

Table 6. Effect of four variables on the presence v. absence of aflatoxin and fumonisin in major maize varieties that were grown in Bungoma, Western province, Kenya in 2009. Likelihood ratio chi-square tests based on a nominal logistic regression in which mycotoxin levels were binary coded as follows: "1" for samples with toxin level above the detection limits and "0" for toxin values below the detection limits. Mycotoxin detection limits were 1 ppb (µg/kg) for aflatoxin and 0.1 ppm (mg/kg) for fumonisin.

		Aflatoxin[1]		Fumonisin[2]	
Source	DF	L-R Chi-Square	Prob>ChiSq	L-R Chi-Square	Prob>ChiSq
Sampling period	2	3.97	0.138	3.57	0.167
Variety	3	13.31	0.004	12.72	0.005
Village	6	11.47	0.075	3.02	0.806
Preservative	1	0.15[k]	0.697	3.69[y]	0.055

[1]Grain (100 gram) from farmers' stores during a 4-month storage period (Aug. 2009 – Feb 2010).
[2]Subset of samples that had been previously analyzed for aflatoxin were analyzed for fumonisin.
[k]Based on a subset of samples (*n*=119) that were included in the grain preservative experiment.
[y]Based on a subset of samples (*n*=84) that were included in the grain preservative experiment.

Table 7. Mode of maize acquisition and percent of samples with detectable aflatoxin and fumonisin at different sampling periods of the longitudinal survey of maize flour at the posho mills in Bungoma, Western Province, Kenya. Sampling involved collection of maize flour samples (100 gram) from individuals who processed maize at a major posho mill in a village where storage shed surveys were ongoing. Maize was sampled at two-month intervals, at harvest (August 2009) to four months at storage. Fumonisin was analyzed on a subset of samples that had been analyzed for aflatoxin. Lower limits of mycotoxin detection were 1 ppb (µg/kg) for aflatoxin and 0.1 ppm (mg/kg) for fumonisin.

Mycotoxin/at sampling periods:	Percentage of samples with detectable mycotoxin		Fisher's exact test. Prob.(mycotoxin category=detectable) is different based on the method of acquisition	Chi-Square test for difference in proportion of contaminated v. clean samples across methods of acquisition	
Aflatoxin	Home-grown (%)	Purchased (%)	*P*-value	χ^2(*P*-value), *n*	Df
At harvest	30	43	0.09	3.07(0.08), 192	1
2 months after harvest	54	54	1	0.01(0.91), 194	1
4 months after harvest	42	42	1	0.73 (0.70),187	1
Fumonisin					
At harvest	93	85	0.58	0.69(0.41), 41	1
2 months after harvest	76	89	0.45	1.21(0.27), 47	1
4 months after harvest	100	86	0.24	2.49(0.11), 37	1

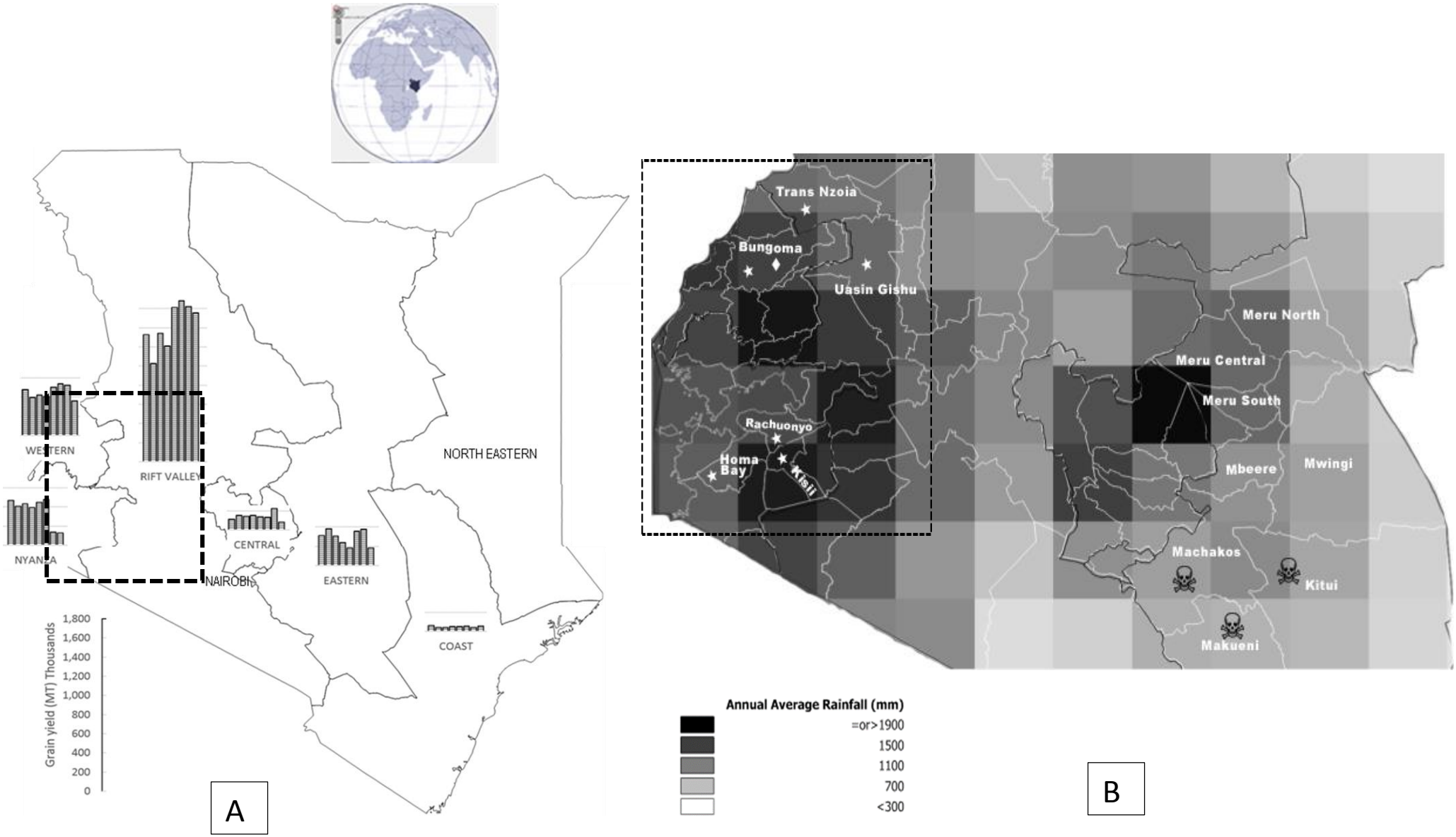

Fig. 1. Map of Kenya showing (**A**) maize grain yield (vertical bars in metric tons, MT. available at http://fsg.afre.msu.edu/gis/) from 2001 to 2008 for six administrative provinces, (**B**) districts in western Kenya, where studies were conducted in 2009-2010. The approximate location of the Kenya's grain basket region is shown by the triangle with a dotted outline in both maps. Districts where a cross-sectional survey was conducted are indicated by one (1) white star, while a white star and white diamond at Bungoma indicates the two studies (cross-sectional and longitudinal surveys) that were conducted in that district. Districts in Eastern Kenya where fatal aflatoxicosis has previously been reported, Machakos, Makueni and Kitui are shown with a hazard label. Gray-scale geographical information systems raster-assigned pixels for long-term average annual rainfall (mm), available at http://harvestchoice.org/products/data/263.

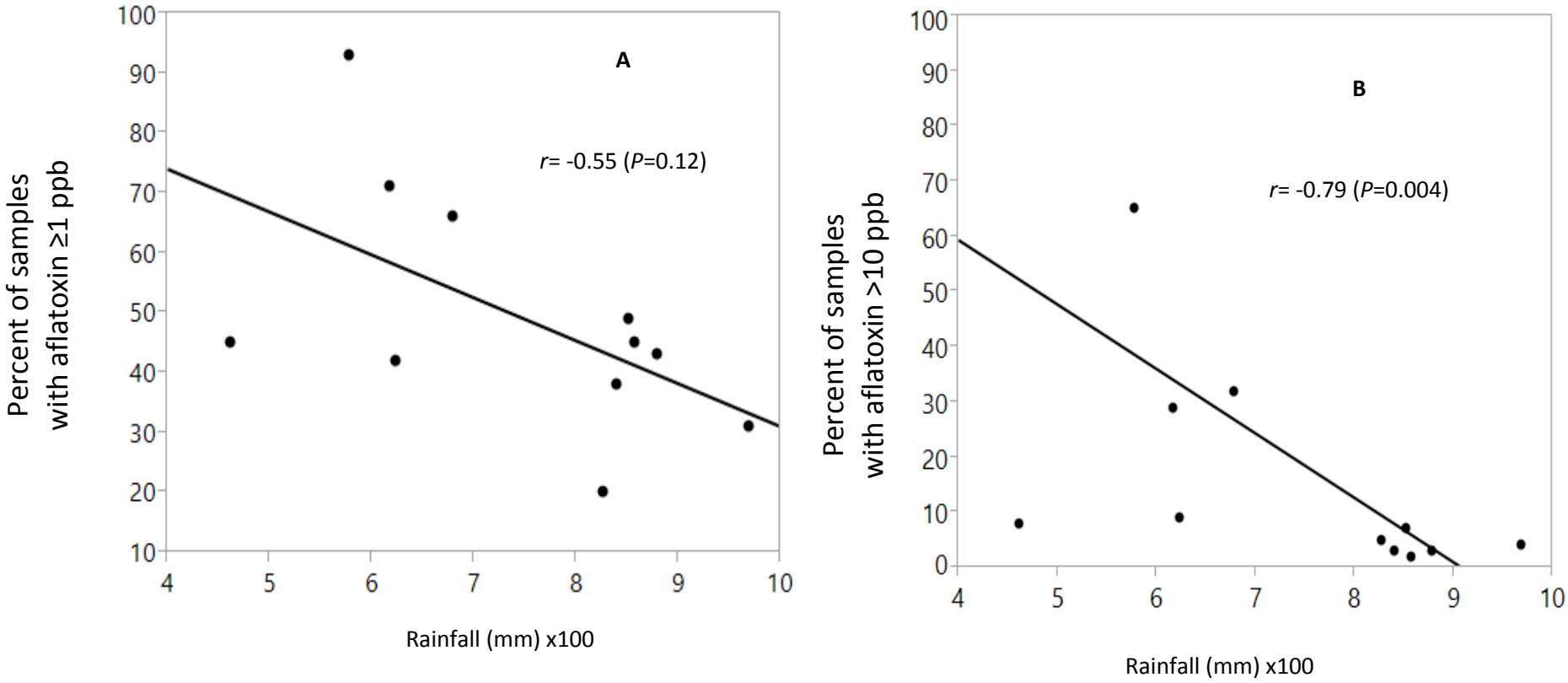

Fig. 2. Relationship between amount of rainfall (mm) and percentage of (**A**) aflatoxin-contaminated (≥1 ppb), and (**B**) above regulatory limit (10 ppb) samples. Percentage of samples in different categories of aflatoxin contamination in 2009 western Kenya survey (Tables 1&2). Rainfall estimate data was retrieved at the World Climate repository http://www.worldclim.org.

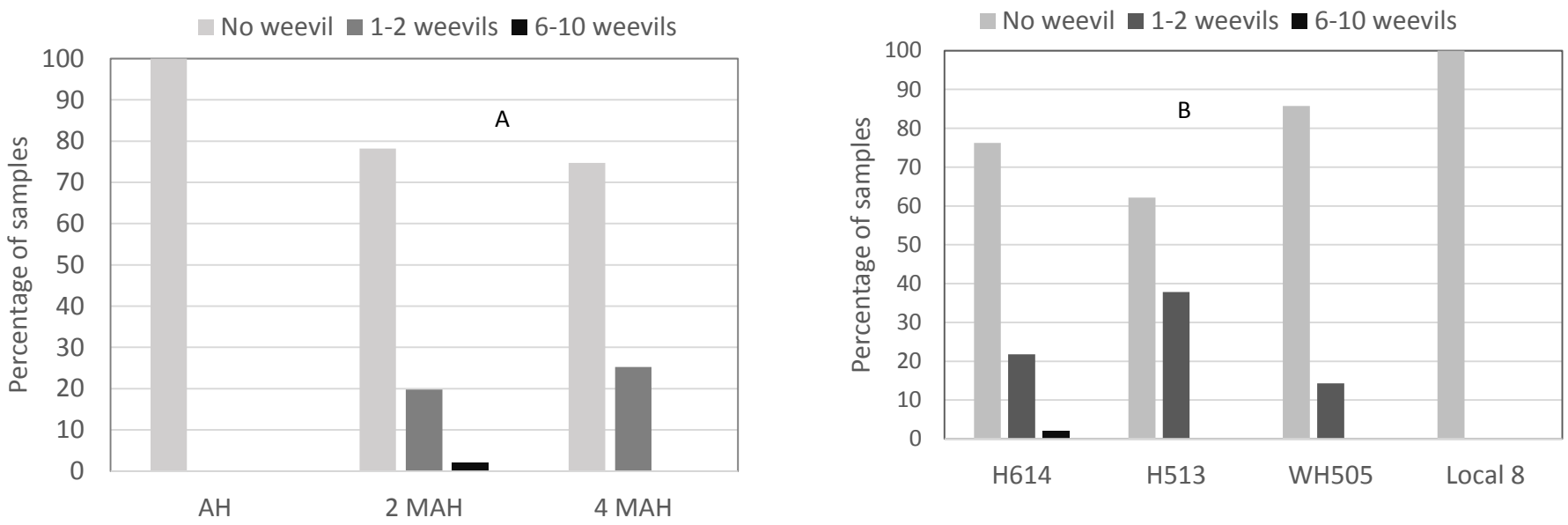

Fig. 3. Percentage of samples with different levels of weevil infestation at three sampling times (**A**): after harvest (AH), two months after harvest (2 MAH) and four months after harvest (4 MAH) in samples (*n*=196) of four popular maize varieties (**B**) in Bungoma district, Western Province, Kenya. Weevils were scored as follows: 0 = no weevils; 1 = 1-2 weevils; 2 = 3-5 weevils; 3 = 6-10 weevils; 4 = >10 weevils.

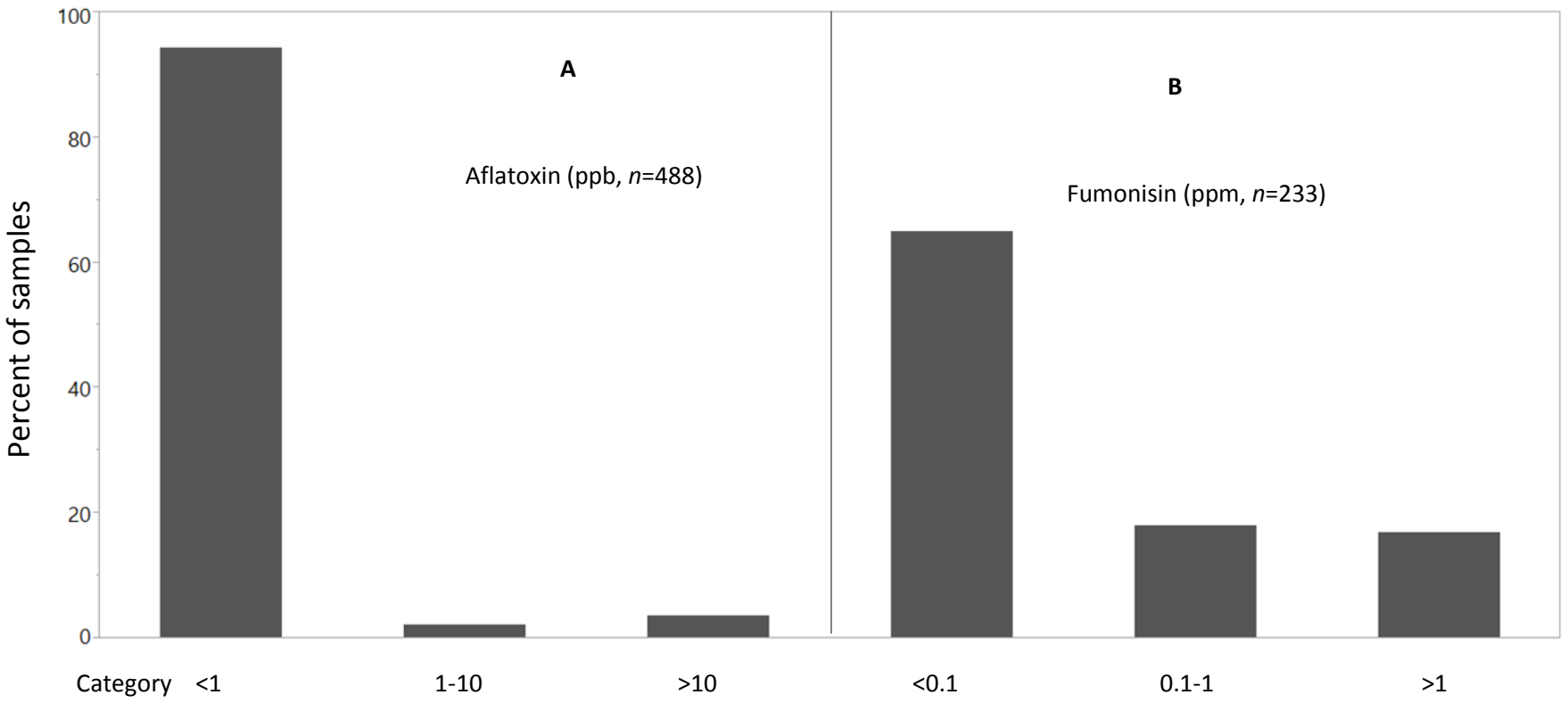

Fig. 4. Percentage of samples showing different levels of aflatoxin (**A**) and fumonisin (**B**) contamination at the storage sheds. Maize was sampled at two month intervals from harvest (August 2009) to four months at storage. Sampling involved collection of whole grain (100 gram) samples from a bulk (approximately 3 kg) that had been drawn from multiple points of up to three 90-kg bags from the store of each of the participating farmers. Sampling period was excluded because of lack of significant influence on the number of samples with detectable mycotoxin levels. Fumonisin was analyzed on a subset of samples that had been analyzed for aflatoxin. Mycotoxins were detected using ELISA kits whose limits ranged from 1-20 ppb (µg/kg), aflatoxin and 0.1-6 ppm (mg/kg), fumonisin. Kenyan regulatory limits for the mycotoxins in maize are 10 ppb for aflatoxin and 1 ppm for fumonisin.

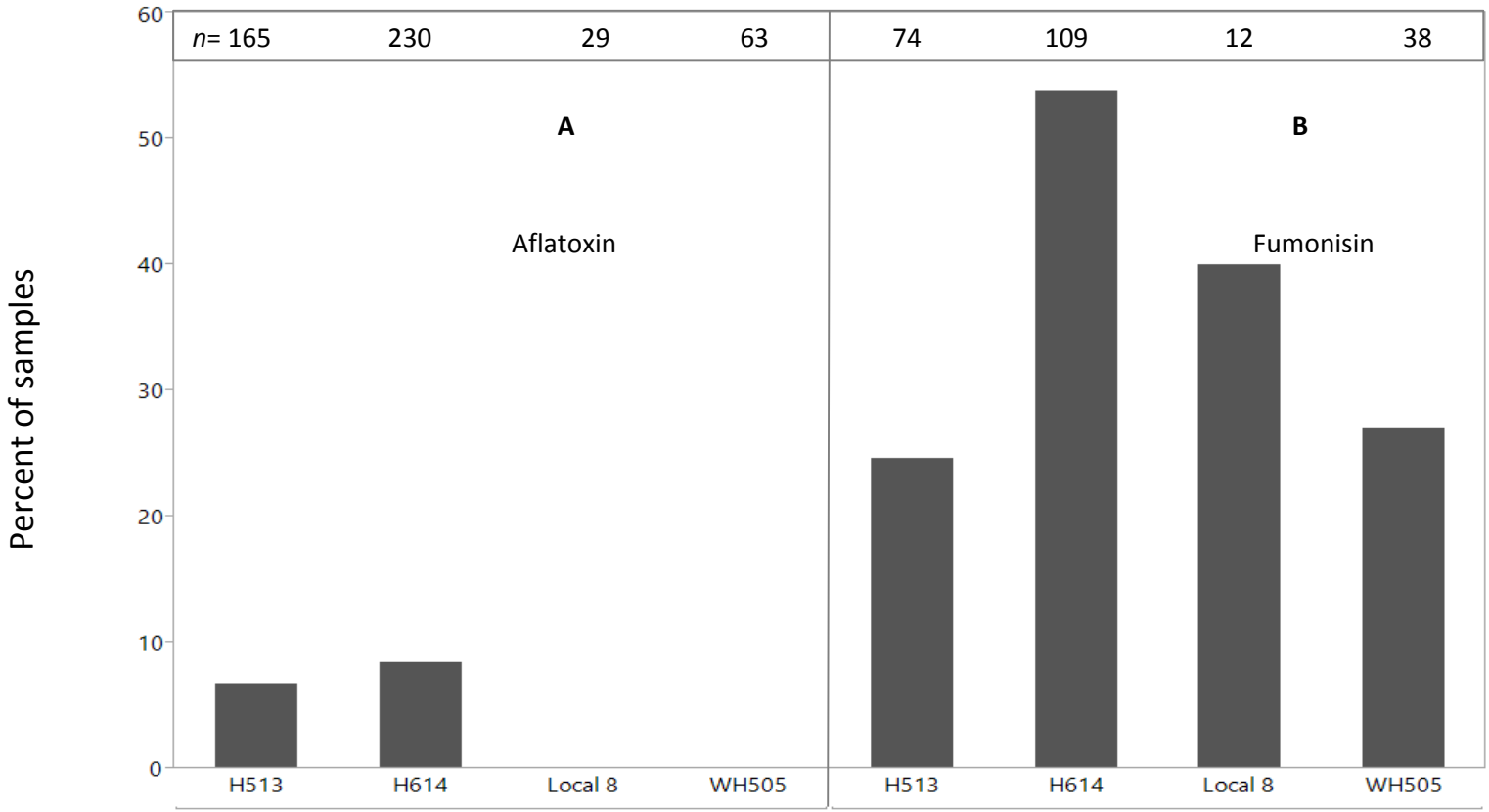

Fig. 5. Percentage of samples with aflatoxin (**A**) and fumonisin (**B**) contamination during a 4-month storage period of four major maize varieties in Bungoma, Western Province, Kenya in 2009. Maize was sampled at two month intervals from harvest (August 2009) to four months at storage. Sampling involved collection of whole grain (100 gram) samples from a bulk (approximately 3 kg) that had been drawn from multiple points of up to three 90-kg bags from the storage shed of each of the participating farmers. The varieties are shown on the x-axis. The number of samples (*n*) analyzed for each variety is shown on top of the graph. Fumonisin was analyzed on a subset of samples that had been analyzed for aflatoxin. Lower limits of mycotoxin detection were 1 ppb (µg/kg) for aflatoxin and 0.1 ppm (mg/kg) for fumonisin.

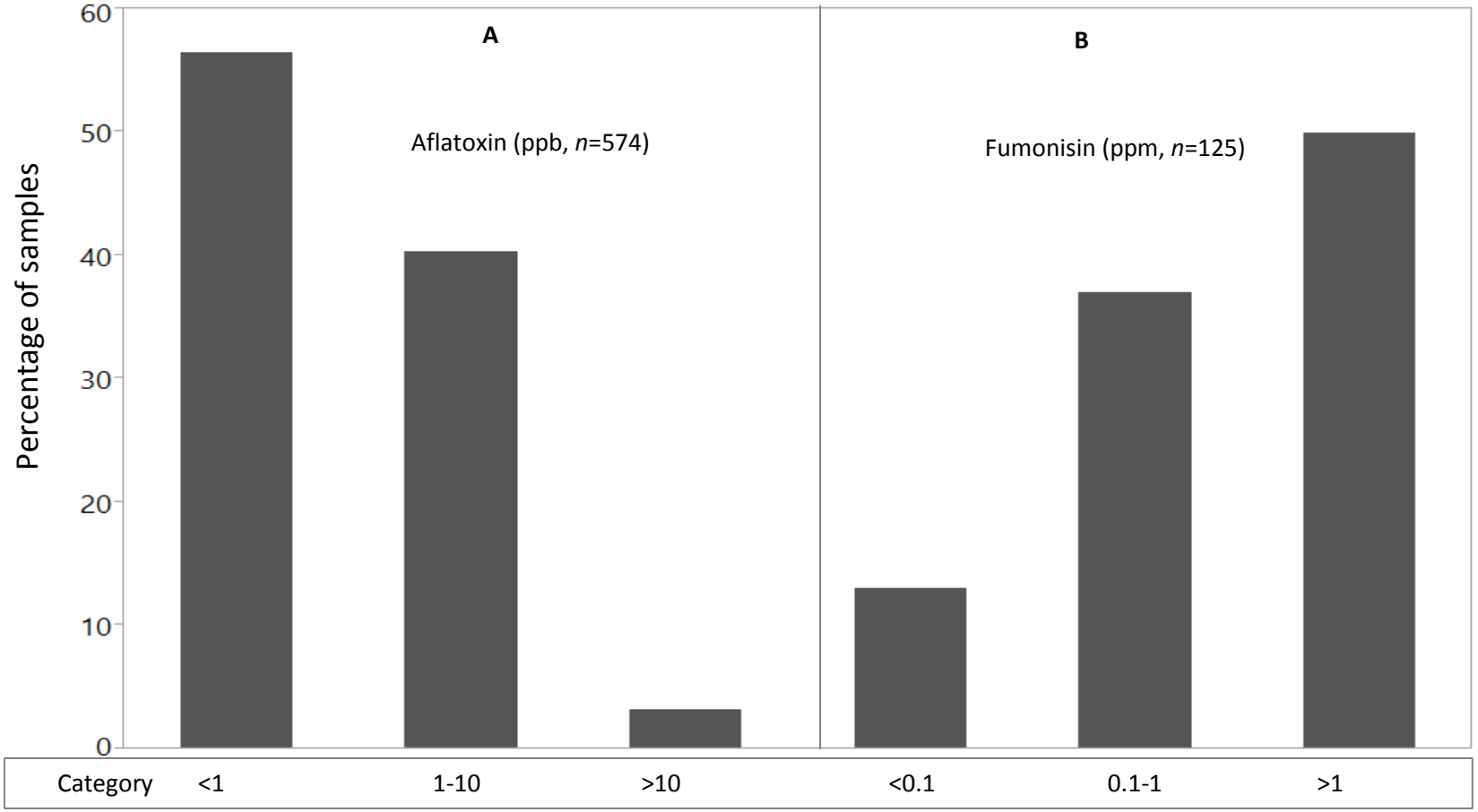

Fig. 6. Percentage of samples showing different levels of aflatoxin (**A**) and fumonisin (**B**) contamination of different categories at the mills in Bungoma, Western Province, Kenya. Sampling involved collection of maize flour samples (100 gram) from individuals who processed maize at a major posho mill in a village where storage shed survey were ongoing. Maize was sampled at two month intervals from harvest (August 2009) to four months at storage. Data included samples from all varieties; not restricted to the four major varieties. Fumonisin was analyzed on a subset of samples that had been analyzed for aflatoxin. Mycotoxins were detected using ELISA kits whose limits ranged from 1-20 ppb (µg/kg), aflatoxin and 0.1-6 ppm (mg/kg), fumonisin. Kenyan regulatory limits for the mycotoxins in maize are 10 ppb for aflatoxin and 1 ppm for fumonisin.

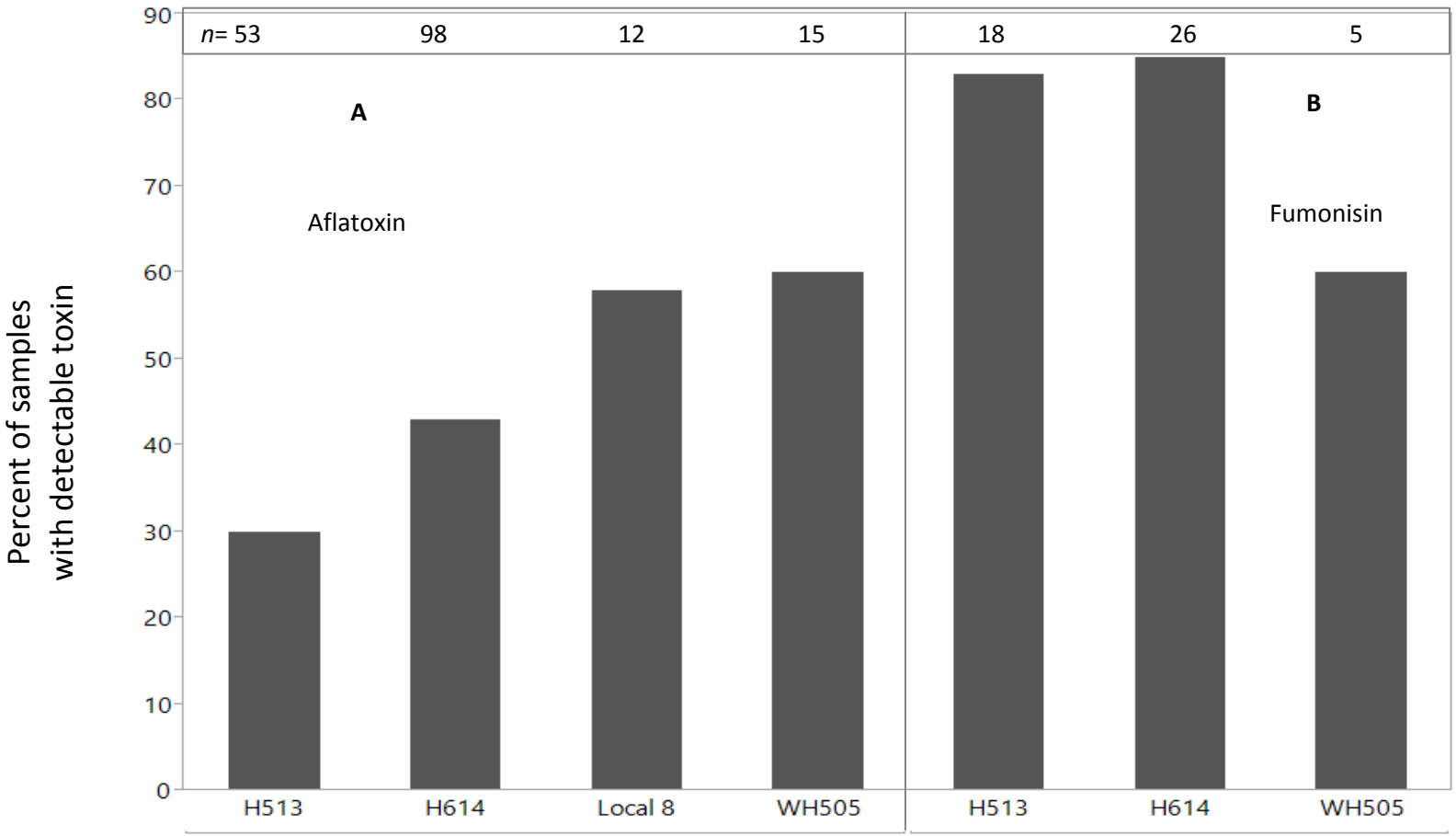

Fig. 7. Percentage of samples of four major maize varieties with aflatoxin (**A**) and fumonisin (**B**) contamination at the mill in Bungoma, Western Province, Kenya in 2009. Sampling involved collection of maize flour samples (100 gram) from individuals who processed maize at a major posho mill in a village where storage shed survey were ongoing. Maize was sampled at two month intervals from harvest (August 2009) to four months at storage. Data included was for samples of the four major maize varieties. The number of samples (*n*) of each variety that were analyzed is shown on top of the graph. Sampling period was excluded because of lack of significant influence on the number of samples with detectable mycotoxin levels. Fumonisin was analyzed on a subset of samples that had been analyzed for aflatoxin. Samples of Local 8 from the mill were not analyzed for fumonisin. Lower limits of mycotoxin detection were 1 ppb (µg/kg) for aflatoxin and 0.1 ppm (mg/kg) for fumonisin.

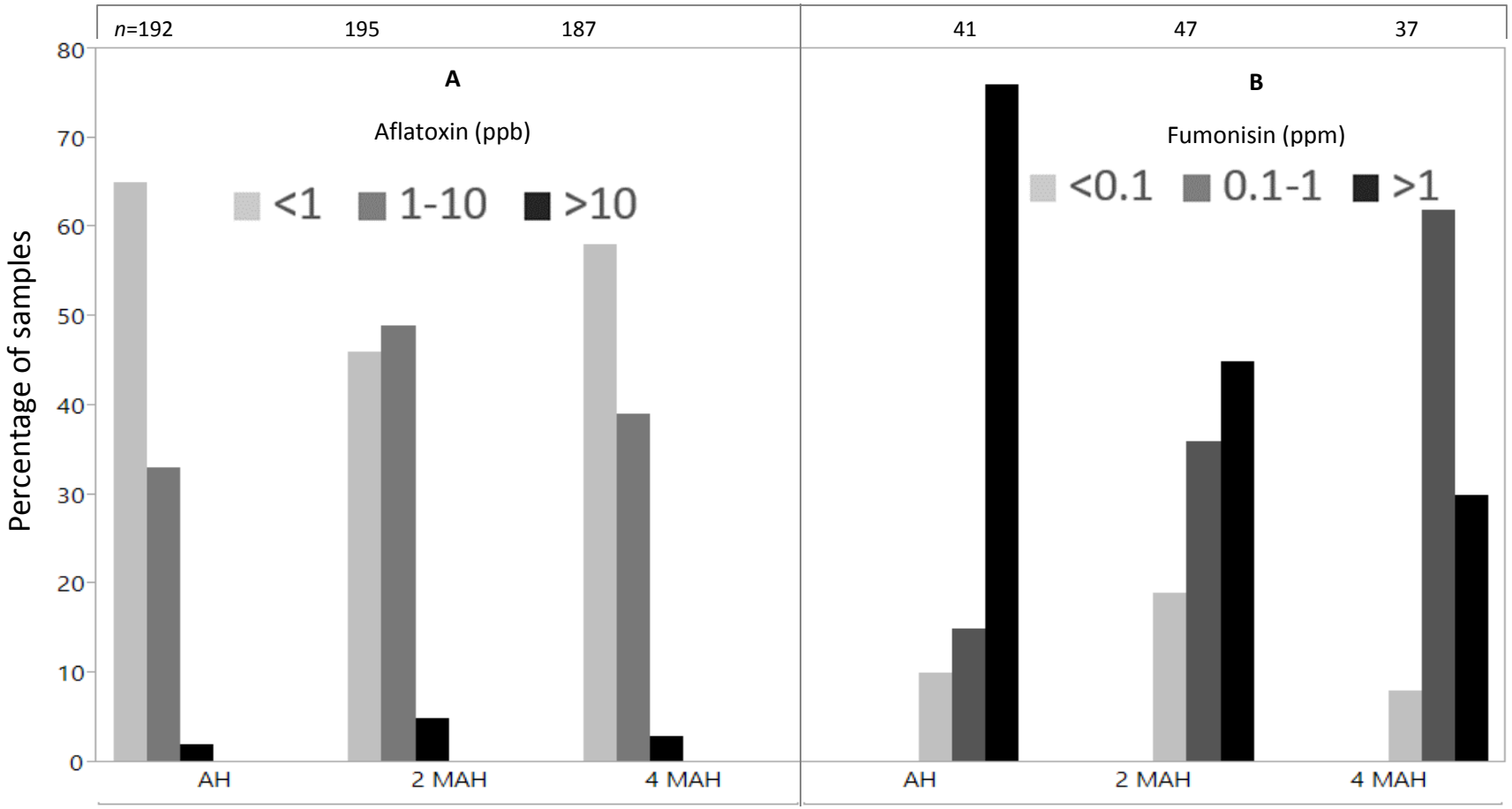

Fig. 8. Percentage of samples from the mills showing different levels of aflatoxin (**A**) and fumonisin (**B**) contamination at three different sampling times: at harvest (AH), two months after harvest (2 MAH), and four months after harvest (4 MAH) between Aug. 2009 and Feb. 2010. Sampling involved collection of maize flour sample (100 gram) from individuals who processed maize at mills in seven villages within Bungoma district, Western Province, Kenya. Data included samples from all varieties; not restricted to the four major varieties. The number of samples (*n*) analyzed at each sampling period is shown on the top of graph. Fumonisin was analyzed on a subset of samples that had been analyzed for aflatoxin. Lower limits of mycotoxin detection were 1 ppb (µg/kg) for aflatoxin and 0.1 ppm (mg/kg) for fumonisin.

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CHAPTER 3

EXTENT AND DRIVERS OF MYCOTOXIN CONTAMINATION: INFERENCES FROM A SURVEY OF KENYAN MAIZE MILLS[2]

Abstract

The prevalence of aflatoxin and fumonisin was investigated in maize intended for immediate human consumption in eastern Kenya at a time in 2010 when an aflatoxin outbreak was recognized. Samples were collected from people who brought their maize for processing at local commercial mills. Sites were selected using a geographical information system overlay of agroecological zones and Kenya's administrative districts. Interviews and collection of maize flour samples was conducted from 1,500 people who processed maize at 143 mills in 10 administrative districts. Mycotoxins were analyzed using enzyme-linked immunosorbent assays for aflatoxin and fumonisin, leading to detection at levels above the respective maximum tolerable limits in 39% and 37% of the samples, respectively. Samples with aflatoxin contamination above the regulatory limit ranged between 22 and 60% across the districts. A higher occurrence of aflatoxin was associated with smaller maize farms, lower grain yield and monocropping system, while a larger magnitude of the toxin was observed in the sub-humid agroecological zone, in samples with more broken kernels, and curiously, less maize ear damage at harvest. Analysis of paired grain samples (visually sorted and unsorted) showed that sorting reduced fumonisin by 65%, from above to below the regulatory limit of 1 ppm. Sorting did not, however, reduce aflatoxin levels. While the aflatoxin problem is widely acknowledged, the high prevalence of fumonisin has not previously been reported. There is need for surveillance of the two mycotoxins and establishment of intervention strategies to reach vulnerable small-scale farmers.

[2] Mutiga, S. K., Were, V., Hoffmann, V., Harvey, J. W., Milgroom, M. G., and Nelson, R. J. in press. Extent and drivers of mycotoxin contamination: Inferences from a survey of Kenyan maize mills. Phytopathology

Introduction

Maize (*Zea mays* L.) is the staple food of millions of people in sub-Saharan Africa, including 96% of the Kenyan population (9,16). There has been increasing concern that African populations dependent on maize are at high risk of exposure to toxic fungal metabolites called mycotoxins (50,41). Mycotoxigenic fungi are endemic in tropical and sub-tropical environments, and maize is an excellent substrate for the aflatoxin-producing fungal species, *Aspergillus flavus* and *A. parasiticus*, and for the fumonisin-producing *Fusarium verticillioides*. Aflatoxin B1 (AFB1) and fumonisin B1 (FB1) are carcinogens (34,41). Acute exposure to AFB1 is lethal, while chronic exposure can cause immunosuppression, liver cancer, impairment of nutrient absorption, and fetal and infant growth retardation (31). Chronic exposure to FB1 has been associated with esophageal cancer, growth retardation and immunosuppression (51). Kenya has been shown to have a high prevalence of maize contamination with aflatoxin and fatal aflatoxicosis is frequently reported in the country (36).

To reduce human exposure to mycotoxins, the United Nations' World Food Program (WFP) and Kenya Bureau of Standards (KEBS) have stipulated maximum tolerable limits (MTL) for aflatoxin in human food at 10 parts per billion (ppb or mg/kg), and that of fumonisin at 1,000 ppb (or 1 part per million, ppm) (45,13). In practice, such standards are rarely operationalized in the food systems of developing countries, such that 25% of the world's foodstuffs are suspected of being contaminated with mycotoxins (10). Surveillance of mycotoxins and other threats to food safety is difficult to implement in the context of Africa's semi-subsistence agriculture. Small-scale farmers produce 75% of Kenya's maize, using it for both home consumption and for trade (37,48). The majority of the grain that is traded goes through informal markets consisting of small-scale traders and a few wholesalers who move the grain within the country (37). The maize value chain lacks a well-established mechanism to systematically test grain quality or safety. Most maize consumers practice visual inspection and sorting of grain prior to milling, as the only quality check approach.

Hell and Mutegi (19) reviewed the pre- and post-harvest maize management practices that influence mycotoxin accumulation in sub-Saharan Africa. Pre-harvest aflatoxin contamination has been associated with factors that overcome physical barriers to colonization (e.g. damage by birds and insects), that stress the plant (e.g. pest pressure, climatic and soil-related stress factors), and/or that cause spatiotemporal coincidence of a vulnerable host and the mycotoxigenic fungi (e.g. silking and harvesting time) (43,49,17,21,46,47). The extent of post-harvest toxin accumulation is influenced by the amount of inoculum and successful colonization at pre- and post-harvest infection, timing of harvest, grain moisture (initial drying; protection and ventilation during storage), and kernel integrity (due to grain handling and pest damage) (20,1,29,28,12,24). Among the many recognized drivers of aflatoxin accumulation, pre-harvest drought and post-harvest moisture are considered the most critical.

Eastern Kenya is a drought-prone region that is recognized as an aflatoxin hotspot (36,3). Acute aflatoxicosis has been observed mainly after periods of moisture stress during maize crop development, when rainfall occurs after the crop has attained physiological maturity and before it is harvested, and when a bumper crop is stored inadequately (35,36). Although multiple factors could have influenced the observed aflatoxin contamination and aflatoxicosis, previous reports emphasized improper post-harvest grain handling as causal factors (36,3,30,13). Between 2004 and 2011, 477 cases of acute aflatoxicosis were reported in eastern Kenya, with 40% resulting in death (13). These tragic incidents are correlated with the area's favorable environmental conditions for *A. flavus* growth and mycotoxin production, as well as presence of a highly toxigenic *A flavus* population (39). An aflatoxin outbreak occurred in 2010, a season of abundant rainfall and bounteous grain yield in

eastern Kenya, and was recognized in the national and world news media (BBC, 2010). To protect the population, the government of Kenya prohibited the consumption or trade of approximately 200,000 tons of maize grain (BBC, 2010) and offered to buy the contaminated grain at a reduced price. The 2010 aflatoxin outbreak was the context for the present study, which was part of a larger effort to assess the extent and drivers of mycotoxin contamination across diverse contexts.

For the majority of maize consumed in Kenya, the main point of value addition is the village-based hammer mill, known locally and hereafter as the *posho* mill (15,27). Maize processed at these local mills may be from farmers' own stores, be purchased from grain dealers, be received as gifts from friends or relatives, or be received as food aid. We focused our sampling on these micro-scale commercial mills because they process approximately 60% of the maize consumed in Kenya and are convenient locations for collecting maize intended for immediate human consumption (26). We reasoned that posho mills represent suitable sites for mycotoxin surveillance and intervention. To test this idea, we investigated whether information gathered from posho mill customers would provide relevant insights related to mycotoxin contamination of maize. The issues of relevance included the extent of self-provisioning of maize, the drivers of contamination, and the effectiveness of pre-consumption grain sorting.

Materials and Methods

Study sites and sampling. Study sites were selected based on an overlay of administrative boundaries and agroecological zones (AEZs) for eastern Kenya using publicly available datasets that were retrieved by using ArcGIS® release 9.3 (ESRI, Redlands, CA) and Manifold System® ver. 8 (Manifold Software Limited, Wanchai, Hong Kong) geographical information systems (GIS) software. Ten study districts were randomly selected and their headquarters identified as the study hubs (Fig. 1). Maize-growing villages were identified with guidance from the local agricultural officers at their district headquarters. A subset of maize-growing villages was randomly selected across the AEZs. In each village, local commercial maize mills were randomly selected for sampling. The key descriptors of the AEZs, indicated in Table 1, are soil moisture availability index (%) and annual rainfall range (22). The 10 administrative districts, AEZs, number of posho mills and sample size are shown in Table 1. Machakos and Kitui districts in lower eastern Kenya, where high case-fatality rates of aflatoxicosis had been previously reported, were included.

Research assistants were recruited and centrally trained to conduct sampling and interviews. They then collected samples from people who came to process their maize at the posho mills. A survey instrument, which was exempted under study protocol number 100800162 of Cornell University Institutional Review Board (IRB) for human participants in research, was used to collect information from the people who provided their maize samples. The survey covered how maize grain was acquired; the maize variety; intended use of flour; awareness about health problems associated with consumption of moldy grain; and pre- and post-harvest maize cultivation and handling practices. Samples were collected from 10-30 customers at each of 143 posho mills. Maize flour samples, each weighing approximately 35 grams, were collected into 50 mL Falcon tubes (BD, Franklin Lakes, NJ). To reduce moisture content in the samples and thus avoid further fungal growth, silica gel packs (Control Company, Houston, TX), were added to the sample tubes, which were then capped and held at ambient temperature for a maximum of 72 hours before they were transported to BecA-ILRI Hub, Nairobi, where they were stored at 4°C until analysis.

Sample preparation and mycotoxin analysis. Aflatoxin was extracted from 5-gram sub-samples of maize flour using 70% methanol and analyzed with a commercially available enzyme-linked immunosorbent assay (ELISA) kit following manufacturer's protocol (Helica Biosystems Inc., Fullerton, CA). The solid-phase direct competitive aflatoxin ELISA kit (Helica Cat. No. 941BAFL01-96) consisted of a polystyrene 96-well micro-plate coated with an antibody that was optimized to cross-react with the four aflatoxin types at the following rates: B1 (100%), B2 (77%), G1 (64%) and G2 (25%). The lower and upper limits of quantification of the kit were 1 and 20 ppb, respectively, based on information provided by the supplier; samples with toxin values below the limit of quantification were considered as containing no detectable toxin.

Fumonisin was extracted from 10-gram sub-samples of maize flour using 90% methanol and analyzed with a commercially available ELISA kit following manufacturer's protocol (Helica Biosystems, Inc., Fullerton, CA). The solid-phase direct competitive fumonisin ELISA kit (Helica Cat. No. 951FUM01C-96) consisted of a 96-well micro-plate coated with an antibody that was optimized to cross-react with three fumonisin subtypes (B1, B2, and B3). The lower and upper limits of quantification of the kit were 100 and 6,000 ppb, respectively, based on information provided by the supplier.

Optical densities of the reactions for the two mycotoxins were quantified using a micro-plate reader (BioTek Instruments, Inc., Winooski, VT) with an absorbance filter of 450 nm. Test values were interpreted with reference to standards that were included in each experiment. Samples with estimated aflatoxin concentration higher than 20 ppb were diluted with the extraction solvent and re-assessed. A sub-set of samples (628 out of 1,500), previously analyzed for aflatoxin, were selected for fumonisin analysis based on the following criteria: all samples (n=333) with aflatoxin greater than 20 ppb (the Kenyan aflatoxin regulatory limit in food at the time of the study; the regulatory limit was recently revised to 10 ppb) were included, while the remainder was selected to equally represent the aflatoxin bins 0–5, 5–10, 10-15 and 15–20 ppb. For each aflatoxin bin, about 70 samples were chosen to represent equal proportions of samples collected from each AEZ. Samples with estimated fumonisin above the upper limit of quantification of the kit were not re-assessed, but were reported as "above 6,000 ppb".

Aflatoxin ELISA validation. The aflatoxin ELISA method was validated by re-analyzing 35 samples using ELISA and the Vicam AflaTest® (Watertown, MA, USA), a flourometric – immunocapture assay. As the two methods have different protocols, a harmonized extraction protocol was established as follows: NaCl was added to maize flour sample in a ratio of 1:10 per the AflaTest protocol, and 80% methanol was used as the extraction solvent with a ratio of maize flour to extraction solvent of 1:5 (w/v) (per Helica Biosystems's ELISA protocol). The slurry was mixed in an incubator shaker at 225 rpm for 4 minutes at 25°C, and the mixture was allowed to settle for 15 minutes prior to filtering through a Whatman filter paper #1. From the above extract, a 100-µL aliquot was directly analyzed per ELISA protocol, while 2 mL was diluted in distilled water (1:4) and analyzed using Vicam AflaTest® method (Watertown, MA, USA).

Scoring for maize kernel type, quality and grain sorting. To investigate the effect of grain sorting based on visual quality, we scored all grain before and after sorting at a mill, prior to sample collection. We developed a grain score scale and a corresponding pictorial guide. The grain characteristics scored were kernel texture or type e.g., flint or dent (5,6); percent of broken kernels in a sample (kernel breakage); and moldiness, discoloration and rottenness (moldiness). The kernel texture score scale was: 1=flint, 2=semi-flint, 3=semi-dent, 4=dent and 5=mixed. The scale for kernel breakage was: 1=<1%; 2=1 to 10%; 3= 10 to 50%; 4=50 to 90%; 5=>90% broken kernels.

The scale for moldiness was: 1=less than 1%; 2=1 to 10%; 3= 10 to 50%; 4=50 to 90%; 5=>90% of grain moldy/discolored or rotten.

To investigate whether sorting maize grain before milling reduced aflatoxin and fumonisin, we collected an additional 62 pairs of unsorted and sorted maize grain in the year 2012 at five posho mills in Machakos district. These samples were scored for kernel breakage and moldiness prior to milling using a Romer Mill (Union, MO) and conducting mycotoxin analysis.

Statistical analysis. For establishment of aflatoxin predictors, a two-part analysis was adopted. First, aflatoxin values below 1 ppb were coded 0; while those above 1 ppb were coded 1 and these scores were used in a generalized linear mixed model "PROC GLIMMIX" with a LOGIT function for prediction of presence or absence of aflatoxin in SAS (SAS Institute Inc., Cary, NC). Second, aflatoxin values above 1 ppb were logarithmically transformed and the data were used in a mixed model for accumulation of aflatoxin in "PROC MIXED" in SAS. In the two models, "Mill", "Village" and "District" were considered as random effects, as the chosen mills were a sample from a larger population of mills within villages and districts. All other factors were considered fixed in the models. As the questionnaire had many variables, fixed factors were entered and removed sequentially until the model with statistically significant factors was identified. For the "PROC MIXED" model, least square means (LSM) of the statistically significant factors were separated by Tukey's HSD method, and back-transformed to geometric means of the aflatoxin levels. For a subset of samples, we used a paired *t*-test and correlation analysis to compare the aflatoxin and fumonisin levels, kernel breakage and moldiness before and after grain sorting. Spearman's correlation tests were done in pairwise comparisons of highly skewed data and/or data that did not meet the criteria of Pearson's correlation test.

Results

In this study, milled maize samples (*n*=1,500) were collected from posho mills (*n*=143) in 2010. For each maize sample, a corresponding questionnaire was completed, providing data on biophysical and socio-economic context. Among the 1,500 samples collected, 867 (57%) of the samples were said to have been produced by the household that would consume the grain ("home-grown"). Majority of the home-grown maize (n=728, 84%) had been cultivated on farms of up to 1 ha. This paper reports mycotoxin prevalence on maize samples (aflatoxin, *n*=1500; fumonisin, *n*=628), and the associations between aflatoxin and farming conditions and practices for data from home-grown maize. Each sample was analyzed for aflatoxin content and a subset of samples was analyzed for fumonisin content. The questionnaire factors that were not found to be statistically associated or did not improve the model for aflatoxin presence or level were dropped. Supplemental samples of whole grain (*n*=62) were collected (before and after sorting) at five mills in 2012 and were used to evaluate the effectiveness of grain sorting for reducing mycotoxin levels. The validity of the commercial ELISA kit for aflatoxin quantification was confirmed by comparison of results with an independent assay procedure based on flourometric-immunocapture. Pearson correlation analysis (using a harmonized extraction protocol) of the ELISA assay with the Vicam AflaTest showed a highly significant correlation (r=0.96, P<0.0001) between the two methods, for samples with aflatoxin levels between 0 and 1,500 ppb.

Prevalence and relationship of occurrences of aflatoxin and fumonisin. All districts and AEZs had samples with aflatoxin and/or fumonisin levels above the limit allowed in human food (Table 2, Fig. 2). The percentage of samples with detectable aflatoxin ranged from 46% (Kathiani) to 85%

(Mwala) (Table 2). The proportion of samples over 10 ppb ranged from 22 to 60% across districts. Districts with previously reported cases of aflatoxicosis fatalities such as Machakos, Mwala and Kitui had lower aflatoxin ranges than those found in the three Meru districts (Table 2). For fumonisin, the proportion of detectably contaminated samples ranged from 69% (Mwala) to 87% (Meru North), and the proportion of samples exceeding the regulatory limit of 1,000 ppb ranged from 22 to 59% across districts. Interestingly, Mwala had the highest proportion of aflatoxin-contaminated samples (85%) and the lowest proportion of samples exceeding 1,000 ppb of fumonisin-contamination (22%) (Table 2).

The levels of mycotoxin contamination varied across AEZs (Fig. 2). The two mycotoxins showed contrasting distributions. Levels of aflatoxin contamination over the regulatory limit showed a bimodal pattern relative to moisture level; the highest were observed in the sub-humid and semi-arid zones. In contrast, fumonisin levels showed a unimodal pattern relative to moisture levels, with the highest levels observed in the semi-humid zones, which had the lowest level of aflatoxin contamination above the regulatory limit (Fig. 2). A low but significant positive correlation was detected between aflatoxin and fumonisin occurrences (ρ=0.15, P<0.001). A test of independence conducted using paired levels of aflatoxin and fumonisin (coded as undetectable; between detectable and regulatory limit; and over the regulatory limit) returned a significant chi-square value (χ^2=18.87, df=4, P<0.001), indicating a significant interaction of the occurrence of the two mycotoxins (Fig. 3).

Factors associated with the presence and quantity of aflatoxin in maize. Aflatoxin data were not normally distributed; out of 1,500 samples, one third (n= 499) did not have detectable aflatoxin. A two-part analysis of factors for contamination was conducted, first for the binary presence/absence, and the other for continuous data (quantitative, detectable aflatoxin levels). Factors that were significantly associated with aflatoxin presence included grain yield, cropping system and size of maize farm (Table 3). Maize obtained from people who intercropped their maize had significantly lower likelihood of aflatoxin contamination compared to those who practiced monocropping (odds ratio=0.65, P=0.048) (Table 3). Having more land cultivated with maize was associated with reduced likelihood of aflatoxin contamination. A one-hectare increase in land size cultivated with maize was associated with a 6% decrease in probability of contamination (Table 3). Farmers who had higher grain yields had lower probabilities of aflatoxin contamination. A one ton/ha increase in grain yield was associated with a 15% reduction in probability of contamination (Table 3). Intercropping was not dependent on land size or grain yield. The 24% of the farmers who grew maize in a monocrop had less land cultivated with maize and higher grain yield (1.3 ha; 1.9 t/ha) compared to the 67% who practiced intercropping (1.8 ha; 1.6 t/ha). Although we hypothesized that agroecological zones would influence aflatoxin presence, the association was only marginal at a significance level of α=0.1 for the joint effect of the five AEZ indicators. The odds ratio for aflatoxin presence differed significantly (P=0.013) between sub-humid and semi-humid zones.

Major factors influencing the amount of aflatoxin in maize were predicted in a mixed model whose power was 23% (r^2=0.23; N=520). The estimated mean aflatoxin (in logarithms and geometric levels), as well as confidence intervals by category for the factors found to be significant predictors of contamination are shown in Table 4. Random variables, "District", "Village [District]" and "Mill [Village, District])" accounted for 11% of random variation. The key factors that were significantly associated with the observed aflatoxin levels included agro-ecological zones, kernel breakage, and percent of damaged maize ears at harvest (Table 4). We observed a significant association between aflatoxin level and AEZ (P<0.05). The highest geometric mean (GM) aflatoxin (44 ppb) was observed in the sub-humid zone, which differed significantly (P<0.05) from the rest of

the zones. The drought-prone semi-arid zone had the second highest aflatoxin contamination (GM = 24 ppb), which did not differ significantly from the rest of the AEZs. All agroecological zones had GM aflatoxin above the regulatory limit of 10 ppb (Table 4).

Aflatoxin level was significantly associated with kernel breakage ($P<0.05$). The majority of the samples had <1% broken kernels (n=-319, or 71%). This category had significantly lower aflatoxin levels (GM=18 ppb) than the two higher breakage categories (1-10% broken kernels, n=121, or 26%, and 10-50% broken kernels, n=12 or 3%). The samples scored as having 1-10% broken kernels were not significantly more contaminated than those scored as having 10-50% broken kernels (GM= 27 and 30 ppb, respectively for the two sets). No samples were found to have more than 50% of broken kernels. Samples from people who reported fewer damaged maize ears at harvest had the highest aflatoxin (GM=43 ppb), while samples from those who reported the highest maize ear damage had least aflatoxin (GM=14 ppb). Aflatoxin levels in samples obtained from consumers who reported fewer damaged maize ears at harvest differed significantly ($P<0.05$) from the rest of the ear damage categories. Geometric mean aflatoxin levels for all categories of kernel breakage and ear damage were above 10 ppb (Table 4).

Grain visual assessment and sorting for mycotoxin removal. The visual scores of grain moldiness before and after sorting were highly correlated ($\rho=0.92$, $P<0.0001$), as was the correlation between scores of kernel breakage before and after grain sorting $\rho=0.94$ ($P<0.0001$) (Table 5). Samples with most broken kernels were not the moldiest, as was evidenced by a marginally significant correlation between scores of kernel breakage and moldiness (Table 5). Grain sorting reduced the percent of broken kernels $t= -3.35$ ($df=810$, $P<0.001$), but did not significantly reduce the percent of moldy grains $t= - 1.02$, ($df=810$, $P=0.308$) (Table 6). Sorting did not cause a significant shift in kernel breakage score. Grain sorting caused a general change in the visual appearance of the grain, decreasing the proportion of moldy/broken samples (Fig. 4). Kernel texture was scored in all whole-grain maize samples prior to sampling of the corresponding flour, but no significant association was observed between this maize trait and aflatoxin.

Neither moldiness nor kernel breakage were correlated with aflatoxin (Table 5). A pre-sorting score of moldiness was correlated with pre-sorting fumonisin contamination $\rho=0.42$ ($P<0.001$). A pre-sorting score of kernel breakage was only marginally associated with pre-sorting fumonisin level ($\rho=0.17$, $P<0.05$) (Table 5). Grain sorting significantly reduced fumonisin ($t = -3.4$, $df=50$, $P=0.001$), but not aflatoxin levels $t = -0.07$ ($df=50$, $P=0.943$) (Table 6). Grain sorting caused a 65% (900 ppb) reduction in fumonisin levels, from above to below the regulatory limit of 1000 ppb, and significantly reduced samples above the limit from 48 to 22% (Table 6; Fig. 5).

Discussion

This study used a novel approach to explore the extent of an aflatoxin outbreak in eastern Kenya and to assess its drivers during the course of a recognized outbreak by assessing aflatoxin contamination at local mills. We also provide evidence for widespread fumonisin contamination in the same maize samples. This region's frequent outbreaks of aflatoxicosis have caught the attention of policy makers, researchers and other stakeholders, but the fumonisin problem has not been recognized (16, 28). The most thorough previous analysis of aflatoxin contamination in eastern Kenya was conducted by Daniel et al. (13), who collected between 165-300 samples per year from households in two districts (Makueni and Kitui) over a three-year period (Table 7). High levels of contamination were found in all years, with the proportion of contaminated samples higher when a

recognized outbreak was in progress. Sampling from local hammer mills in the present study allowed us to extend the spatial and temporal coverage of the aflatoxin analysis. We analyzed 1,500 maize samples from 10 districts for aflatoxin levels over a two-month period, and determined fumonisin levels for a subset of *n*=628. Our findings confirm and extend the picture of aflatoxin contamination, with some of the new areas sampled showing the highest levels of aflatoxin contamination (Table 7). *In-vitro* studies with model organisms have shown that a combined ingestion of aflatoxin and fumonisin can increase chances of liver cancer (16). If the results are applicable to humans, the observed widespread fumonisin contamination means that the maize-consuming population of E. Kenya faces more of a health threat than originally thought. Taken together, these findings confirm the need for surveillance and management of mycotoxins in maize in this region.

Mycotoxin surveillance and management strategies should target the most relevant points in the maize value chain. Given the high costs of aflatoxin analysis and the skewed distribution of contamination, sampling is inherently difficult for aflatoxin (32,19). Surveillance and management is particularly problematic in the African context, where the majority of smallholder farmers consume maize produced on their own farms. In this survey, 57% of the samples collected at local mills had been grown by the household that would consume the grain, and the majority of the samples (84%) had been cultivated on farms of less than 1 ha. A high proportion of self-provisioned maize was previously reported for eastern Kenya (59% in 2006 and 100% in 2007) (13). In the semi-subsistence maize system typical of east Africa, the local hammer mill is the only site of value addition in the system, and it has been reported that approximately 60% of Kenyan maize is processed at these mills, with the remaining maize processed at larger mills or consumed in whole-grain preparations (26). Our personal observations suggest that home-grown maize is mainly processed at posho mills, with very little home-based milling. Posho mills can thus permit surveillence and application of mycotoxin strategies in maize destined for immediate human consumption.

Our study reveals alarmingly high proportions of samples above the regulatory limit for the two toxins across eastern Kenya. We provide data on the prevalence and inferences regarding the drivers of aflatoxin contamination. The study design, involving collection of samples and survey responses at posho mills, presents obvious weaknesses with regard to analysis of drivers. First, the design could not differentiate between pre- and post-harvest aflatoxin drivers. Second, inferences regarding drivers relied on responses to questionnaires presented to posho mill customers, while the individuals interviewed may not have perfect knowledge or recollection of these factors. Nonetheless, the findings of our statistical analysis appear credible and relevant in aflatoxin management, and this design allowed us to expand the relative scope of our study, enabling us to report on a wider geographical area than has been included in previous studies. This study was conducted in the year with second highest amount of rainfall since the last worst known acute fatal poisoning case, in 2004, and complements the previous studies with more factors (both pre- and post-harvest drivers) and insights into maize contamination.

In this dataset, districts with the sub-humid AEZ had the highest proportion of aflatoxin contamination, followed by the districts representing the semi-arid zone. The sub-humid AEZ was predominantly represented by samples collected from the three Meru districts in the upper eastern Kenya, where no cases of aflatoxicosis have been reported (13). The Meru districts had the highest proportion of samples contaminated above the regulatory limit, the highest mean aflatoxin levels, and the highest variance in aflatoxin levels. The sub-humid regions of Meru region are not drought-prone, and the maize harvest in 2010 was bounteous. The high levels of aflatoxin recorded in this study were therefore not likely to be attributable to pre-harvest moisture stress, but rather to poor

drying and storage conditions. The sub-humid AEZ is characterized by erratic weather conditions, and should also be considered in surveillance and management efforts. Efforts to address this expanded hotspot must include both attention to pre-harvest stress and to improving drying and storage conditions. Although previous studies have described post-harvest grain handling as the major cause of contamination and aflatoxicosis, this study reports other factors such as size of the farm, grain yield, and intercropping to be associated with the problem. There is need for consistent season-to-season mycotoxin surveillance and analysis of drivers to facilitate better response to protect public health and knowledge of management strategies.

A lower proportion of aflatoxin contamination was observed in samples from farmers who grew their maize in an intercrop. Cultivation of maize in an intercrop system is a common practice in eastern Kenya (23). Previous reports on the influence of intercropping on aflatoxin contamination have been inconsistent. Tédihou et al. (44) observed that intercropping maize with cowpea reduced aflatoxin in stored maize, while Hell (18) observed a higher likelihood of aflatoxin contamination when maize was intercropped with cowpea. Intercropping has been documented to reduce plant disease pressure. In support of their observation, Tidehou et al. (33) argued that intercropping protects the host crop by providing a barrier to inoculum spread. An additional mechanism through which intercropping could have reduced aflatoxin accumulation is via improved soil fertility, as nutrient stress is a known driver of aflatoxin susceptibility in maize (8,4). We acknowledge a need for follow-up experiments to confirm the effect of intercropping on mycotoxins, to investigate the mechanism(s), and to assess the effects of crop species used in the intercrop system.

Our current finding that farmers with larger maize farms and higher grain yield had lower likelihood of having contaminated maize could be explained in two ways. Firstly, stress during crop growth has been positively correlated with aflatoxin levels (11,17). Farmers with larger parcels of land and with higher yield returns can afford better crop prodution inputs that effectively manage pre-harvest stress-related vulnerability factors, and may have better post-harvest handling and storage facitilites that prevent further deterioration of produce. Use of insecticides and fertilizers have been documented to reduce aflatoxin contamination (7). Secondly, farmers with larger harvests have a wider choice of what grain to consume, could be more rigorous in sorting, and may sell what they perceive lower qualiy grain, so they would not bring this through the posho mill for milling.

Maize with the most broken kernels was found to be most contaminated with aflatoxin. This presumably reflects the greater fragility of grain that has been colonized by fungi. In contrast, apparent moldiness and peri-harvest maize ear damage were not correlated with aflatoxin contamination. Previous studies have reported an increase in aflatoxin contamination with maize damage by insects (25,52,53). Susceptibility of maize to infection by *A. flavus* and aflatoxin contamination was reported to increase with different forms of maize ear and kernel damage (38). Kernel breakage creates an infection court for the opportunistic pathogen (11). It is difficult to reconcile with existing knowledge the observation that farmers who had least perceived peri-harvest maize ear damage had most contaminated maize, and that the least aflatoxin contamination was associated with worst peri-harvest ear damage. It should be noted that the sample size for the group perceived fewer damaged ears than usual was relatively small (n=14) compared to that for ears that were perceived to have had more damage than usual (n=254). The latter findings are likely if farmers with perceived higher peri-harvest damage sorted their maize immediately after harvest, as opposed to those who thought that their maize was clean. An immediate post-harvest removal of damaged maize ears was reported to have reduced aflatoxin contamination (21). However, if farmers shelled their grain before sorting, then the process would not remove aflatoxin as our current study shows no correlation between apparent moldiness and aflatoxin.

This study reports a strong correlation between visually apparent moldiness and fumonisin, and concurs with previous studies that reported positive correlations between the toxin and kernel or ear rot (40,2). We further show that grain sorting reduced fumonisin from above to far below the limit acceptable in human food. These findings imply that Kenyan maize consumers can effectively use their visual assessment to remove moldy grain, and hence significantly lower the fumonisin contamination burden in their maize. *F. verticillioides* kernel rot is restricted to the pericarp, and the starburst symptoms are well correlated with fumonisin production (14). In contrast, we did not detect a difference between sorted and unsorted samples for aflatoxin. This could be because aflatoxin level was not reduced by grain sorting, and/or because the effect of sorting was overwhelmed by sampling error. We speculate that both of these factors contributed to our observations. It is known that *A. flavus* colonization can be internal to a maize kernel, hindering the effectiveness of visual inspection (42). The extreme skewness of aflatoxin contamination among individual kernels within maize sample means that our design was not powerful for detecting the effect of sorting; it would have been more relevant to test the unsorted v. sorted kernels.

While the high proportion of aflatoxin-contaminated maize was expected in this region, this study provides the first report of maize contamination by fumonisin in eastern Kenya (13,19). The low correlation observed between aflatoxin and fumonisin suggests that management efforts should be focused on both mycotoxins. Since drivers for fumonisin were not assessed in this study, a detailed survey is now required to establish the factors associated with the widespread fumonisin contamination.

In conclusion, we have provided evidence that aflatoxin and fumonisin are major threats to the maize-consuming populations in eastern Kenya. Further, we have identified the major factors that drive aflatoxin presence and accumulation. Some of the factors identified to influence aflatoxin accumulation can be mitigated using inexpensive and sustainable approaches. For example, if intercropping is validated through experimentation to reduce aflatoxin, small-scale farmers could be advised to practice intercropping of maize with leguminous crops. This would not only reduce likelihood of aflatoxin-contamination, but also improve soil nutrient levels and also provide an alternative dietary source, which subsequently reduces exposure to aflatoxin and fumonisin if less maize is eaten. Creation of awareness of grain sorting as a method to reduce exposure to fumonisin, and/or alternative use for the moldy and mycotoxin contaminated maize would serve best to manage mycotoxin problems if effective grain sorting methods were in place.

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Tables and Figures

Table 1**.** Descriptors of agroecological zones (AEZs) and number of posho mills and samples across AEZs and districts in eastern Kenya

	Agroecological Zone (AEZ)				
	Humid	Sub-Humid	Semi-Humid	Semi-humid to Semi-Arid	Semi-Arid
Rainfall (mm/PA)	1,100 – 2,700	1,000 – 1,600	800 – 1,400	600 -1,100	450 - 900
Soil moisture availability index (%)	>80	65-80	50-65	40-50	25-40
Number of mills sampled	18	29	20	31	45
District	Number of samples collected per AEZ within districts				
Embu	50	10	80	-	10
Kathiani	-	-	40	110	-
Kitui	-	-	-	-	150
Machakos	-	-	60	60	30
Mbeere	20	-	20	80	30
Meru central	60	90	-	-	-
Meru north	10	140	-	-	-
Meru south	30	80	-	40	-
Mwala	-	-	-	10	140
Mwingi	-	-	-	40	110
Total	170	320	200	340	470

Table 2. Percentage of samples with aflatoxin and fumonisin at different contamination categories per district. Lower limit and upper limits of aflatoxin and fumonisin quantification, 1 and 100 and 20 and 6,000 ppb, respectively; Limits of aflatoxin and fumonisin allowed in human food, 10 and 1,000 ppb.

	Aflatoxin					Fumonisin				
	Top quartile range	Percent of samples of in aflatoxin (ppb) category				Top quartile range	Percent of samples in fumonisin (ppb) category			
District	ppb	<1	1 - 10	>10	n	ppb	<100	100 - 1,000	>1,000	n
Meru central	52 - 4,839	20	20	60	150	2,040 - >6,000	27	36	37	86
Mwala	13 - 1,110	15	30	55	150	842 - 4,809	31	46	22	54
Meru north	23 - 1,373	27	27	45	150	2,300 - >6,000	13	39	48	54
Meru south	25 - 2,285	23	35	43	150	1,596 - >6,000	18	48	34	60
Mwingi	16 - 395	35	27	38	150	2,562 - >6,000	16	25	59	56
Kitui	15 - 1,024	27	36	37	150	1,459 - >6,000	30	36	35	61
Mbeere	15 - 989	39	28	33	150	1,711 - >6,000	14	41	44	70
Embu	15 - 695	42	27	31	150	1,300 - >6,000	21	47	32	78
Machakos	9 - 1,135	50	27	23	150	974 - >6,000	20	57	24	51
Kathiani	8 - 1,133	54	24	22	150	1,346 - 3,679	17	44	39	54

Table 3. Estimates of effects of factors associated with presence of aflatoxin in maize. A generalized linear mixed model (PROC. GLIMMIX in SAS system) was used with binomial distribution (0/1) of data was coded follows: samples with aflatoxin<1 ppb were assigned 0; those with aflatoxin≥1 ppb were assigned 1. "District", "Village" and "Mill" were included in the two models as random, and "Mill" was nested in "Village" within a "District".

		Estimate (SE [j])	*df*	*t* Value	Pr > |*t*|	Odds Ratio
Agroecological zone	Humid	-0.41 (0.50)	74.75	-0.82	0.412	0.66
	Semi-Humid	-1.33 (0.50)	27.85	-2.65	0.013	0.26
	Semi-humid to Semi-Arid	-0.60 (0.46)	25.53	-1.32	0.199	0.55
	Semi-Arid	-0.07 (0.45)	13.15	-0.16	0.878	0.93
Cropping system [k]	Intercrop	-0.42 (0.21)	813	-1.98	0.048	0.65
Land size [l]		-0.06 (0.03)	341.1	-1.98	0.049	0.94
Yield_(T/Ha) [m]		-0.16 (0.06)	813	-2.48	0.014	0.85

[j]SE=standard error of the mean. [k, l, m] Questionnaire information about whether maize was grown in intercrop or monocrop, size of maize farm, and grain yield associated with sampled maize. Model reference categories: AEZ, Sub-Humid; Cropping system, Monocrop.

Table 4**.** Factors associated with amount of aflatoxin in home-grown maize. A mixed model (PROC MIXED in SAS system) was used with logarithmically transformed aflatoxin (AFL) data (aflatoxin ≥1 ppb only). "District", "Village" and "Mill" were included in the two models as random. "Mill" was nested in "Village" within a "District".

			Aflatoxin (ppb)			
				Geometric mean (GM) and confidence interval (95%)		
Factor	Factor category	*n*	Log AFL	GM	Lower	upper
Ear damage at harvest [q]	Fewer damaged ears than usual	14	3.8 A	43.1	20.1	92.3
	About the same damage as usual	29	3.0 AB	19.6	11.6	33.3
	More damaged ears than usual	254	2.6 B	14.1	10.3	19.3
	Don't know	218	3.4 A	28.9	16.5	50.6
Kernel breakage after sorting	<1%	319	2.9 B	17.5	13.2	23.3
	1 to 10%	121	3.3 A	26.9	19.0	38.2
	10 to 50%	12	3.4 AB	30.1	13.3	68.4
Agroecological zone	Humid	61	3.0 B	19.9	11.7	33.8
	Sub-Humid	123	3.8 A	43.9	27.9	69.1
	Semi-Humid	46	2.9 B	17.7	9.5	33.1
	Semi-humid to Semi-Arid	104	3.1 B	21.2	14.7	35.5
	Semi-Arid	180	3.2 B	23.6	15.4	36.2

[q] People rated ear damage at harvest in the current maize crop relative to their experience in previous three seasons in 1 to 3 scale, where 1=fewer damaged than usual; 2= About the same damage as usual; 3= More damaged ears than usual. Those who could not recall/provide the estimate were coded as "don't know". Logarithms of aflatoxin least square means (LSM) were separated by Tukey's HSD; identical letter suffix implies a lack of statistical difference.

Table 5**.** Pair-wise correlations among grain quality scores and mycotoxin levels before and after visual sorting of maize.

		Moldiness [x]	Kernel breakage [x]		Aflatoxin (ppb) [y]		Fumonisin (ppb)[y]	
		Before	Before	After	Before	After	Before	After
Moldiness	Before	-	-	-	0.05		0.42**	-
	After	0.92***	0.31***	0.27***	-	0.17	-	0.27
Kernel breakage	Before	0.40***	-	-	0.08		0.17	-
	After	0.32***	0.94***	-	-	0.22	-	0.17*

[x] Trained research assistants scored grain for moldiness and kernel breakage prior to collection of milled samples of the sorted grain during a cross-sectional survey in eastern Kenya, 2010. Grain was scored before and after sorting using a 1 to 5 score scale as follows: 1=less than 1%, 2=1 to 10%, 3=10 to 50%, 4=50 to 90%, 5=greater than 90% broken or moldy/discolored/rotten kernels in a sample.

[y] Paired unsorted and sorted grain samples (*n*=51) were collected from posho mills (*n*=5) within an aflatoxin hotspot area, Machakos district in eastern Kenya, 2012. Grain was scored using the described scale. Mycotoxins were analyzed using ELISA.

*, **, *** represent significance of the correlation coefficient at α=0.05; 0.01; 0.001, respectively

Table 6**.** Pair-wise comparisons of means of grain quality scores and mycotoxin levels before and after visual sorting.

	df	Mean BS (±SE)	Mean AS (±SE)	Mean difference	*t*-ratio	Prob > |*t*|
Moldiness[x]	810	1.45 (±0.01)	1.44 (±0.01)	-0.01	-1.02	0.308
Kernel breakage[x]	810	1.35 (±0.01)	1.33 (±0.01)	-0.02	-3.35	0.0008
Aflatoxin (ppb)[y]	50	15.9 (±5.5)	15.4 (±5.4)	-0.6	-0.071	0.943
Fumonisin (ppb) [y]	50	1,386 (±189)	487 (±185)	900	-3.402	0.001

[x] Grain were scored for moldiness and kernel breakage before (BS) and after sorting (AS) using a 1 to 5 score scale, where 1=less than 1%, 2=1 to 10%, 3=10 to 50%, 4=50 to 90%, 5=greater than 90% broken or moldy/discolored/rotten kernels in a sample. Scoring was done by trained research assistants using the same grain quality pictorial guides employed during a cross-sectional survey in eastern Kenya, 2010.

[y] Mycotoxins were quantified from samples (*n*=51) that were collected from posho mills (*n*=5) in within aflatoxin hotspot area Machakos district, in eastern Kenya, 2012. Data for samples with fumonisin level above 6,000 ppb was excluded (see Fig 4). SE, standard error.

Table 7: Chronology and conditions for aflatoxin contamination in eastern Kenya.

Year[2]	Rainfall (mm) in Eastern province[1]		Outbreak	# Districts; sample size (n); fatality cases (fc)	Aflatoxin (ppb)	#, AEZs	[5]Factors associated with aflatoxin occurrence or level of contamination	Reference
	Upper Eastern (UE)[3]	Lower Eastern (LE)[4]			Maximum (Max); Geometric mean (GM)			
2003 - 2004	678	395	Yes	3 LE and I Central Kenya; n=350; fc=125	46,400; 52.91	2, Semi-arid and Semi-humid to Semi-arid	Post-harvest handling	(36,3,30)
2004 - 2005	518	349	Yes	2 LE; n=298; fc=?	48,000; 12.92	1, Semi-arid	Post-harvest handling	(13)
2005 - 2006	700	334	Yes	2 LE; n=165; fc=53	24,400; 26.03	1, Semi-arid	Post-harvest handling	(13)
2006 - 2007	961	640	No	2 LE; n=253; fc=?	2,500; 1.95	1, Semi-arid	Post-harvest handling	(13)
2007 - 2008	601	372	No	2 LE	136; NA	1, Semi-arid	Post-harvest handling	(26)
2008 - 2009	532	239	No	-	-	-	-	-
2009 - 2010	732	518	Yes	10 (LE and UE); n=1,500, fc=1	4,839; 5.60	All maize-growing AEZs in E. Kenya	Several drivers identified	BBC 2010 and This paper

[1]Rainfall estimates (mm) data was retrieved from The Famine Early Warning Network http://earlywarning.usgs.gov/. [2]Bimodal rainfall, short season, October – Feb; Long season, March – July. Short season rainfall is more reliable for crop production in the region. [3]Districts: Meru north, Meru central, Meru south, Tharaka, Mbeere and Embu. [4]Districts: Machakos, Makueni, Kitui, and Mwingi. [5]Based on author's claim(s). NA, not reported. AEZ, agroecological zones. ?, no report of fatal case of aflatoxicosis.

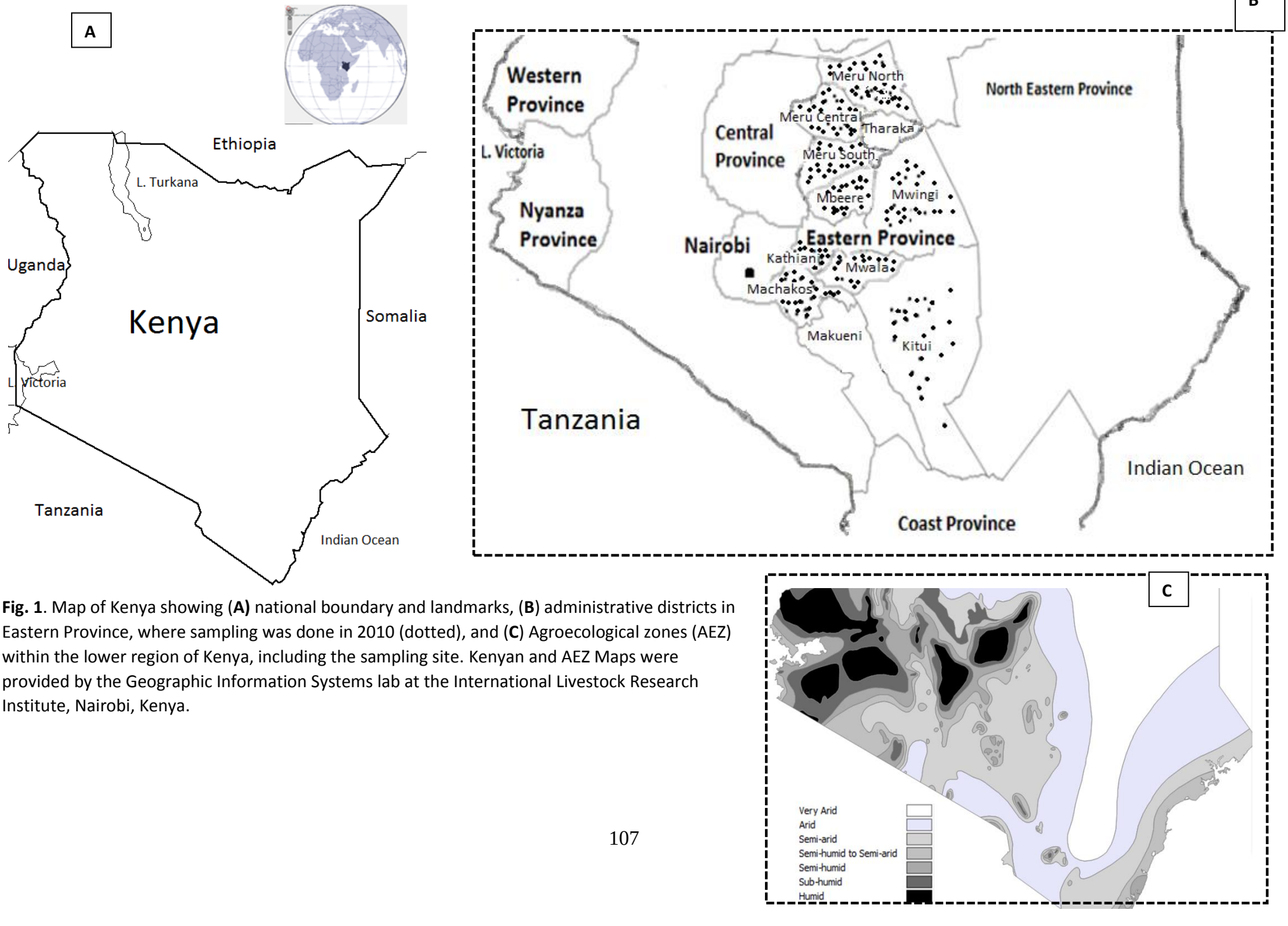

Fig. 1. Map of Kenya showing (**A)** national boundary and landmarks, (**B**) administrative districts in Eastern Province, where sampling was done in 2010 (dotted), and (**C**) Agroecological zones (AEZ) within the lower region of Kenya, including the sampling site. Kenyan and AEZ Maps were provided by the Geographic Information Systems lab at the International Livestock Research Institute, Nairobi, Kenya.

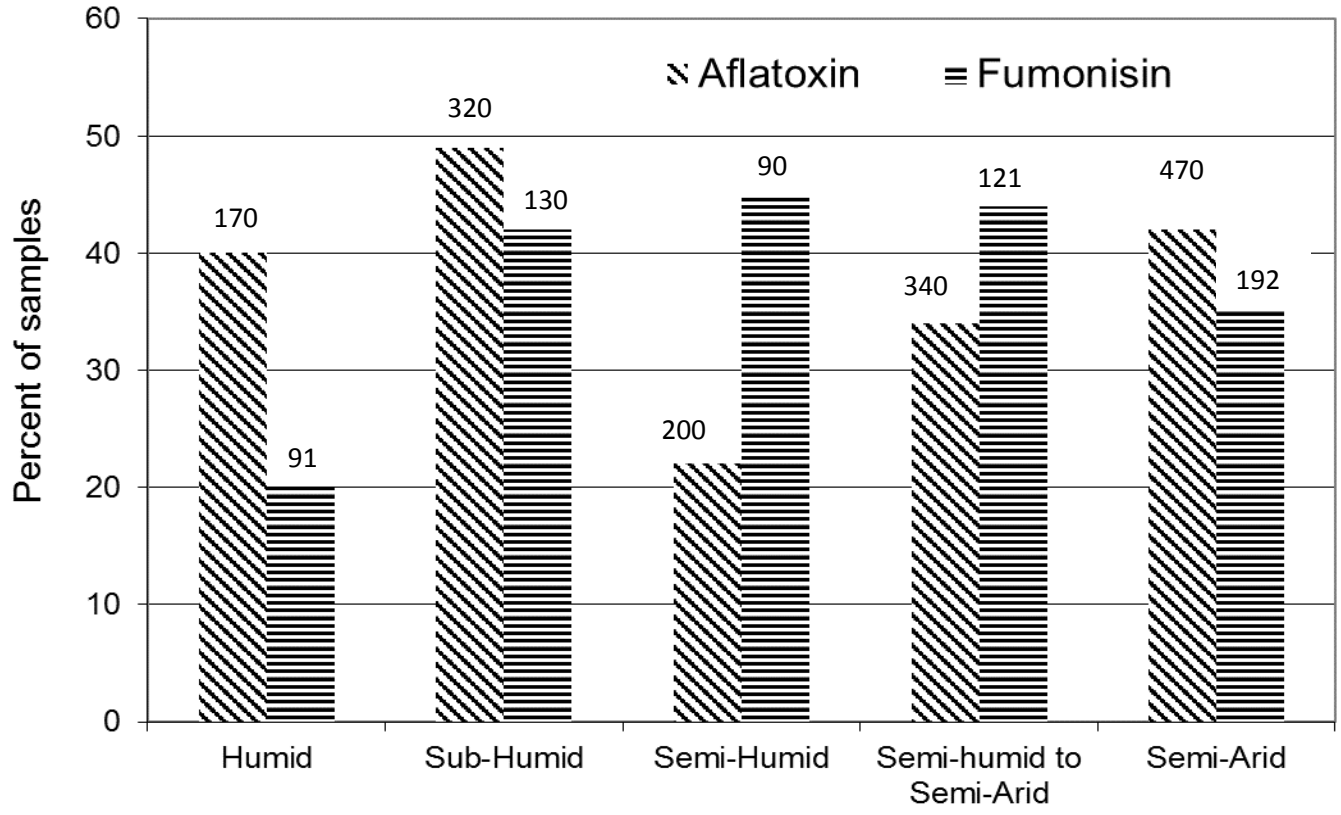

Fig. 2. Percentage of samples with mycotoxins above the regulatory limits in the five agroecological zones. Number of samples analyzed for each toxin in each agroecological zone is shown over the bars.

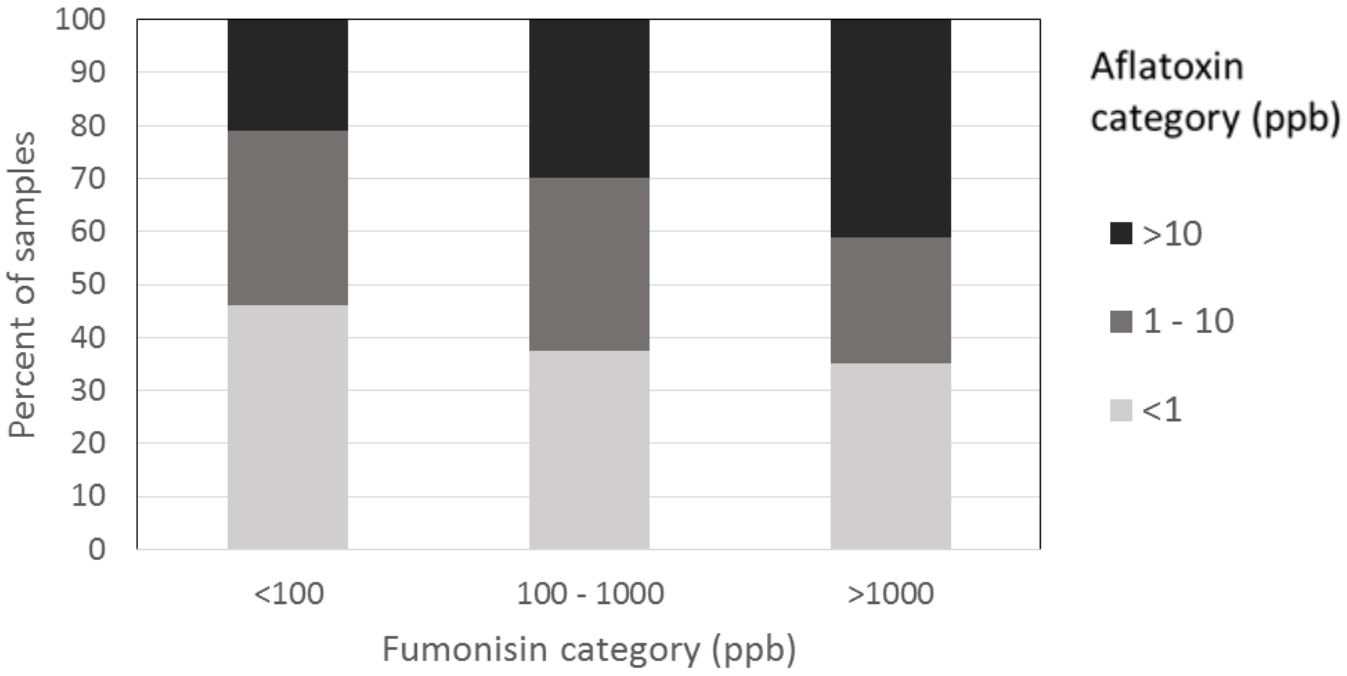

Fig. 3. Mosaic plot of percentage of samples with aflatoxin and fumonisin at different contamination categories (*n*=628).

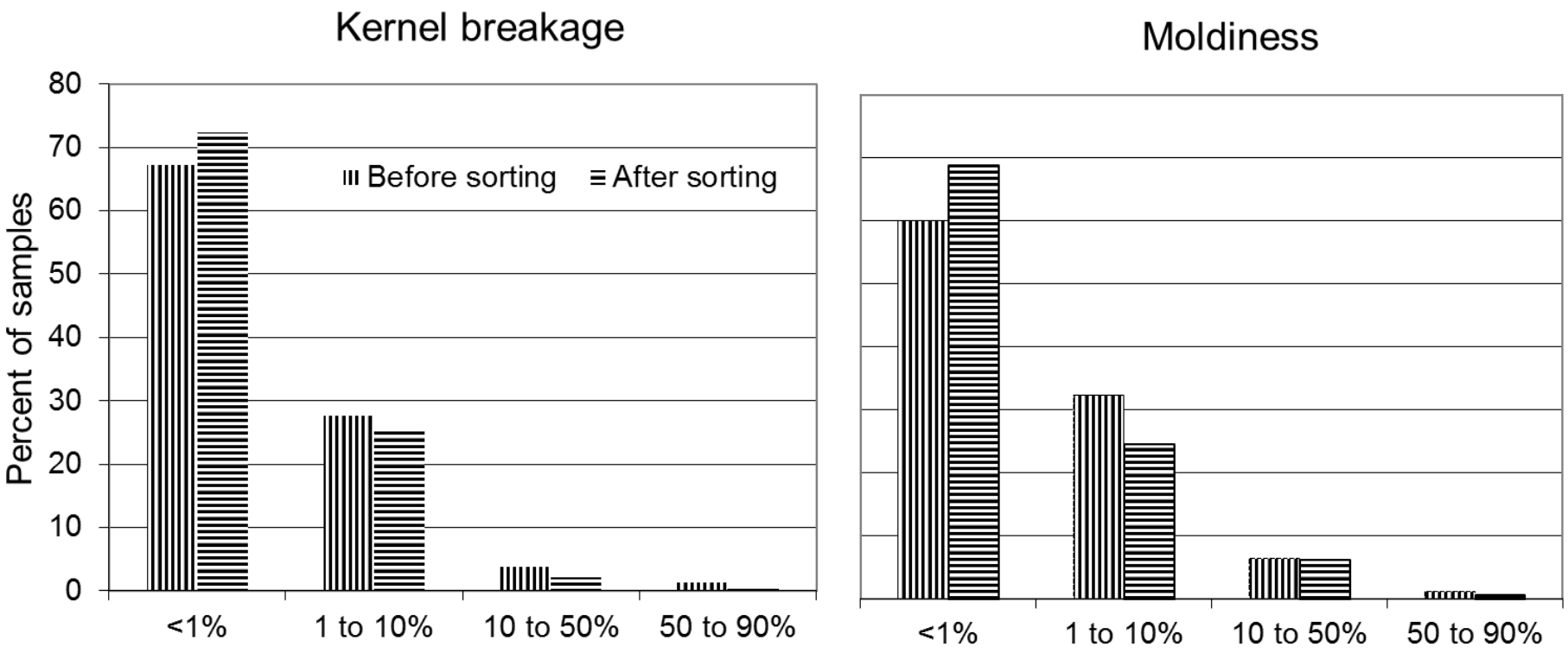

Fig. 4. Effect of sorting on kernel breakage and moldiness. Grain samples were visually scored at sampling sites. Y- axis, percentage of samples in grain quality category; N=822. X-axis, grain score scale: 1=less than 1%, 2=1 to 10%, 3=10 to 50%, 4=50 to 90%, 5=greater than 90% broken or moldy/discolored/rotten kernels in a sample.

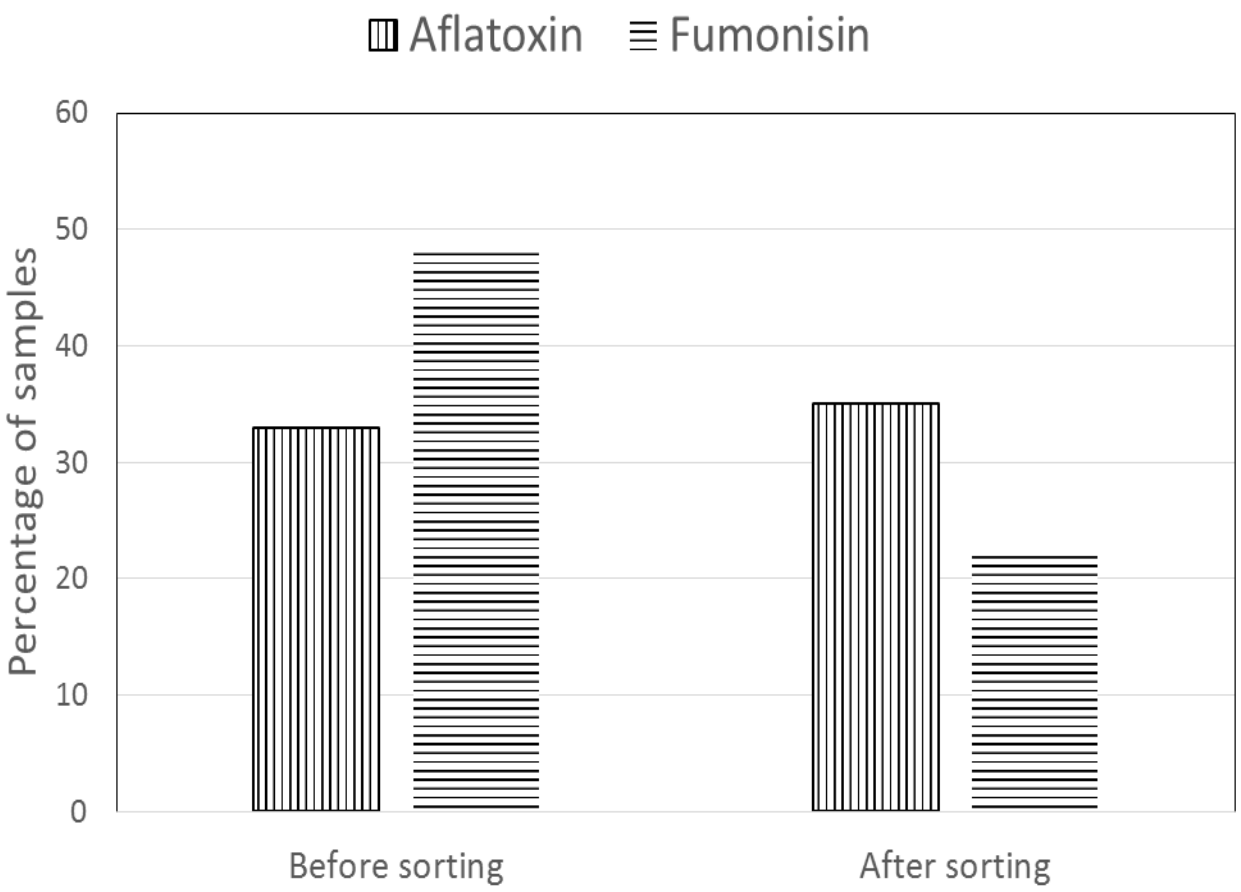

Fig. 5. Percentage of samples with mycotoxin above limits allowed in human food before and after grain sorting. Mycotoxins were quantified from samples (*n*=62) that were collected from posho mills (*n*=5) in within aflatoxin hotspot area Machakos district, in eastern Kenya, 2012. Data includes samples with fumonisin above 6,000 ppb

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CHAPTER 4

INSIGHTS INTO SUSCEPTIBILITY OF MAIZE TO AFLATOXIN ACCUMULATION: INFLUENCES OF PLANT STRESS, AGRONOMIC TRAITS AND *ASPERGILLUS FLAVUS* POPULATIONS

Abstract

Aflatoxin resistance is a complex trait with low heritability. We used diverse maize test-crosses that were grown and naturally colonized by *Aspergillus flavus* in the aflatoxin hotspot of eastern Kenya to investigate the relationships between agronomic traits and aflatoxin accumulation under two soil nitrogen levels. Maize traits and aflatoxin were analyzed on a random subset (n=205) of the 421 entries in the Improved Maize for African Soils association mapping panel which was being evaluated for tolerance to low soil nitrogen (N) at two locations in 2011-2012. The proportion of plots with detectable aflatoxin was higher under low soil N than under the regionally-recommended N level. Under low N, aflatoxin accumulation level differed significantly (P<0.05) among maize maturity groups, but no significant genotypic differences were observed within the maturity groups. Aflatoxin did not differ significantly (P>0.05) between the early (geometric mean, GM=3.1 ppb) and intermediate (GM=3.9 ppb), but was significantly (P<0.05) higher in the late maturity group (GM=8.2 ppb). Kernel bulk density was negatively correlated with aflatoxin level (r=-0.21, P<0.0001). Kernel bulk density and kernel percent protein were higher in early and intermediate groups than in the late maturity group. Grain yield did not differ (P≤0.05) among the maturity groups. The association between the level maize colonization by *A. flavus*, fungal aflatoxigenicity, and pre-harvest aflatoxin accumulation was tested in maize samples with known aflatoxin levels. *A. flavus* biomass (estimated by using real-time PCR) was correlated with pre-harvest aflatoxin (r=0.53, P≤0.05). However, the aflatoxin production potential of *A. flavus* was uncorrelated with pre-harvest aflatoxin level. We conclude that maize maturity should be considered in aflatoxin resistance breeding, and evaluations should be carried out in well-replicated multi-season trials of artificial inoculation and natural infection.

Introduction

Contamination of food by mycotoxins, toxic fungal secondary metabolites, is a major global food security and safety threat, especially in the tropical developing countries of Sub-Saharan Africa (76,27). In the tropics, maize is commonly infected by *Aspergillus flavus,* which causes Aspergillus ear rot and produces aflatoxin B1 (AFB1), the most potent naturally-occurring carcinogen (72,37). In developed countries, aflatoxin regulation leads to economic losses for farmers. In most tropical developing countries, aflatoxin regulation is non-existent or not consistently enforced (27). Acute aflatoxin exposure is fatal, and chronic exposure causes immunosuppression, increased morbidity, nutrient absorption impairment, retardation of fetal growth, and liver cancer (82). Kenya is one of the tropical countries with an inadequate food regulation system, and has been globally recognized for acute aflatoxicosis (20). Breeding for resistance to *A. flavus* and aflatoxin accumulation in maize is one approach for reducing exposure in maize-consuming populations (78).

Maize can be infected by aflatoxigenic fungi before or after harvest (27,36,51). Aflatoxin accumulation occurs in the field and increases if the grain is stored under conditions conducive to fungal growth and aflatoxin production. Pre-harvest infection mainly occurs when *A. flavus* invades and grows on silks within the silk channel during grain filling, and could also occur after physiological maturity if delayed harvest coincides with unexpected rain (59,14,60). Further inoculum exposure peri- and post-harvest increases infection of maize grain (27). Peri-harvest infection occurs if predisposing harvesting techniques are used under conditions that favor fungal entry into the maize ears e.g., heaping the harvested maize ears on the ground. Post-harvest infections occur if grain is dried or stored under conditions and environment that favor fungal entry. At each stage of infection, aflatoxin contamination can reach thousands of parts per billion (ppb) (29,60). The importance of each stage of infection and aflatoxin contamination is determined by climatic conditions and the nature of maize value chain.

A complex of plant stress and spatiotemporal cropping factors influence pre-harvest infection and aflatoxin accumulation (59). Plant stress factors that have been associated with aflatoxin contamination include drought, insect damage, other diseases and low soil fertility (14,34,81). Plant stress increases vulnerability to colonization by weak pathogens like *A. flavus*. The adaptation of *A. flavus* to grow well in dry medium makes it successful in colonizing maize during drought (66,61). Depending on their feeding habits and movement within the plants, insects can either spread *A. flavus* conidia on the crop, or they can cause wounds that facilitate entry of *A. flavus* into the maize ear

(45,79). Cultivation of maize under low soil fertility leads to nutrient deficiency, and hence improper development of important parts of the plant such as the seed (68). The interaction among the plant stress factors is likely to increase the likelihood of aflatoxin accumulation in maize.

Factors that drive pre-harvest infection and aflatoxin accumulation in maize have been studied, but their interaction with other crop production factors under natural conditions has not, especially in African aflatoxin hotspots (28,27). The nature of crop production systems and the abundance of *A. flavus* inoculum in tropical climates lead to persistent aflatoxin contamination of African maize. For example, in East African maize value chains, over 50% of the crop is produced by resource-poor farmers under sub-optimal conditions (70,30). For example, in the aflatoxin hotspot of eastern Kenya, maize is cultivated under drought-prone and nitrogen-depleted soils (71,73). The crop is cultivated in soils that are deficient in nitrogen and phosphorous, essential crop production nutrient elements (71). Although studies conducted in other parts of the world have shown that application nitrogenous fertilizer reduces aflatoxin accumulation in maize, the majority of resource-poor farmers often cannot afford the high prices of the inputs (9,8,27).

Breeding for aflatoxin resistance in maize is a potential approach for reducing aflatoxin exposure (7,13). Resistance to *A. flavus* and aflatoxin accumulation exists in maize, but has not been consistently bred into African commercial varieties due to the complex nature of the pathosystem (27,13). The estimates of heritability for aflatoxin accumulation trait in maize have ranged from 0 to 0.6, with strong interaction between maize genotype and environment (GxE) (77,11,49). Abiotic factors such as soil fertility and climate influence the extent of maize contamination (3,9). Factors influencing kernel growth and development (including their morphology and chemical composition) also affect susceptibility to infection and contamination (26). Among the multiple maize traits that have been associated with aflatoxin resistance are ear morphology (e.g. husk coverage and tightness) (75,5), kernel structure and chemical composition (e.g. hardness of the endosperm, waxy pericarp, antifungal proteins etc.) (25,5,6,10), silk characteristics (51), early maturity and adaptation (5,16), insect resistance (16), kernel integrity (56), and tolerance to drought and heat (58). These traits are mainly tested individually or in combinations in aflatoxin resistance studies. Due to the quantitative nature of resistance to aflatoxin, development of resistant varieties requires breeding strategies that would combine multiple traits of interest into the final resistant varieties.

In eastern Kenya, highly aflatoxigenic isolates of *A. flavus* have been reported and associated with lethal aflatoxicosis (64,65). The interaction between *A. flavus* aflatoxigenicity and maize genotype could influence the extent of aflatoxin accumulation, but has not been investigated. Previous studies

have shown strong correlations between maize infection (estimated by fungal biomass) and aflatoxin accumulation (21,50,80). In contrast to the majority of published studies that have used artificial inoculation, we investigated the relationships among aflatoxin production of *A. flavus* populations, kernel colonization and aflatoxin accumulation in maize under natural infection in the Kenyan aflatoxin hotspot. These interactions may play a major role in the GxE hurdle that has hindered the aflatoxin resistance breeding effort (24).

We aimed to gain insights into the relationships among kernel traits, soil fertility and aflatoxin accumulation under natural colonization of maize by *A. flavus* in field trials in the Kenyan aflatoxin hotspot. We collaborated with the International Maize and Wheat Improvement Center's (CIMMYT) Improved Maize for African Soils (IMAS) project, wherein test-crosses of a diverse maize association mapping panel were being evaluated for tolerance to low soil nitrogen in a Kenyan aflatoxin hotspot. Because nitrogen depletion is a major plant stress linked to increased susceptibility to *A. flavus* and subsequent aflatoxin accumulation (9), we used the IMAS association mapping (IMAS-AM) panel and experimental design to study aflatoxin accumulation (based on natural infection by *A. flavus*), to investigate the relationship between aflatoxin and agronomic traits, and to gain insights on the aflatoxigenicity of the *A. flavus* populations.

Materials and Methods

Germplasm. The Improved Maize for African Soils association mapping (IMAS-AM) panel is a diverse maize germplasm which was assembled by CIMMYT for use in breeding for low nitrogen (low N) tolerance in African maize. The germplasm consists of 421 inbred lines that were sourced from the CIMMYT's global tropical breeding programs, the Kenya Agricultural and Livestock Research Organization (KALRO) and the Agricultural Research Council (ARC) of South Africa. All inbred lines were test-crossed to a common tester, CML539 (CML312SR), a white, sub-tropical, dent of intermediate maturity, good combining ability and moderate adaptation to drought and low N (81).

Study sites. The IMAS-AM test-crosses were evaluated at two Kenya Agricultural and Livestock Research Organization (KALRO) experiment stations: Embu (latitude 00° 30'S and longitude 37°42'E; elevation 1,510 M asl) and Kiboko (latitude 02° 12' S, longitude 37°43' E, altitude 975 M asl), in eastern Kenya. The Embu station receives an average of 1200-1495 mm rainfall annually and its temperature ranges 14–25°C. Soils at the station are humic nitrisol, slightly acidic (pH of 5.6) and deficient in both nitrogen (0.2%) and phosphorus (26 ppb). The Kiboko station receives an average

rainfall of between 400 and 600 mm annually and its temperature ranges 17-32°C. Soils at Kiboko are haplic lixisol, slightly acidic (pH of 5.7), and deficient in nitrogen (0.15%) and phosphorus (15 ppb). Both sites are located in drought-prone areas, rainfall was supplemented by irrigation during the growing periods. Both sites are located within the known Kenyan aflatoxin hotspot (41,20), trials were naturally infected by aflatoxigenic fungi.

Experimental design. Test-crosses of the IMAS-AM panel were grown under the regionally-recommended amount of nitrogen fertilizer (henceforth referred to as the recommended N) at Kiboko (2011 and 2012), and under low N at Kiboko and Embu (2012). Both soil N level trials were planted in two replicates in an alpha lattice experimental design. In the low N trials, 100 kg of triple super phosphate (TSP – 0:46:0) fertilizer was applied per hectare at planting to promote root development and early stand establishment. In addition, 100 kg of calcium ammonium nitrate (CAN – 26:0:0 + 8 Ca) was applied per hectare as top dressing (42 days after planting). This is reflective of average nitrogen application amongst small holder farmers in sub-Saharan African which averages 20 kg of N per hectare (54). In the recommended N trials, the regionally recommended fertilizer doses (54) were applied as follows: 200 kg of di-ammonium phosphate (DAP: 18:46:0) was applied per hectare at planting followed by a top dressing of 300 kg of CAN per hectare 42 days after planting.

Post-harvest agronomic trait analysis. Data was collected on flowering period (anthesis: days to 50% pollen shed), ear rot percentage, grain weight and grain moisture content. Grain yield was estimated from the shelled grain weight per plot and was adjusted to 12.5% moisture. Grain moisture (also termed as grain moisture at harvest or GMAH) was determined as a percentage of the shelled grain weight. Trials were not treated with fungicide. Kernel samples (100 and 200 grams from trials conducted in 2011 and 2012, respectively) delivered to the Biosciences eastern and central Africa (BecA)-ILRI hub, Nairobi, approximately one week after harvest, and were kept in cold storage at 4^0C until further analysis.

Selected kernel traits and aflatoxin accumulation (based on natural colonization of the maize lines by *A. flavus*) was analyzed on kernels of a random subset of the test-crosses (genotypes) that were grown under low N (n=205) at the two sites in the long rain season of 2012 and under the recommended N (n=123) during the short[3] and long[4] rain seasons of 2011 and 2012 at Kiboko. The

[3] Short season: In eastern Kenya, maize is planted in October and is harvested in February.
[4] Long season: In eastern Kenya, maize is planted in April and is harvested in September.

entries that were analyzed from recommended N were a subset of those grown under low N. The differences in the number of entries analyzed for low and recommended N resulted from differences in availability of the seed stock of the individual maize genotypes. Due to limited seed stock from each replicate in Kiboko, kernels of the two field replicates of each entry were bulked to obtain the sample for mycotoxin analysis. Kernels of the two field replicates of the 205 entries from Embu low N were analyzed separately.

Kernel trait laboratory analysis. Selected morphological and chemical composition kernel traits that have been previously implicated in resistance to aflatoxin accumulation were analyzed (6,51). The analyzed kernel characteristics included bulk density, texture, protein, size and moisture. Kernels were oven-dried overnight at 40°C and cooled at room temperature for 6 hours prior to analysis of moisture (also termed as grain moisture after oven-drying or GMAD) and kernel bulk density. For determination of moisture, two subsamples, approximately 50 grams, were scanned with two repacks in a Fourier-Transformed Near-Infra-Red spectrophotometer (NIRS), and predictions were based on an existing calibration (r^2=98%) (Unpublished). To measure kernel bulk density, containers of known mass and volume were filled with kernels to the brim in triplicate. Each container was weighed and the density was calculated. Kernel texture was visually scored as follows: 1= flint; 2=semi-flint; 3=semi-dent; 4=dent. Kernel size was visually scored in reference to a 1-cm square sieve on three subsamples as follows: 1=small (kernel length <1 cm) 2=medium (kernel length of approximately 1 cm), and 3=large (kernel length > 1 cm). Percent grain protein was estimated using a NIRS method that has been calibrated and validated using the Kjeldahl method (AOAC, 2000).

Ear rot scoring. Individual ears were scored for ear rot based on the percentage of the ear exhibiting visible symptoms. Ear rot symptoms were not analyzed or categorized for the causal organism, but were assumed to have been caused by different fungal species. Plot-means of ear rot were computed from ear rot scores of the individual ears.

Aflatoxin quantification. Each sample was thoroughly mixed and approximately half of its content was ground into a fine flour and divided into two sub-samples using a Romer mill (Union, MO). For the entries whose field replicates had been bulked, aflatoxin was analyzed on four sub-samples that were obtained by splitting about half of the original sample into two random whole grain samples, and a subsequent milling of each sub-sample as described earlier using the Romer mill. Pre-harvest aflatoxin contamination was extracted from 5 grams of each flour sub-sample using 70% methanol and analyzed using a commercially available enzyme-linked immunosorbent assay (ELISA) (Helica

Biosystems Inc., Fullerton, CA). The solid phase direct competitive ELISA kit (Helica Cat. No. 941BAFL01-96) consisted of an antibody-coated 96-well micro-plate whose micro-wells were coated with an antibody optimized to cross-react with the four aflatoxin types at the following rates: B1(100%), B2(77%), G1(64%) and G2(25%). The lower and upper quantification limits of the kit were 1 and 20 ppb, respectively. The ELISA method had been previously validated using the Vicam AflaTest method (Waters, Watertown, MA) (see chapter 3). Samples with greater than 1000 ppb of aflatoxin were re-analyzed by Vicam AflaTest. A subset (10%) of the latter samples was purified via Vicam AflaTest columns and then analyzed using Ultra-Performance Liquid Chromatography (UPLC) as a reference method.

Fungal biomass quantification. Maize samples whose aflatoxin levels had been established using ELISA were selected for analyzing the amount of *A. flavus* per unit maize DNA. Quantitative PCR (qPCR) was used to quantify the amount of fungal and maize DNA in each sample as described by Klosterman (39). Total genomic DNA was extracted from 5 grams of maize flour samples using a modified cetyl trimethylammonium bromide (CTAB) protocol as described by Mideros et al. (50). DNA for the *A. flavus* standard curve was extracted using the modified CTAB method from three-day-old pure cultures of *A. flavus* mycelium that were grown on malt extract agar (MEA) media (50). DNA for the maize standard curve was extracted from aseptically-grown young maize shoots (50). *A. flavus*-specific primers (AF2) designed by Mideros et al. (50) for the internal transcribed spacer 1 (ITS1) were used to estimate the amount *of A. flavus* DNA present in the sample. A maize-specific primer pair for a cell wall invertase gene sequence (INCW_2), previously described in Murray et al. (53), was used for quantification of maize DNA in the sample. The concentration of both primers was 2 pmol/µL. For standard curves, primers were diluted as follows: AF2 at ten-fold serial dilutions between 5 and 0.0005 ng/µL, and INCW_2 at 50, 25, 5, 0.5 and 0.05 ng/µL.

A. flavus and maize DNA were quantified separately in 96-micro-well fast optical plates with each sample of milled maize. Quantitative PCR (qPCR) conditions for *A. flavus* DNA quantification were: 95°C (10 min) for initial denaturation, followed by 40 cycles of 95°C (30 sec) and 57°C (1 min), and a melt curve analysis at 95°C (15 sec), 60°C (15 sec), 95°C (15 sec) prior to holding at 10°C. Quantitative PCR conditions for maize DNA quantification were: 94°C (5 min) for initial denaturation, followed by 40 cycles of 94°C (20 sec) and 55°C (20 sec), and a melt curve analysis at 95°C (15 sec), 60°C (15 sec), 95°C (15 sec) prior to holding at 10°C.

Each milled sample was split into two sub-samples and the sub-samples were randomized prior to DNA extraction. DNA was extracted from each subsample and was normalized to 20 ng/µL. Two replicates of the template DNA of each subsample and standards were included in random micro-wells in a single run in a 96-microwell fast optical plate. DNA was quantified in a 20 µL reaction of infected maize DNA mix consisting of 1 µL of each primer pair, 5 µL template DNA, 10 µL Power SYBR® Green Master Mix (2x) and 3 µL of nuclease-free water. There were two no-template controls (NTCs) in each plate. Absolute quantification of DNA was done using an ABI 7900HT in a Sequence Detection System ver. 2.3, which uses the fluorescence of the DNA-binding dye, SYBR green, to estimate the DNA copy number based on cycle thresholds (ct) values (Applied Biosystems, Foster City, CA). Logarithmically transformed concentrations of the DNA calibration standards were regressed against the Ct values in a standard curve that were used to statistically predict the amount of sample DNA in each 96-well plate. The amplification efficiency (E) of each run of qPCR reaction was computed based on the slope of the calibration standard curve using the equation $E = 10^{(-1/slope)}$, as per ABI recommendation. The pathogen-host ratio (P:H) was computed for each of the sub-samples (80).

Fungal isolation and species confirmation. Maize whole-grain samples (*n*=12) were selected across aflatoxin contamination levels such that each of the aflatoxins bins 0, 1 – 100, 100 – 500 and above 500 ppb was represented by 4 samples from maize grown under low N soils. Three replicates of kernels (*n*=10) of each sample were surface sterilized in 10% commercial bleach (NaOCl) solution, washed with two changes of sterile distilled water and plated directly on a Modified Rose Bengal Agar media (18,62). Kernels were incubated at 30°C for 4 days. From each kernel, *Aspergillus* species were sub-cultured onto Potato Dextrose Agar (PDA) prior to growing of pure cultures and identification of section *Flavi* based on cultural and morphological characteristics (38,4).

We selected representative *A. flavus* isolates (*n*=32) for further confirmation by PCR amplification and sequencing of internal transcribed spacer (ITS) region of the ribosomal DNA. Genomic DNA was extracted using a modified CTAB method (50) from 3-day-old mycelia that had been grown on MEA. PCR was done using ITS1_F or ITS1 (forward) and ITS4 (reverse) primer pairs (74). PCR products were purified using GeneJet® (ThermoFisher Scientific Inc., Waltham, MA) and sequenced using ABI 3730 genetic analyzer (Life technologies, Grand Island, NY).

Aflatoxigenicity analysis. Pure cultures that had been confirmed to be *A. flavus* by using PCR-sequencing method were grown on aflatoxin-production inducing yeast extract sucrose agar (YESA)

media at 28°C for 5 days. Aflatoxin was extracted from 10-gram YESA slabs using 80% methanol and analyzed using a modified Vicam AflaTest method as follows. Agar was added to methanol in a ratio of 1:2 into a 50 mL falcon tube (BD, Franklin Lakes, NJ) containing steel beads and the tube was shaken in an incubator shaker (New Brunswick Scientific Co, Edison, NJ) for 10 min at 300 rpm. The slurry was filtered twice using fluted filter papers, and then by using microfiber filter papers (Waters, Watertown, MA). The filtrate was diluted with sterile water at 1:4. The extract (2 mL) was passed through the AflaTest column at 1-2 drops/sec. The column was washed twice with 5m mL of deionized distilled water prior to eluting with 1 mL of HPLC grade methanol into a cuvette. Vicam AflaTest developer was added to the eluate and the aflatoxin level was read in a flourometer (Waters, Watertown, MA).

Sclerotial production analysis and strain classification. Sclerotia formation and strain classification was assessed by growing the isolates in three replicates in V8-agar medium (5% V-8 juice and 2% agar, adjusted to pH 5.2 prior to autoclaving) at 30°C for 12 days (47). Sclerotia production was scored under a dissecting microscope (45X) on a per plate basis as follows: 0=no sclerotia, 1=few sclerotia (<500 sclerotia), and 3=Many sclerotia (>500 sclerotia) (47). Conidia and sclerotia were washed from the surface of media by adding 5 mL of 0.2% Tween 20 solution. Sclerotia diameter was estimated in a compound microscope (400X). Isolates with any sclerotia with a diameter >400 µm were designated strain L, while those that produced numerous sclerotia of diameter <400 µm were designated strain S (67,17).

Statistical analyses. JMP Pro ver. 11 (SAS Institute Inc., 2013, Cary, NC.) was used for data analysis. Because some trials had samples of field replicates pooled into one sample prior to delivery for laboratory analysis, descriptive statistics were computed on an entry basis using the values of the sub-samples. For all statistical comparisons, aflatoxin (ppb) was transformed as log (ppb+1) prior to input into models. Least square means (LSM) of the logarithmically transformed data were back-transformed and reported as geometric means (GM). Repeatability of traits across the locations was assessed using intra-trait correlations between the locations. Best Linear Unbiased Predictors (BLUPs) of the maize genotypes for days to anthesis (DTA) were computed using a mixed model in which location and maize genotype were included as random factors in a restricted maximum likelihood (REML) method of variance component estimation. The DTA BLUPs were ordered and the values were used in identifying maize maturity groups. A chi-square test was conducted to assess association between kernel texture score and aflatoxin categories. The latter included presence or absence (with samples

coded as 0 if they had aflatoxin less than 1 ppb, the ELISA detection limit, or coded as 1 if aflatoxin was greater than 1 ppb) and above or below regulatory limit (coded as 0 if below 10 ppb, or coded as 1 if equal to or above 10 ppb).

DNA sequences were compared with those in the gene bank by using BLAST. Pairwise nucleotide differences per site between *A. flavus* isolates were computed based on National Biomedical Research Foundation /Protein Information Resource (NBRF/PIR) DNA sequence alignment in Clustal 2.1 (40)

Results

We analyzed agronomic and kernel traits that have been previously implicated in resistance to aflatoxin accumulation in maize. The pedigrees of the maize genotypes studied, and their maturity groups are shown in Supplemental Table 1. The descriptive statistics of the analyzed traits are shown in Table 1. Grain yield, protein, and kernel bulk density were higher in maize grown under recommended N compared to maize grown under low N, with grain yield twice as high in entries from the optimal treatment (Table 1).

Aflatoxin levels and the percentage of maize entries with detectable aflatoxin were higher under low N compared to the recommended N. Based on the aflatoxin levels detected in the entries (n=123) that were common under the two soil nitrogen levels, aflatoxin accumulation did not differ significantly (P=0.891) between the two locations (geometric mean, GM=4.7±0.1 ppb, Embu; GM=4.5±0.2 ppb, Kiboko) under low N. Aflatoxin accumulation was four-fold higher under low N than under the recommended N (GM=1.1±0.2 ppb) in the long season, 2012. Under low N in the long season of 2012, 55% of the entries showed detectable aflatoxin for both locations, while 36% of entries had detectable aflatoxin under the recommended N in the same season. Under the recommended N in the short season of 2011/2012, 1% of entries showed detectable aflatoxin (Table 1). Similarly, plots with low N treatment had a higher percentage of entries with aflatoxin above the regulatory limit (>10 ppb), 30% at Embu and 36% at Kiboko, compared to 15% of entries under the recommended N at Kiboko. No entries had aflatoxin above 10 ppb in the short season under the recommended N (Table 1). Because of the low aflatoxin frequency under the recommended N, aflatoxin mean and variance comparisons among the maize genotypes were only analyzed with data from low N.

Maize flowered (days to anthesis) approximately 22 days earlier at Kiboko than at Embu, but there was a strong rank correlation between the flowering time of genotypes at the two sites (ρ=0.53, P<0.0001) (Table 1). Late flowering was associated with higher grain moisture at harvest and more aflatoxin accumulation across the two sites under low N (Table 2). High grain moisture at harvest was associated with increased aflatoxin accumulation (Table 2). High kernel bulk density was associated with high protein content, and the two traits were negatively correlated with flowering time. Aflatoxin was negatively correlated with kernel bulk density under low nitrogen conditions. Kernel bulk density was not correlated with either grain moisture or grain yield (Table 2). Grain moisture at harvest and after oven-drying were uncorrelated across the two sites under low N (Table 2).

Ear rot was not correlated with aflatoxin and did not differ between low N at Embu and recommended N at Kiboko in the long season 2012. Correlations between ear rot and other agronomic traits were inconsistent across the two environments under low N (Table 2). Early maturity and high protein content were associated with higher incidence of ear rot under low N at Kiboko. Additionally, higher ear rot incidence was associated with low grain yield under low N at Embu. Ear rot and grain moisture at harvest were uncorrelated but the magnitudes of the two traits followed the same trends across the three environments (Table 1).

A mixed model under low N with aflatoxin transformed to log (ppb+1) as the response, location and maize genotype as random effects, and days to anthesis (DTA), grain protein, kernel bulk density, kernel size and kernel texture as fixed effects identified DTA and kernel bulk density as significant (Table 3). Maize genotype only explained 3% of the variance in aflatoxin but was insignificant when included instead as a fixed effect in the model. No significant genotypic best linear unbiased predictors (GBLUPs) for aflatoxin were identified in the model. We further estimated GBLUPs for DTA in a mixed model with location and genotype as random effects, and used the GBLUPS to group genotypes into three maturity groups (Table 3). The three maturity groups consisted of early (n=63), intermediate (n=79) and late (n=63) genotypes. Analysis of variance showed that the maturity groups differed significantly (P<0.0001) in aflatoxin level. Aflatoxin did not differ significantly (P>0.05) among the maize genotypes or kernel texture within individual maturity groups (Table 3). Aflatoxin level did not differ significantly (P>0.05) between the early (geometric mean, GM=3.1 ppb) and intermediate (GM=3.9 ppb) groups, but was significantly (P<0.05) lower in the early and medium maturity groups than than in the late maturity (GM=8.2 ppb) group (Table 4).

Grain moisture increased with maturity (early, 26%; intermediate, 27%; late, 28%) (Table 4). Kernel bulk density and protein were significantly (P<0.05) less in the late maturity (density, 0.745 grams/cm^3; protein, 8.5%) group than in the early (density, 0.754 grams/cm^3; protein, 8.7%) and intermediate (density, 0.754 grams/cm^3; protein, 8.7%) groups (Table 4). A negative correlation was observed between aflatoxin and kernel bulk density in the intermediate and late maturity groups, but not in the early maturity group (Table 5). The early maturity group had twice as much ear rot than the late maturity group (Table 4). Grain yield did not differ significantly (P=0.267) among the maturity groups (Table 4). High grain moisture was significantly associated with increased aflatoxin accumulation in the early and late maturity groups, but not in intermediate category (Table 5). An increase in grain yield was associated with high grain moisture at harvest and protein content in all maturity groups (Table 5).

The agronomic traits that were associated with kernel bulk density were included in separate mixed models for the low and the recommended N treatments with DTA, kernel texture, protein, and size as fixed effects; the low N model included genotype and location as random effects, and the recommended N model included genotype and season as random effects (Table 6). Maize genotype accounted for at least 24% of the variance in kernel bulk density. Flowering time and grain protein were consistently associated with kernel bulk density under both low and the recommended N, while kernel texture was significant only in the low N model (Table 6).

We investigated whether kernel bulked density influenced aflatoxin accumulation. Because we expected that maize kernel density would differ in genotypes of varying kernel texture, we investigated the trends of aflatoxin accumulation among maize of different kernel types. A comparison of kernel bulk density means among the genotypes (n=85) that did not have detectable aflatoxin under the recommended N and with matching samples under low N (with or without aflatoxin) showed similar trends among the kernel types (Table 7). Although kernel types did not differ in density under recommended N, the expected decreasing trend was observed as follows: flint>semi-flint>semi-dent > dent. An almost similar trend in kernel density was observed under low N, flint>semi-flint>dent>semi-dent (Table 6). Interestingly, the percentage of samples with either detectable aflatoxin or above the regulatory limit showed a trend that was almost reverse of that observed in the kernel bulk density. Furthermore, a chi-square test for the proportion of samples with either detectable (>1 ppb) or above regulatory limit (10 ppb) under low N showed that none of the proportions differed from the non-target categories (<1 or <10 ppb, respectively) (Table 7).

The relationships between kernel bulk density or protein and days to anthesis were determined in a subset of entries that did not have detectable aflatoxin under the recommended N (n=85) and low N (n=27). A negative correlation was observed between kernel bulk density and days to anthesis under the recommended N (r= -0.26, P=0.0008) (Fig. 1). However, protein and days to anthesis were uncorrelated (r=0.12, P=0.27) under recommended N (Fig. 1). Kernel bulk density or protein and days to anthesis were not correlated under low N in the two sites (P>0.05).

To investigate whether colonization of maize by *A. flavus* reduced kernel density under low N, the following tests were conducted. First, we conducted a paired t-test and a correlation test with the kernel bulk density between the two field replicates of genotypes (n=17) that did not have aflatoxin. We found that the replicates did not differ significantly (t-ratio=-1.579, P=0.134) in kernel density, and were marginally correlated (r=0.46, P=0.06). Second, we compared the means of kernel bulk density from two field replicates of a subset of maize genotypes (n=22) for which one replicate had detectable aflatoxin while the other did not have detectable aflatoxin. We observed a significantly lower kernel density in the toxin-contaminated samples than in the non-toxic (0.737 grams/cm^3 v. 0.755 grams/cm^3; t-ratio= -2.345, P=0.029), while the densities were uncorrelated (r=0.28, P=0.199). To see whether the differences in colonization could be attributed in grain moisture, we compared the differences in densities of the toxic and non-toxic paired replications with the differences in grain moisture of the corresponding entries at harvest. The differences in the density and grain moisture were uncorrelated (r=0.1, P=0.762).

The relationships among kernel colonization, density and aflatoxin were investigated by estimating the amount of *A. flavus* DNA (biomass) in maize samples with varying levels of aflatoxin under low N (Table 5 and Fig. 2). Pre-harvest aflatoxin was positively correlated with fungal biomass (r=0.4, P=0.0286). No correlation was observed between fungal biomass and kernel bulk density (r= -0.28, P=0.128). Similarly, grain moisture at harvest was uncorrelated with fungal biomass (r=0.11, P=0.556) (Table 5). A comparison of fungal biomass among different pre-harvest aflatoxin categories showed that samples with non-detectable aflatoxin had the least colonization. Biomass did not differ significantly (P>0.05) between the pre-harvest aflatoxin categories 1 – 10 and 10 – 100 ppb (Fig. 2). Fungal biomass was highest in the upper pre-harvest aflatoxin category, 100 ppb (Fig. 2).

Maize was colonized by sclerotigenic (those that produced sclerotia) and non- sclerotigenic *A. flavus* strains of varying toxigenicity potential (Table 8). Based on the ITS sequence data, the pairwise haplotypic variation of the isolates ranged 3 to 34 nucleotides (Supplemental Table 2). Isolates of the

three S strains were toxigenic, but their toxigenicity was not higher than that of the other strains (Table 8). Toxigenicity potential ranged 0 to 7,400 ppb and was not correlated with mean pre-harvest aflatoxin (r=0.17, *P=0.538*). The isolate with the highest aflatoxigenicity did not produce sclerotia, and was identified in a plot which had a high pre-harvest aflatoxin (4,500 ppb) accumulation in maize. Maize from 42% of the sampled plots (5/12) had low or no detectable pre-harvest aflatoxin, but the isolates identified in the plots had aflatoxigenicity potential ranging from 252 to 2,050 ppb. Forty-four percent (7/16) of isolates did not produce aflatoxin (atoxigenic) or produced low (<10 ppb) in the aflatoxin-production inducing media. Twenty-five percent (3/12) of the plots that did not have or had low aflatoxin contamination (<10 ppb) were only colonized by atoxigenic strains (Table 8). Interestingly, two plots had 350 and 500 ppb aflatoxin levels, but the only isolates identified in each of them were atoxigenic or produced little aflatoxin in media (Table 8).

Discussion

The current sought to provide insights into the *A. flavus*-maize pathosystem by analysis of a set of diverse maize germplasm that had been grown under natural infection at an aflatoxin hotspot in Kenya with two levels of nitrogen fertilization. Although the use of natural inoculum meant that there was considerable heterogeneity in fungal colonization, the diverse maize germplasm used allowed us to make inferences about the relationship between agronomic traits and aflatoxin accumulation. We provide evidence on the influence of soil nitrogen on aflatoxin accumulation, as well as on the association between maize maturity (flowering time) and susceptibility to *A. flavus* colonization and aflatoxin accumulation.

Higher aflatoxin accumulation was observed in maize grown under low soil nitrogen conditions compared to that under the recommended N. Similar findings were reported by Blandino et al. (9) in a three-year trial in northwest Italy, wherein N-dosage was varied under natural *A. flavus* infection, and by Jones et al. (33) in their evaluation of pre-harvest aflatoxin contamination in corn hybrids grown in North Carolina under artificial *A. flavus* inoculation. In the current study, soil analysis prior to the low N experiments had shown that the soils across the two sites had a deficit of nitrogen and phosphorus, which is typical of the majority of maize farms in eastern Kenya and is a known cause for low maize yield (71,32). Soil nitrogen deficiency is a major plant stress factor. Nitrogen is required for synthesis of proteins, including those involved in plant defense, such as resistance-associated proteins (RAPs) (12,13) and zein, which is responsible for endosperm hardness (23). General improvement in crop vigor and resistance to fungal infection has been reported from soil N amendment (75,16,15); here this was further evidenced by the higher grain protein content, kernel bulk density and grain yield under recommended N.

Aflatoxin accumulation was higher in late-maturing maize compared to early and intermediate genotypes. The majority of studies carried out in the USA have reported a positive correlation between flowering time and aflatoxin accumulation (5,51), with the exception of Henry (31), who reported that aflatoxin resistance co-segregated with early maturity. Early maturity can enable a crop to escape late season stressors such as drought and low N, and has been hypothesized to be associated with reduced aflatoxin accumulation (5,46). Plant stress at flowering and during kernel development could cause decreased crop vigor and kernel integrity, as well as general susceptibility to biotic stress factors (58,56). Here late maturing lines had the lowest grain protein and kernel bulk density, implying that stress could have led to poor kernel development. Grain yield did not differ among the maturity groups,

suggesting maturity-associated stress factors might have caused quality but not quantity differences. Alternatively, the observed higher grain moisture at harvest in late-maturing genotypes may have compensated for the grain yield. Although multiple stress factors might have existed, increased N stress in late maturing maize genotypes might have led to less crop vigor and increased susceptibility to *A. flavus*. Loss of soil nitrogen over time through leaching, denitrification and volatization during crop development could be a disadvantage in late maturing maize because of a possible occurrence of critical nitrogen shortage during kernel development stages (22).

We observed a negative correlation between kernel bulk density and pre-harvest aflatoxin levels. To explore this further, we examined the correlation between density and *A. flavus* fungal biomass with a subset of samples. We found that fungal biomass was not correlated with kernel density. In a separate test, we compared the density of paired sets of field replicates where one replicate was contaminated and other was not, assuming that some entries had escaped colonization. We observed an overall higher kernel density in the non-toxic replicates. However, some replicates with detectable aflatoxin had higher density than their corresponding non-contaminated replicates. While it is possible that colonization caused a decrease in kernel density by lowering the biomass of the kernel, the low density in the toxic replicate could have also be due to stress factors which might have as well made maize more vulnerable to fungal colonization. The observed lack of correlation between fungal biomass and kernel density implies that kernel density and/or toxin levels were influenced by other factors (e.g., plant genotype and stress for density; fungal toxigenicity for toxin levels). Given that aflatoxin accumulation was not correlated with ear rot scores, maize was possibly colonized by other ear rot causing fungi such as *Fusarium verticillioides*. We did not find a significant correlation between ear rot and kernel density. Other biotic stress factors such as insects and other maize pathogens might have caused the observed reduction in kernel bulk density.

There are several interactions that can be hypothesized to explain the negative correlation between kernel density and aflatoxin levels. First, colonization of a maize kernel by *A. flavus* or any other fungus is likely to reduce its biomass and thus its density. Secondly, kernel textural features (influenced both by genotype and environment) could affect fungal colonization. Flint maize genotypes have compact, vitreous endosperm, and have previously been reported to be more resistant to aflatoxin accumulation than dent maize (6,55). Nitrogen stress is known to influence kernel development; stressed plants produce kernels with lower density and increased susceptibility to fungal colonization and aflatoxin accumulation. Because soil N diminishes over time, late maturing maize genotypes are expected to experience more low N stress than the earlier maturing lines. Given these complex

interactions of genotype, environment and fungal colonization processes, it was difficult to determine the extent to which low kernel density led to increased colonization v. colonization caused a decrease in kernel density.

While aflatoxin levels were associated with kernel density, we were not able to support the hypothesis that aflatoxin accumulation was lower in flint maize. While we found that flint maize kernels were denser than dent maize kernels, kernel texture was not associated with aflatoxin accumulation. The highest proportion of samples with detectable and > 10 ppm aflatoxin levels were, however, in dent maize. Guo et al. (25) hypothesized that aflatoxin resistance was correlated with endosperm hardness, which could enhance kernel integrity and reduce penetration by fungal pathogens. Bétran et al. (6) reported lower pre-harvest aflatoxin in flint maize hybrids compared to the dent. Flint endosperm is characterized by vitreousness, high compactness of starch, high kernel bulk density and high protein content compared to dent endosperm (63). A lack of consistent association between endosperm texture and aflatoxin was previously reported by Lillehoj et al. (44) in their multi-location evaluation of diverse maize lines for pre-harvest aflatoxin resistance. Xenia effects caused by open-pollination could influence endosperm chemical composition, and thus may have led to inconsistencies in the role of kernel texture in pre-harvest aflatoxin resistance (69,42).

We observed a negative correlation between kernel bulk density and flowering time in non-contaminated maize that was grown under recommended N. Furthermore, aflatoxin was not correlated with kernel density in the early maturing maize lines under low N. This finding could imply that kernel density reduction was caused by maturity-related stressors e.g., nitrogen or other biotic stressors at later stages of kernel development. Alternatively, the grain of late-maturing genotypes may not have fully reached maturity at harvest, and the incomplete starch conversion would have led to lower density. The decline in soil N towards the end of the season might have influenced kernel development, reducing kernel density over time. Early-maturing maize lines would thus have better kernel development, and higher density compared to later-maturing lines because the latter would encounter N deficiency and critical stages of kernel development (22). Although other biotic maize stressors may have been involved and could increase over time, and significantly affect kernel development, we only analyzed *A. flavus* colonization and aflatoxin contamination in the current study.

Maize was colonized by *A. flavus* strains of varying aflatoxigenicity. Variability in aflatoxin production potential among *A. flavus* populations from eastern Kenya has been reported previously (57,65). This study shows that diverse maize grown in the same field was colonized by *A. flavus* populations of varying aflatoxin production potential. Colonization of one maize ear with multiple

isolates of varying toxin production potential is a likely impediment to screening for aflatoxin resistance in aflatoxin hotspots because they introduce a complexity in GxE. Besides the expected interaction between maize and individual *A. flavus* isolates, intra-species interaction between fungal isolates could occur, possibly influencing growth and metabolic activities of individual fungal isolates (47). Intra-specific competition for nutrients has been reported among *A. flavus* genotypes (43,48). The observed lack of significant correlation between pre-harvest aflatoxin accumulation and mean aflatoxigenicity of isolates in a plot suggests the interaction among fungal isolates within maize ears. The observed lack of aflatoxin production in the media by isolates that had colonized maize with detectable aflatoxin was unexpected, but could be due to sampling error. Previous studies have shown a high success rate of aflatoxin-production induction in YESA medium (1).

We observed a lower correlation between fungal biomass and pre-harvest aflatoxin than what was previously reported in artificially inoculated trials (50). The interaction between *A. flavus* strains of varying aflatoxigenicity within a maize ear could influence pre-harvest aflatoxin accumulation. The concept of interaction is demonstrated here by the observed aflatoxigenicity of isolates that had colonized 42% of the sampled plots with low or undetectable pre-harvest aflatoxin levels. Certain strains of atoxigenic *A. flavus* are known to inhibit aflatoxin production of the toxigenic strains, and they have in turn been applied as biocontrols (19). These findings show that maize response to *A. flavus* and the observed pre-harvest aflatoxin accumulation are highly influenced by the biology of the prevailing *A. flavus* populations in addition to other factors. The observed lack of correlation between ear rot and aflatoxin suggests that the disease was not cause by *A. flavus*. We suspect that the observed ear rot was mainly caused by *Fusarium verticillioides*, another major mycotoxigenic fungal species which is endemic to maize-growing zones (52,35). Previous studies have shown a higher correlation between ear rot and incidence of *F. verticillioides* compared to *A. flavus* in the tropics (2,49).

This study shows that aflatoxin accumulation differed among maize maturity groups, but no significant maize genotypic differences were reported based on low N trials. The general lack of correlations between aflatoxin or ear rot values of the two locations under low N was likely caused by variation in stress factors, exacerbated by the non-uniformity of natural infection. We have shown that soil N amendment reduced aflatoxin accumulation in maize under natural infection in the Kenyan aflatoxin hotspot areas. In addition to synthetic fertilizer application, which is costly to resource-poor farmers, other sustainable methods such as intercropping with leguminous crops could be used to improve soil nitrogen fertility. Our previous studies in eastern Kenya had shown reduced aflatoxin in intercropped maize compared to levels in monocropped maize (see chapter 3). Breeding for nitrogen

use efficiency could also reduce maize stress and hence reduce aflatoxin accumulation. Improved experimental designs including artificial inoculation, fungicide control treatment, and naturally inoculated treatments with replication over years, seasons, and location are necessary for effective evaluation of aflatoxin resistance in diverse maize germplasm. Maize maturity should also be considered in aflatoxin resistance breeding programs.

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Tables and Figures

Table 1. Descriptive statistics of agronomic traits and aflatoxin contamination in maize test-crosses grown under two levels of nitrogenous fertilizer; low nitrogen (20 kg/ha) and regionally-recommended rate (60 kg/ha), at two locations in eastern Kenya. Trait means were compared using univariate analysis of variance. Trait mean values followed by similar letters across environments do not differ significantly (Tukey's HSD, α=0.05).

		Embu	Kiboko		
		Soil nitrogen status/ season			
	Statistics	Low N / Long season	Low N / Long season	Recommended N / Long season	Recommended N/ Short season
Trait	Lines or entries (*n*)	123	123	123	123
Aflatoxin (ppb)	Top quartile range	21-18,725	18-766	1.9-552	0
	% with toxin	55	55	36	1
	% above 10 ppb	30	36	15	0
Days to anthesis	Range	71-99	60-74	58-67	56-69
	Median	83	67	63	63
	Mean (±SD)	83±5 A	67±3 B	63±2 C	63±3 C
Ear rot (%)	Range	0-46.7	0-22.7	0-51.5	
	Mean (±SD)	9.8±4.6 A	3.9±1.3 B	8.2 ±1.9 A	
[1]Grain moisture after oven-drying (%)	Range	10.3-13.4	6.1-8.7	8.1-9.6	8.3-9.7
	Mean(±SD)	11.4±0.5 A	8.1±0.3 D	8.8±0.3 C	9.0±0.3 B
Grain moisture at harvest (%)	Range	20.3-36.4	11.7-24.9	16.7-23.8	
	Mean(±SD)	30.0±3.0 A	20.1±2.6 C	21.6±1.0 B	
Grain yield (t/ha)[3]	Range	0.8-7.4	2.2-5.9	6.8-13.6	4.8-18.0
	Median	4.2	3.3	9.8	8.6
	Mean(±SD)	4.3±0.1 C	3.4±0.1 D	10.0±0.1 A	8.7±0.2 B
[2]Kernel bulk density (grams/cm^3)	Range	0.639-0.803	0.691-0.817	0.716-0.810	0.723-0.816
	Median	0.756	0.750	0.770	0.768
	Mean(±SD)	0.754±0.025 B	0.750±0.028 B	0.769±0.018 A	0.769±0.018 A
Percent kernel protein (%)	Range	7.6-10.6	6.7-9.7	9.0-11.9	
	Median	9.1	8.1	10.6	
	Mean(SD)	9.1±0.7 B	8.2±0.5 C	10.5±0.6 A	

[1]Grain moisture after kernels were oven-dried at 40°C for 12 hours in the laboratory.
[2]Kernel bulk density based on a mass to volume ratio after oven-drying of the grain at 40°C for 12 hours.
[3]Grain yield was adjusted to 12.5% moisture

Table 2. Pearson correlations within (gray, diagonal) and among agronomic traits, ear rot (ER) and aflatoxin (AF) in maize test-crosses (*n*=205) that were grown and naturally infected by aflatoxin-producing fungi under low nitrogen soils (20 kg/ha) at Embu (above the diagonal) and Kiboko (below the diagonal), Kenya. DTA: days to anthesis; GY: grain yield estimate (t/ha); GMAH: percent grain moisture at harvest; GMAD: percent grain moisture after oven-drying at 40°C for 24 hours; KD: kernel bulk density (grams cm^{-3}). *, **, ***: significant at 0.05, 0.001 and <0.0001, respectively.

	AF(ppb)	ER (%)	DTA	GY (%)	Protein (%)	GMAH (%)	GMAD (%)	KD(grams/cm^3)
AF	0.06^{ns}	-0.10(0.088)	0.15 (0.011)*	0.05 (0.392)	-0.04 (0.539)	0.22 (0.0001)***	0.004 (0.945)	-0.16 (0.007)**
ER	-0.01 (0.880)	-0.07^{ns}	-0.43 (<0.0001)***	0.08 (0.126)	0.42 (<0.0001)***	-0.17 (0.0006)**	0.06 (0.299)	0.21 (0.0002)**
DTA	0.14 (0.044)*	0.01 (0.892)	0.53***	-0.26 (<0.0001)***	-0.32 (<0.0001)***	0.36 (<0.0001)***	-0.07 (0.217)	-0.24 (<0.0001)***
GY	0.38 (<0.0001)***	-0.2 (0.004)*	-0.09 (0.191)	0.16*	0.30 (<0.0001)***	0.14 (0.005)**	0.19 (0.0008)**	0.09 (0.120)
Protein	-0.03 (0.694)	-0.06 (0.364)	-0.15 (0.035)*	0.13 (0.072)	0.30***	-0.10 (0.035)*	0.15 (0.010)*	0.30 (<0.0001)***
GMAH	0.40 (<0.0001)***	-0.16 (0.019)	0.21 (0.002)*	0.47 (<0.0001)***	0.03 (0.654)	0.22*	-0.07 (0.232)	-0.06 (0.269)
GMAD	-0.03 (0.692)	-0.06 (0.404)	-0.05 (0.454)	0.04 (0.532)	0.20 (0.003)*	-0.05 (0.505)	0.14*	0.11 (0.063)
KD	-0.26 (<0.0001)***	-0.03 (0.709)	0.05 (0.468)	-0.01 (0.899)	0.14 (0.05)*	-0.12 (0.099)	0.20 (0.004)*	0.32***

Table 3. Effect tests for association of maize agronomic traits with aflatoxin accumulation in combined maize test-crosses (*n*=205) and within maturity groups of the hybrids. Maize was grown under low soil nitrogen (20 kg/ha) and was naturally infected by aflatoxin-producing fungi at two locations in eastern Kenya.

Source	DF	F Ratio
Combined genotypes (*n*=205)[1]		
Days to anthesis	1	7.745**
Grain protein (%)	1	0.078^{ns}
Kernel bulk density (grams cm^{-3})	1	19.815***
Kernel size	2	2.549^{ns}
Kernel texture	3	0.161^{ns}
Genotypes within maturity groups[2]		
Early(*n*=63)		
Genotype	62	1.270^{ns}
Kernel texture	3	0.345^{ns}
Intermediate (*n*=79)		
Genotype	78	0.729^{ns}
Kernel texture	3	0.125^{ns}
Late (*n*=63)		
Genotype	62	1.206^{ns}
Kernel texture	3	0.718^{ns}

[1]Location and Genotypes were included as random factors in the mixed model.
[2]Genotypes included in the model as fixed factors within each maturity category. Maturity groups were identified based on best linear unbiased predictors of genotypes in the days to anthesis with location and genotype as random factors in the mixed model.
^{ns} not significant at α=0.05. *,**,***, factors significant at α=0.05,0.001 and 0.0001, respectively.

Table 4. Mean comparisons of maize agronomic traits, ear rot and aflatoxin among maturity categories of maize test-crosses that were grown under low soil nitrogen (20 kg/ha) at two locations in eastern Kenya. Aflatoxin was transformed to log (ppb+1) prior to input as a response variable in the model. Geometric means of aflatoxin levels are shown.

Trait	Maturity	*n*	Mean(±SE)	Geometric mean (ppb)	95% Confidence interval (CI)	
					Lower	Upper
Aflatoxin (ppb)	Early	153	0.61±0.07 B	3.1	2.0	4.6
	Intermediate	196	0.69±0.06 B	3.9	2.7	5.5
	Late	152	0.97±0.07 A	8.4	5.8	11.9
Kernel bulk density (grams/cm^3)	Early	153	0.754±0.002 A		0.750	0.758
	Intermediate	196	0.754±0.002 A		0.750	0.758
	Late	152	0.745±0.002 B		0.740	0.749
Grain moisture at harvest (%)	Early	187	26.0±0.4 B		25.2	26.8
	Intermediate	234	27.0±0.4 AB		26.3	27.6
	Late	188	27.7±0.4 A		27.0	28.5
Grain Yield (t/ha)	Early	187	3.9±0.1 A		3.7	4.1
	Intermediate	234	4.1±0.1 A		3.9	4.2
	Late	188	3.9±0.1 A		3.7	4.1
Protein (%)	Early	187	8.7±0.05 A			
	Intermediate	233	8.7±0.05 A			
	Late	188	8.5±0.05 B			
Ear rot (%)	Early	187	9.2±0.6 A		8.6	8.8
	Intermediate	234	7.1±0.6 B		8.6	8.8
	Late	188	4.0±0.6 C		8.4	8.6

Means followed by similar letters within a trait do not differ significantly (Tukey's HSD, α=0.05).

Table 5**:** Correlations of agronomic traits and aflatoxin-associated factors in maize test-crosses grown under low N (20 kg/ha), and naturally infected by aflatoxin-producing fungi at two locations (Kiboko and Embu) in eastern Kenya. Correlations among agronomic traits, ear rot and aflatoxin in early (above first diagonal), intermediate (above second diagonal) and late (below third diagonal) maize maturity groups. Correlation among agronomic traits, aflatoxin and *A. flavus* biomass (bottom).

	GMAH	Protein	Ear rot	KD	GY	Aflatoxin
	Correlations among traits within maturity groups[1]					
GMAH	**Early**	0.48***	0.35***	0.16*	0.47***	0.22**
Protein			0.39***	0.34***	0.39***	0.18*
Ear rot				0.09^{ns}	0.08^{ns}	0.01^{ns}
KD					0.09^{ns}	-0.06^{ns}
GY						0.33**
	Intermediate					
GMAH		0.43***	0.18**	0.08^{ns}	0.35***	0.06^{ns}
Protein			0.47***	0.17*	0.39***	-0.08^{ns}
Ear rot				0.21**	0.13*	-0.09^{ns}
KD					0.07^{ns}	-0.16*
GY						0.09^{ns}
Aflatoxin						
GMAH	**Late**	0.38***	-0.06^{ns}	-0.04^{ns}	0.33***	0.12^{ns}
Protein			0.27**	0.20*	0.40***	-0.14*
Ear rot				0.06^{ns}	0.07^{ns}	-0.17*
KD					0.09^{ns}	-0.40***
GY						0.02^{ns}
	Correlation of selected agronomic traits with fungal biomass (q-PCR, *n*=30)[2]					
KD						-0.46*
GMAH				-0.42*		0.29^{ns}
Fungal biomass	0.11^{ns}			-0.28^{ns}		0.40*

[1]Number of samples of each trait within maturity categories: Late (aflatoxin and KD or kernel bulk density, 152; ear rot, grain yield (GY), grain moisture at harvest (GMAH) and kernel percent protein, 188), Early (Aflatoxin and kernel bulk density, 153; Protein, GMAH, GY and ear rot, 187). Intermediate (aflatoxin and kernel bulk density, 196; kernel percent protein, 233, GMAH and GY, 234. [2]Fungal Biomass assessed using real-time PCR on a subset of samples (*n*=30) that were selected to represent different levels of aflatoxin contamination in maize grown at Embu.

Table 6**.** Effect tests for association of maize agronomic traits with kernel bulk density in combined maize test-crosses (*n*=205) grown under low soil nitrogen (20 kg/ha), and in a subset of genotypes (*n*=85) that were grown under the regionally-recommended nitrogen level (60 kg/ha) and did not have detectable aflatoxin. Model under low N included maize genotype and location as random effects. Model under the recommended N included season and genotype as random effects because maize was grown under the recommended N in two seasons at one location, Kiboko, eastern Kenya.

Kernel bulk density (grams/cm^3) combined genotypes (*n*=205) under low N			Kernel bulk density (grams/cm^3) in a subset of genotypes (*n*=85) under the recommended N	
Source	DF	F Ratio	DF	F Ratio
Days to anthesis	1	9.570**	1	7.8157**
Grain protein (%)	1	42.012***	1	5.0763*
Kernel size	2	1.027^{ns}	1	1.8161^{ns}
Kernel texture	3	15.115**	3	2.1038^{ns}

Table **7.** Test of association between kernel texture, kernel bulk density and aflatoxin accumulation in maize. Maize genotypes (*n*=85) which did not have detectable aflatoxin under the recommended nitrogen (60 kg/ha) in the long and short seasons of 2011/2012 at Kiboko, eastern Kenya were identified. Corresponding samples which were grown under low N (20 kg/ha) at Kiboko and Embu, eastern Kenya (long season of 2012) and contaminated with aflatoxin were used for comparison of aflatoxin and kernel bulk density among kernel types.

		Low nitrogen			Recommended nitrogen
Kernel type	*n*	% with aflatoxin	% > RL	LSM(±SE) of Kernel bulk density (grams/cm3)	LSM(±SE) of Kernel bulk density (grams/cm3)
Flint	97	49	29	0.757±0.003 A	0.772±0.003A
Semi-Flint	40	45	25	0.746±0.004 AB	0.770±0.003 A
Semi-Dent	26	58	35	0.732±0.005 B	0.771±0.003 A
Dent	19	74	47	0.740±0.006 AB	0.768±0.003 A
χ^2 (df=3)		4.947^{ns}	2.949^{ns}		

Least squares of means, LSM, followed by similar letters in the same column do not differ significantly (Tukey's HSD, α=0.05).

^{ns,} chi-square test not significant (α=0.05). RL, aflatoxin regulatory limit per world food program (WFP) regulation, 10 ppb. Chi square is based on proportion of samples with (>1 ppb) or without aflatoxin (<1ppb) or below (<10 ppb) or above (>10 ppb) the WFP aflatoxin B1 limit in human food.

Table 8. Aflatoxigenicity of *Aspergillus flavus* isolates that were identified in maize that was colonized before harvest in random field trial plots under low soil nitrogen (20 kg/ha) at Embu, eastern Kenya in the long season of 2012.

Isolate	Field Plot	Pre-harvest aflatoxin (ppb)	Aflatoxigenicity (ppb)	Strain[1]
AF14	E391	0	0	NA
AF04	E390	0	0	L
AF20	E579	7	0	NA
AF21	E579	7	0	NA
AF08	E379	500	0	NA
AF12	E580	20	2	NA
AF03	E380	350	2	NA
AF15	E318	350	384	S
AF19	E522	22,050	935	NA
AF22	E580	11	964	NA
AF05	E393	0	990	S
AF23	E580	11	1,136	NA
AF31	E390	0	1,309	L
AF29	E603	20	1,500	NA
AF17	E393	0	2,050	S
AF33	E362	4,500	7,400	NA

[1]Assessed based on number and size of sclerotia that were produced when the isolates were grown in three replicates in V8-agar (67,47). NA, means that isolate did not produce sclerotia and could not be classified as either S or L (47).

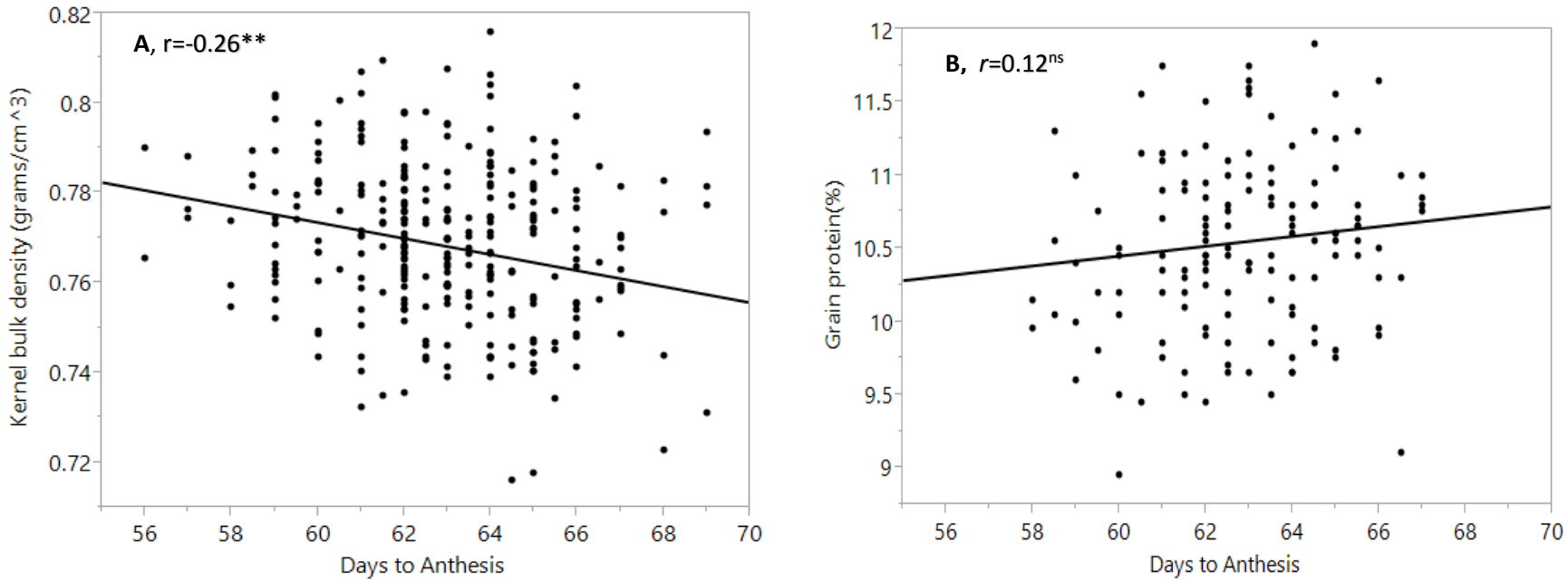

Fig. 1. Relationship between kernel bulk density (grams/cm^3) (**A**) or grain protein (%) (**B**) and days to anthesis for a subset of maize test-crosses (*n*=85) that did not have detectable aflatoxin when grown under the recommended soil nitrogen (60 kg/ha) during the long season of 2012 at Kiboko, eastern Kenya.

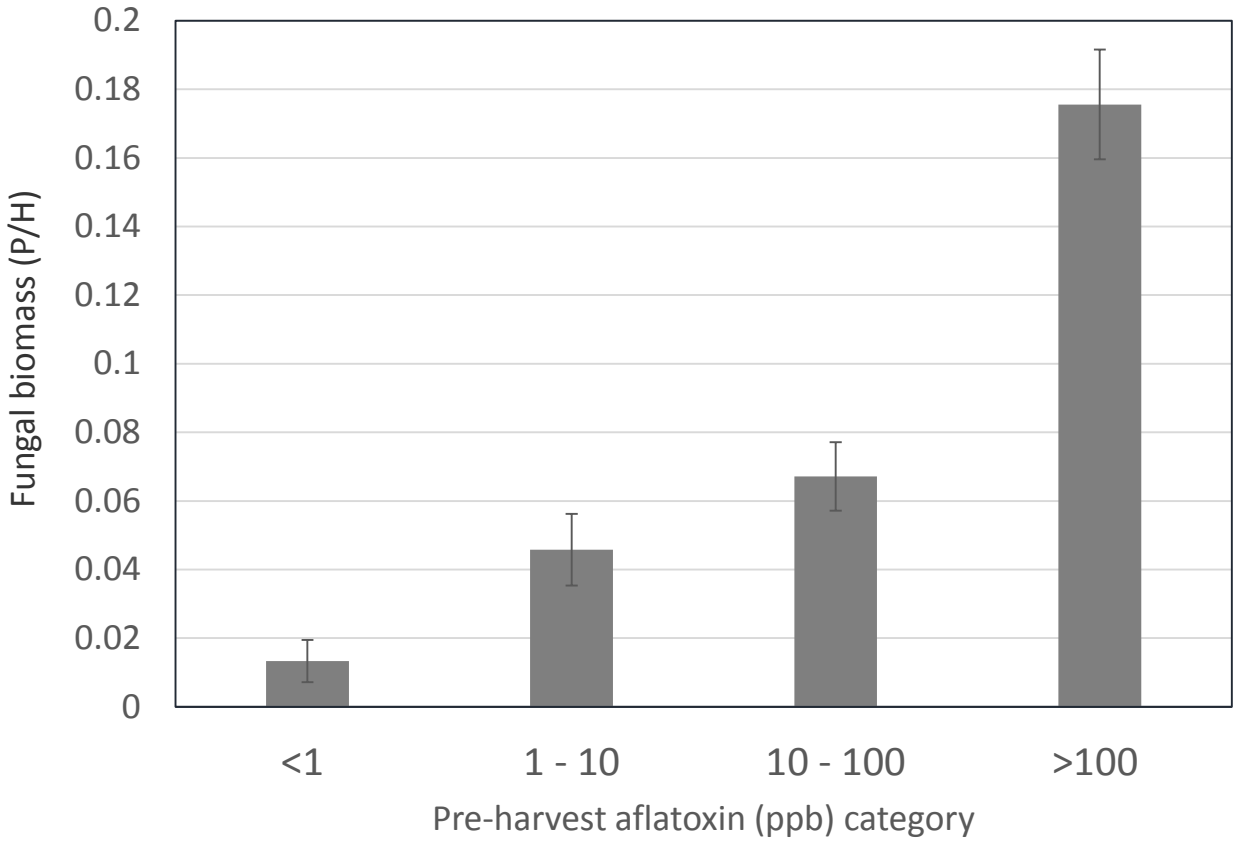

Fig 2. Fungal biomass in maize samples (*n*=42) of varying level of pre-harvest aflatoxin contamination under low nitrogen (20 kg/ha) conditions. Fungal biomass represents the ratio of *A. flavus* to maize DNA as estimated using real-time PCR with maize samples whose pre-harvest aflatoxin levels had been analyzed using ELISA method at a lower detection limit of 1 ppb.

SUPPLEMENTAL TABLE 1**:** Maize genotypic codes, pedigree and maturity groups.

Code	**Pedigree**	**Maturity**
IMAS-006	CKL05009/CML312 z SR	Early
IMAS-009	CKL05017/CML312 z SR	Early
IMAS-014	CKL05024/CML312 z SR	Early
IMAS-017	INTA/INTB-B-41-B-1-1-B/CML312 z SR	Early
IMAS-025	CZL03007/CML312 z SR	Early
IMAS-033	CZL068/CML312 z SR	Early
IMAS-034	CZL03011/CML312 z SR	Early
IMAS-040	VL05119/CML312 z SR	Early
IMAS-042	VL055065/CML312 z SR	Early
IMAS-070	CML379/CML312 z SR	Early
IMAS-087	CML505/CML312 z SR	Early
IMAS-092	CLWQ240/CML312 z SR	Early
IMAS-118	CLQRCWQ26/CML312 z SR	Early
IMAS-122	CLWQ254/CML312 z SR	Early
IMAS-127	(200-6xGUAT189)(51-2-1)F1-B-xP84c1F26-2-2-4-B-2-B]F102-1-2-2-3x[KILIMAST94A]-30/MSV-03-2-10-B-1-B-B-xP84c1F27-4-1-6-B-5-B]-2-2-B/CML312zSR	Early
IMAS-130	[(P86S.F*P.S.P.A.AxP.S.P.A.A.TL91A44-3-1-18-2P-2-1-1-3-1)xA.I.R.L.TL91A2(3)-1-4-2-2TL-1-1-B]-3-2-3-1-B/CML312 z SR	Early
IMAS-131	[[KILIMAST94A]-30/MSV-03-1-10-B-1-B-B-1xP84c1F27-4-1-6-B-5-B]F8-3-2-2-1xG16SeqC1F47-2-1-2-1-BBBB-B-xP84c1F26-2-2-6-B-3-B]-3-1-B/CML395]-1-1-B/CML312 z SR	Early
IMAS-132	[200-7xMAIZSANANDRES-F1-B-xP84c1F26-2-2-4-B-2-B]F93-3-2-1-3xP43C9-1-1-1-1-1-BBBB-1-xP84c1F26-2-2-6-B-3-B]-1-1-BxCML486]-1-1-B/CML312 z SR	Early
IMAS-134	[CML440/[[[K64R/G16SR]-39-1/[K64R/G16SR]-20-2]-5-1-2-B*4/CML390]-B-38-1-B-7-#-B//ZM303c1-243-3-B-1-1-B]-2-1-B/CML312 z SR	Early
IMAS-140	[M37W/ZM607#bF37sr-2-3sr-6-2-X]-8-2-X-1-BB-B-xP84c1F27-4-3-3-B-1-B]F29-1-1-1-7x[KILIMAST94A]-30/MSV-03-2-10-B-1-B-B-xP84c1F27-4-1-6-B-5-B]-1-3-B/CML312SR]-1-1-BCML312 z SR	Early
IMAS-145	CLA18/CML312 z SR	Early
IMAS-149	CL-G1844/CML312 z SR	Early
IMAS-150	CL-P10201/CML312 z SR	Early
IMAS-158	DTPWC9-F115-1-2-1-2-B/CML312 z SR	Early
IMAS-160	DTPWC9-F17-1-3-1-1-B/CML312 z SR	Early
IMAS-165	DTPWC9-F66-2-1-1-2-B/CML312 z SR	Early
IMAS-166	DTPWC9-F67-1-2-1-2-B/CML312 z SR	Early
IMAS-167	DTPWC9-F67-2-2-1-B/CML312 z SR	Early
IMAS-168	DTPWC9-F73-2-1-1-1-B/CML312 z SR	Early
IMAS-170	DTPYC9-F114-2-4-1-1-B/CML312 z SR	Early
IMAS-171	DTPYC9-F13-2-3-1-2-B/CML312 z SR	Early
IMAS-172	DTPYC9-F143-5-4-1-2-B/CML312 z SR	Early
IMAS-173	DTPYC9-F15-3-4-1-1-B/CML312 z SR	Early
IMAS-175	DTPYC9-F46-1-2-1-2-B/CML312 z SR	Early
IMAS-176	DTPYC9-F46-3-9-1-1-B/CML312 z SR	Early
IMAS-178	DTPYC9-F72-1-2-1-1-B/CML312 z SR	Early
IMAS-179	DTPYC9-F74-1-1-1-1-B/CML312 z SR	Early
IMAS-180	DTPYC9-F74-3-4-1-3-B/CML312 z SR	Early
IMAS-181	DTPYC9-F86-1-1-1-1-B/CML312 z SR	Early
IMAS-198	LaPostaSeqC7-F96-1-5-1-1-B/CML312 z SR	Early

IMAS-202	P502c2-185-3-4-1-3-B-1-BxCML311/CML321xMBRC1BcF114-1-1-1-B-B-B-B-1-1-B/CML312 z SR	Early
IMAS-203	G16 BNSEQ.C4-(F20 x 17)-3-1-4/CML312 z SR	Early
IMAS-204	G16 BNSEQ.C4-(F8 x 17)-4-3-1/CML312 z SR	Early
IMAS-206	G16 BNSEQ.C4-(F8 x 17)-4-2-2/CML312 z SR	Early
IMAS-207	G16 BNSEQ.C4-(F37 x 2)-4-1-3/CML312 z SR	Early
IMAS-226	CL-RCW48/CML312 z SR	Early
IMAS-232	CL-RCW72/CML312 z SR	Early
IMAS-238	CLYN265/CML312 z SR	Early
IMAS-244	CNO7/8-4/CML312 z SR	Early
IMAS-248	CNO7/8-9/CML312 z SR	Early
IMAS-252	CNO7/8-18/CML312 z SR	Early
IMAS-290	CNO7/8-268/CML312 z SR	Early
IMAS-291	CNO7/8-275/CML312 z SR	Early
IMAS-294	CNO7/8-97/CML312 z SR	Early
IMAS-295	CNO7/8-119/CML312 z SR	Early
IMAS-299	CNO8Y/375/CML312 z SR	Early
IMAS-303	CNO8Y/435/CML312 z SR	Early
IMAS-369	SM-189-26/CML312 z SR	Early
IMAS-379	SM-189-36/CML312 z SR	Early
IMAS-395	SM-189-52/CML312 z SR	Early
IMAS-411	SM-189-68/CML312 z SR	Early
IMAS-413	SM-189-70/CML312 z SR	Early
IMAS-421	SM-189-78/CML312 z SR	Early
IMAS-003	CKL05004/CML312 z SR	Intermediate
IMAS-016	INTA/INTB-B-194-B-2-1-B/CML312 z SR	Intermediate
IMAS-018	INTA/INTB-B-86-B-6-1-B/CML312 z SR	Intermediate
IMAS-020	INTA/INTB-B-52-B-1-1-B/CML312zSR	Intermediate
IMAS-021	CZL0619/CML312 z SR	Intermediate
IMAS-028	CZL0617/CML312 z SR	Intermediate
IMAS-031	CZL052 -stockid-C240-27/CML312 z SR	Intermediate
IMAS-032	CZL00001/CML312 z SR	Intermediate
IMAS-037	CZL066/CML312 z SR	Intermediate
IMAS-048	CML130/CML312 z SR	Intermediate
IMAS-052	CML161/CML312 z SR	Intermediate
IMAS-058	CML254/CML312 z SR	Intermediate
IMAS-061	CML312/CML312 z SR	Intermediate
IMAS-064	CML341/CML312 z SR	Intermediate
IMAS-076	CML445/CML312 z SR	Intermediate
IMAS-077	CML451/CML312 z SR	Intermediate
IMAS-079	CML489/CML312 z SR	Intermediate
IMAS-081	CML494/CML312 z SR	Intermediate
IMAS-083	CML497/CML312 z SR	Intermediate

IMAS-088	CML511/CML312 z SR	Intermediate
IMAS-090	CLWN268/CML312 z SR	Intermediate
IMAS-091	CLYN242/CML312 z SR	Intermediate
IMAS-095	CLWQ250/CML312 z SR	Intermediate
IMAS-099	CLYN243/CML312 z SR	Intermediate
IMAS-101	CLYN 226/CML312 z SR	Intermediate
IMAS-105	CLWQ253/CML312 z SR	Intermediate
IMAS-110	CLWN270/CML312 z SR	Intermediate
IMAS-111	CLWN271/CML312 z SR	Intermediate
IMAS-119	CLYN208/CML312 z SR	Intermediate
IMAS-121	CLQRCYQ14/CML312 z SR	Intermediate
IMAS-128	(CML-20XCML-329)-17-3-3-1-B/CML312 z SR	Intermediate
IMAS-133	[CML312/CML444//[DTP2WC4H255-1-2-2-BB/LATA-F2-138-1-3-1-B]-1-3-2-3-B]-2-1-2-B/CML312 z SR	Intermediate
IMAS-142	[P591c4F31-1-1-2-B-B-B/CML312SR]-1-1-B/CML312 z SR	Intermediate
IMAS-143	02SADVL2B-#-17-1-1-B/CML312 z SR	Intermediate
IMAS-146	CLA222/CML312 z SR	Intermediate
IMAS-156	DTPWC9-F104-5-4-1-1-B/CML312 z SR	Intermediate
IMAS-159	DTPWC9-F16-1-1-1-1-B/CML312 z SR	Intermediate
IMAS-174	DTPYC9-F38-4-3-1-1-B/CML312 z SR	Intermediate
IMAS-187	LaPostaSeqC7-F12-2-3-1-2-B/CML312 z SR	Intermediate
IMAS-190	LaPostaSeqC7-F180-3-1-1-1-B/CML312 z SR	Intermediate
IMAS-192	LaPostaSeqC7-F64-2-4-1-1-B/CML312 z SR	Intermediate
IMAS-193	LaPostaSeqC7-F64-2-6-2-2-B/CML312 z SR	Intermediate
IMAS-196	LaPostaSeqC7-F78-2-1-1-1-B/CML312 z SR	Intermediate
IMAS-199	LaPostaSeqC7-F96-1-6-2-2-B/CML312 z SR	Intermediate
IMAS-200	MBRC5BcF4-1-2-1-B/CML312 z SR	Intermediate
IMAS-209	CLRCW106/CML312 z SR	Intermediate
IMAS-215	CLWN201/CML312 z SR	Intermediate
IMAS-216	CLWN240/CML312 z SR	Intermediate
IMAS-222	CLYN231/CML312 z SR	Intermediate
IMAS-224	CL02720/CML312 z SR	Intermediate
IMAS-227	CLRCW88/CML312 z SR	Intermediate
IMAS-228	CLWN211/CML312 z SR	Intermediate
IMAS-236	CLYN260/CML312 z SR	Intermediate
IMAS-242	CLYN262/CML312 z SR	Intermediate
IMAS-243	CL-G2620 /CML312 z SR	Intermediate
IMAS-247	CNO7/8-8/CML312 z SR	Intermediate
IMAS-250	CNO7/8-13/CML312 z SR	Intermediate
IMAS-251	CNO7/8-16/CML312 z SR	Intermediate
IMAS-253	CNO7/8-19/CML312 z SR	Intermediate
IMAS-257	CNO7/8-31/CML312 z SR	Intermediate
IMAS-258	CNO7/8-32/CML312 z SR	Intermediate

IMAS-260	CNO7/8-34/CML312 z SR	Intermediate
IMAS-269	CNO7/8-66/CML312 z SR	Intermediate
IMAS-278	CNO7/8-174/CML312 z SR	Intermediate
IMAS-280	CNO7/8-178/CML312 z SR	Intermediate
IMAS-293	CNO7/8-290/CML312 z SR	Intermediate
IMAS-302	CNO8Y/417/CML312 z SR	Intermediate
IMAS-308	CNO8Y/480/CML312 z SR	Intermediate
IMAS-312	CNO8Y/523/CML312 z SR	Intermediate
IMAS-315	CNO8Y/554/CML312 z SR	Intermediate
IMAS-318	CNO8Y/581/CML312 z SR	Intermediate
IMAS-363	SM-189-20/CML312 z SR	Intermediate
IMAS-387	SM-189-44/CML312 z SR	Intermediate
IMAS-388	SM-189-45/CML312 z SR	Intermediate
IMAS-392	SM-189-49/CML312 z SR	Intermediate
IMAS-410	SM-189-67/CML312 z SR	Intermediate
IMAS-412	SM-189-69/CML312 z SR	Intermediate
IMAS-418	SM-189-75/CML312 z SR	Intermediate
IMAS-428	NAW 5867/CML312 z SR	Intermediate
IMAS-001	CKL05001/CML312 z SR	Late
IMAS-007	CKL05010/CML312 z SR	Late
IMAS-008	CKL05015/CML312 z SR	Late
IMAS-010	CKL05018/CML312 z SR	Late
IMAS-012	CKL05022/CML312 z SR	Late
IMAS-027	CZL03019/CML312 z SR	Late
IMAS-029	CZL076/CML312 z SR	Late
IMAS-041	VL054178/CML312 z SR	Late
IMAS-044	VL5553/CML312 z SR	Late
IMAS-049	CML144/CML312 z SR	Late
IMAS-062	CML339/CML312 z SR	Late
IMAS-063	CML340/CML312 z SR	Late
IMAS-066	CML343/CML312 z SR	Late
IMAS-067	CML344/CML312 z SR	Late
IMAS-074	CML442/CML312 z SR	Late
IMAS-084	CML502/CML312 z SR	Late
IMAS-093	CLRCW105/CML312 z SR	Late
IMAS-096	CLWQ251/CML312 z SR	Late
IMAS-097	CLWQ252/CML312 z SR	Late
IMAS-098	CLWQ225/CML312 z SR	Late
IMAS-104	(CML491xCML150)-B-11-2/CML312 z SR	Late
IMAS-108	(CML495 x CML254)-B-36-1/CML312 z SR	Late
IMAS-109	(CML495 x CML254)-B-36-2/CML312 z SR	Late
IMAS-112	(CML495 x CL-RCW54)-B-14-1/CML312 z SR	Late

IMAS-115	CLQ6315/CML312 z SR	Late
IMAS-123	CLQRCWQ97/CML312 z SR	Late
IMAS-124	CLRCY031/CML312 z SR	Late
IMAS-126	CLQRCWQ52/CML312 z SR	Late
IMAS-148	CLA41/CML312 z SR	Late
IMAS-163	DTPWC9-F31-1-3-1-1-B/CML312 z SR	Late
IMAS-188	LaPostaSeqC7-F153-1-1-1-2-B/CML312 z SR	Late
IMAS-191	LaPostaSeqC7-F64-2-3-1-2-B/CML312 z SR	Late
IMAS-208	CLRCW100/CML312 z SR	Late
IMAS-211	CLWN217/CML312 z SR	Late
IMAS-212	CLWN273/CML312 z SR	Late
IMAS-221	CLYN253/CML312 z SR	Late
IMAS-229	CLWN223/CML312 z SR	Late
IMAS-233	CLWN276/CML312 z SR	Late
IMAS-237	CLYN261/CML312 z SR	Late
IMAS-239	CLRCY034/CML312 z SR	Late
IMAS-240	CLRCY039/CML312 z SR	Late
IMAS-256	CNO7/8-26/CML312 z SR	Late
IMAS-259	CNO7/8-33/CML312 z SR	Late
IMAS-261	CNO7/8-36/CML312 z SR	Late
IMAS-322	L116W/CML312 z SR	Late
IMAS-337	SO503W/CML312 z SR	Late
IMAS-341	DO620Y/CML312 z SR	Late
IMAS-345	SM-189-2/CML312 z SR	Late
IMAS-348	SM-189-5/CML312 z SR	Late
IMAS-354	SM-189-11/CML312 z SR	Late
IMAS-361	SM-189-18/CML312 z SR	Late
IMAS-362	SM-189-19/CML312 z SR	Late
IMAS-365	SM-189-22/CML312 z SR	Late
IMAS-381	SM-189-38/CML312 z SR	Late
IMAS-391	SM-189-48/CML312 z SR	Late
IMAS-396	SM-189-53/CML312 z SR	Late
IMAS-401	SM-189-58/CML312 z SR	Late
IMAS-403	SM-189-60/CML312 z SR	Late
IMAS-416	SM-189-73/CML312 z SR	Late
IMAS-419	SM-189-76/CML312 z SR	Late
IMAS-423	SM-189-80/CML312 z SR	Late
IMAS-425	P1/CML312 z SR	Late
IMAS-426	P2/CML312 z SR	Late

SUPPLEMENTAL TABLE 2. Pairwise comparison of the number of polymorphic nucleotides (below diagonal) among *Aspergillus flavus* isolates (left and top). Except for AF08 and AF21, which were amplified with ITS1(forward) and ITS4 (reverse) primers, genomic DNA of other isolates were amplified using ITS_F (forward) and ITS4 (reverse) (74). The number of polymorphic nucleotides were calculated based on National Biomedical Research Foundation /Protein Information Resource (NBRF/PIR) DNA sequence alignment in Clustal 2.1 (40). AF08 and AF21 were compared separately from the other isolates because they were amplified using a different primer pair. The pairwise NBRF/PIR alignment included 630 nucleotides for ITS_F/ITS4 and 600 nucleotides for ITS1/ITS4 product sequences.

	Isolate list 2														
Isolate list 1	AF12	AF31	AF14	AF23	AF11	AF05	AF04	AF15	AF03	AF33	AF19	AF22	AF17	AF29	AF21
AF31	3														
AF14	11	11													
AF23	10	10	8												
AF11	4	10	7	8											
AF05	7	11	11	7	9										
AF04	9	10	8	12	9	8									
AF15	12	12	12	11	12	17	14								
AF03	11	12	8	6	11	9	12	18							
AF33	11	6	9	12	12	9	11	14	11						
AF19	10	10	10	11	9	17	18	10	11	13					
AF22	9	13	13	14	9	10	8	11	18	10	18				
AF17	12	12	9	10	8	11	12	9	9	12	8	11			
AF29	17	16	18	17	17	19	15	13	22	15	14	16	17		
AF20	26	27	24	23	24	28	29	21	26	31	19	26	22	29	
AF08															34

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CHAPTER 5

INSIGHTS INTO THE ROLE OF EAR ENVIRONMENT IN POST-HARVEST SUSCEPTIBILITY OF MAIZE TO *ASPERGILLUS FLAVUS*

Abstract

Colonization of maize by *Aspergillus flavus* and aflatoxin accumulation cause quality losses and threaten the health of maize consumers globally. Understanding the genetic and environmental factors affecting vulnerability to aflatoxin accumulation would facilitate effective mycotoxin management in maize. Mature kernels of diverse maize lines that had been grown in seven environments in the United States were used in a kernel screening assay to investigate whether the genotypes differed in response to a toxigenic *A. flavus* strain, whether the environment where the parent plant is grown (ear environment) influences colonization and aflatoxin accumulation, and to investigate the association of the two traits with grain mineral elemental content. Kernels of 26 diverse maize inbred lines and of the intermated B73 x Mo17 (IBM) population were inoculated with an aflatoxigenic strain of *A. flavus* and incubated in the dark at 30ºC for 6 days. Data were collected on latent period, percent kernel colonization, sporulation and aflatoxin. The colonization, sporulation and aflatoxin were influenced by the genotypes of the inbred lines, the ear environment and the interactions between the genotype and the ear environment. Aflatoxin level was positively correlated with sporulation in kernels from all environments, and with kernel colonization in kernels from four of the seven environments. Kernel colonization was strongly correlated with sporulation. Low but statistically significant correlations were observed between the IBM grain mineral elemental content and aflatoxin or colonization. Both sulfur and magnesium were negatively correlated with kernel colonization and sulfur was negatively correlated with aflatoxin. This study shows that the kernel chemical composition, which is influenced by the ear environment, modulates susceptibility of maize to *A. flavus* and aflatoxin accumulation. Findings of this study suggest that susceptibility of stored grain to *A. flavus* may be influenced by the climatic and soil nutrient profiles of the environment where the parental plant was grown.

Introduction

Colonization of maize by toxigenic *Aspergillus* species leads to loss of grain quality and causes morbidity and mortality in the tropical and sub-tropical world (48). *Aspergillus flavus* and *A. parasiticus* are the major fungi that contaminate cereal crops and nuts with different types of aflatoxins, including aflatoxin B1, the most potent known natural carcinogen (43,9). Maize grain provides an excellent substrate for the growth of *A. flavus* (43,44). Colonization and toxin accumulation can occur anywhere along the maize value chain when conditions are conducive. The predisposing factors for pre-harvest colonization of maize by *A. flavus* include the fungal prevalence, susceptibility of maize, plant stress factors, and the interaction among these factors (37). Peri- and post-harvest colonization of maize could occur subsequent to pre-harvest colonization, and are determined by the spatiotemporal interactions among the farm practices, existence of fungal inoculum, climatic conditions, and maize genetics. The relationships in the mechanisms of resistance to colonization and aflatoxin accumulation across the stages of maize ear development are unknown. In this paper, we focus on post-harvest colonization and aflatoxin accumulation, and the associated factors. We also investigate the relationship between pre-and post-harvest colonization mechanisms by using data from the current study and publicly available data of artificially-inoculated field trials.

While it is not known whether the mechanisms of pre- and post-harvest resistance are similar, it is reasonable to speculate that distinct mechanisms would operate at different stages of ear development. It is also not known whether the mechanisms of resistance to infection and aflatoxin accumulation are similar. The main infection court for maize at pre-harvest is the silk channel (5). Open silks have been found to be more susceptible than closed (4). However, the fungus can enter into the kernels of the growing ear through wounds caused by insect and bird damage (53). Crop stress factors such as drought or inadequate soil nutrients have been correlated with increased aflatoxin accumulation (20). Post-harvest infection of mature kernels occurs during shelling, drying or storage, if the practices are carried out under conditions that have predisposed the grain to be susceptible, and that expose maize to more A. *flavus* inoculum (2,27). Post-harvest colonization of maize by *A. flavus* involves an initial direct invasion of kernels, and the resistance mechanisms would involve barriers to access or inhibition of fungal growth. Aflatoxin accumulation occurs upon successful colonization of maize kernels by a toxigenic *A. flavus* strain (43).

Most aflatoxin resistance breeding and trait dissection efforts have been focused on pre-harvest aflatoxin accumulation. However, peri- and post-harvest aflatoxin accumulation are also important in value chains where whole grain is stored for several months under conditions that permit colonization and growth of *A flavus* (47,50,41). Owing to the polygenic nature and low heritability of the aflatoxin resistance, evaluation of maize germplasm for the trait across environments has always

shown a strong GxE (37,23). Although the susceptibility of maize genotypes to post-harvest colonization by *A. flavus* could differ across the ear environments (GxE_e), the interaction has not been investigated perhaps because most aflatoxin-related research was conducted by scientists whose focus was in areas where farmers do not store maize under conditions that favor fungal colonization. Post-harvest resistance to maize colonization by *A. flavus* is more relevant to East African maize value chains where the majority of maize farmers store wholegrain maize for up to 8 months under conditions that favor fungal colonization (26).

Factors that contribute to the strong pre-harvest GxE are generally assumed to involve the interactions among the maize and pathogen genotypes, the soil nutrient characteristics, climatic conditions and other biotic factors across environments (21,28,23). The strong GxE causes inconsistencies in aflatoxin accumulation in different maize genotypes across environments, and hinders progress in breeding for resistance (10). The effect of GxE on pre-harvest aflatoxin accumulation could influence the post-harvest aflatoxin accumulation, but such a relationship has not been studied. The GxE_e effect on post-harvest colonization and aflatoxin accumulation could occur through kernel characteristics, which might influence susceptibility of maize to the fungus. Evaluation of diverse maize germplasm could facilitate identification of sources of aflatoxin resistance. To test whether the environment where maize is grown influences post-harvest susceptibility to *A. flavus* colonization and aflatoxin accumulation, we used a mature kernel screening assay. We compared kernel colonization, sporulation, and aflatoxin accumulation in diverse inbred lines that had been grown in multiple environments in the US (54).

Environmental factors (e.g., water stress) that influence kernel development have been reported to influence susceptibility of kernels to *A. flavus* and aflatoxin accumulation (25). Adaptation of maize to different environments has been correlated with resistance to colonization by fungal pathogens (7,33). The chemical composition and the structural characteristics of the kernel have been associated with resistance to colonization of mature kernels by *A. flavus* (14,24). The availability and concentration of nutrients that are required for maize kernel development varies across environments (29). The major nutrients that influence kernel development are: nitrogen, phosphorus and potassium (51,19). A lower aflatoxin accumulation was reported in maize that was grown in soil under high nitrogen (N) compared to low N (11).

Although increased crop vigor could reduce susceptibility of maize to fungal pathogens, the relationship between maize kernel chemical composition and susceptibility to infection by *A. flavus* is not well understood. Mideros et al. (38) reported negative correlations between pre-harvest aflatoxin and kernel fiber and ash content, and a positive correlation between aflatoxin and carbohydrate content. However, the mechanisms through which fiber, ash and carbohydrates influenced aflatoxin levels is not known, and it is not clear whether the findings are of relevance to post-harvest resistance to aflatoxin. Previous studies have reported lower aflatoxin accumulation in intact kernels relative to kernels with low integrity (8,41). Zein proteins are known to regulate kernel integrity by binding

starch granules and enhancing compactness within the endosperm (8,42). Flint maize has more compact endosperm and was previously reported to have lower susceptibility to aflatoxin accumulation compared to dent genotypes (8). Certain antifungal proteins have been reported to play a role in resistance of maize to *A. flavus* colonization, but the findings have not been useful because of the strong GxE (3).

Dissection of aflatoxin resistance in maize has been carried out using a range of methods. Pre-harvest aflatoxin resistance has mainly been investigated using artificial field inoculations through the silk channel (56,7,37). Mideros et al. (37) carried out *in-vitro* inoculations of developing kernels and silks but were not able to demonstrate an association between field aflatoxin accumulation and the response of the tissues to *A. flavus*. Several researchers have used mature kernel screening assays to investigate aflatoxin resistance in different maize varieties, for identification of aflatoxin resistance-associated proteins, and for mapping aflatoxin resistance quantitative trait loci (QTL) (17,13). The assay could be effective in screening maize for post-harvest resistance to *A. flavus* colonization and aflatoxin accumulation. We used the mature kernel assay to investigate sources of resistance to post-harvest aflatoxin accumulation in a diverse maize germplasm.

Aflatoxin resistance is a complex trait that is regulated by multiple genes of small effect and the identified resistance QTL are inconsistent across different environments (52,34,39). Genetic mapping of complex traits requires utilization of mapping populations that have low linkage disequilibrium and hence increased resolution in QTL identification (54). The intermated B73xMo17 (IBM) and the nested association mapping (NAM) are important genetic resources that are widely used for genetic analysis (32,54). We investigated sources of resistance to post-harvest aflatoxin accumulation in the inbred lines which had been used as founders of the NAM population and in an IBM population that was being used for mapping of genes for mineral elemental content in grain (ionomic content). We envisioned that a strong genotypic effect in the IBM population would facilitate mapping for post-harvest aflatoxin resistance. In addition, we correlated the available grain ionomic content with kernel colonization and aflatoxin accumulation.

Previous studies have reported aflatoxin resistance based on mature kernel assays, but it was not clear whether the findings would be relevant to post-harvest resistance to aflatoxin accumulation (15,13). The assay involves an initial direct contact between the kernel and the fungal inoculum, a phenomena that is similar to post-harvest infection. Because it is not known whether the pre- and post-harvest resistance mechanisms are dissimilar at kernel level, we reasoned that a mature kernel assay of the publicly available and most researched maize germplasm could facilitate comparison of the findings with those of previous and future related studies (36). The objectives of this study were to investigate whether ear environment influences post-harvest susceptibility of maize to *A. flavus* and aflatoxin accumulation, to investigate whether grain ionomic content is correlated with susceptibility of the kernels to *A. flavus* and aflatoxin accumulation, and to investigate whether the publicly available IBM population could be useful in mapping of aflatoxin resistance.

Materials and Methods

Germplasm. Mature, dry kernels of 26 maize inbred lines, the nested association mapping (NAM) population founders (54), that were grown in different environments (hereafter referred to as E_es, for ear environments) in the USA were used in this study. The inbred lines included in this study captures up to 90% of the allelic diversity in domesticated maize (32). Dr. Sherry Flint-Garcia kindly provided mature kernels from Puerto Rico and Missouri, while our lab had a stock of kernels from two years of seed increase at Florida, and from trials at Blacksburg, VA and Aurora, NY. In addition, Dr. Owen Hoekenga kindly provided kernels of approximately 190 IBM recombinant inbred lines (RILs) from a trial that was conducted at Clayton, NC in 2005, for which ionomic content had been analyzed.

Fungal inoculum and pilot mature kernel assay. A stock of commonly used *Aspergillus flavus* strain NRRL 3357 (36), which had been preserved in 50% glycerol at -80°C, was used in *in-vitro* inoculation of kernels. Fungal inoculum was multiplied on autoclaved maize kernels as described by Mideros et al. (37). A developing kernel screening assay (DvKA) that had been established in our lab showed differences in susceptibility of diverse maize lines to *A. flavus* when kernels were inoculated with a 10-day-old conidial suspension at 10^7conidia/mL (38). Based on the established assay, data on the visual score of the percentage of the surface of individual kernels that was colonized by *A. flavus* (percent kernel colonization) and conidia count from the colonized kernels (sporulation) were collected after a 7-day incubation of kernels in the dark at 30°C (37). A mature kernel assay was modified from the DvKA and based on the previously reported assays (16,37), and pilot experiments were conducted to establish the protocol. In the pilot experiment, the effect of inoculum concentration and the timing of collection the data on percent kernel colonization were investigated.

Mature, dry kernels of 10 maize inbred lines (a subset of the NAM founders, Fig. 1), grown at Cornell's Robert Musgrave Research Farm, Aurora, NY in 2008, were inoculated with *A. flavus* at three inoculum concentration levels: 10^5, 10^6 and 10^7 conidia/mL. Mock-inoculated kernels that were dipped in sterile water, instead of fungal inoculum, served as control. There were three replicates per inoculum concentration level treatment or control, and each replicate contained four kernels of each inbred line. Prior to inoculation, kernels were oven-dried overnight at 40°C and cooled at room temperature for 6 hours, while sealing to prevent contamination. The four kernels were surface-sterilized using 10% sodium hypochlorite (bleach) solution and rinsed twice in deionized distilled water (DD H_2O), before they were dipped in *A. flavus* conidial suspension (concentration treatments of 10^5, 10^6 and 10^7 conidia/ml) or in deionized H_2O (control) for 30 seconds. While avoiding direct kernel-to-kernel contact, kernels were transferred into uncapped 9-cm diameter petri plates that were placed side-by-side on a moist (moistened with 30 mL of DD H_2O) filter paper in a capped and parafilm-wrapped clear tray (243 x 243 x18 mm, Corning, NY.). Trays were incubated in a dark

chamber at 30°C for seven days, and the percent kernel colonization and the hours to appearance of *A. flavus* mycelia (latent period) were assessed at 24- and 12-hr intervals, respectively.

Mature kernel assay for diverse inbred lines from multiple environments. Following the findings of the pilot experiment, appropriate assay protocols were established and used for screening of a panel of the 26 diverse maize inbred lines. Kernels were inoculated at 10^7 conidia/mL and incubated in uncapped 4-cm diameter petri plates, which were placed on a moist (moistened with 7 mL of DD H_2O) Whatman #1 filter paper, in a stand-alone 9-cm diameter petri-plate. The 9-cm diameter petri plates were capped and wrapped with a parafilm to avoid evaporation and spread of conidia. The number of kernels of each genotype in each replicate assay was reduced to three due to limited seed stock of the inbred lines. CML322 and CML52 inbred lines were used as susceptible and resistant checks, respectively. Data were collected on percent kernel colonization, sporulation, and aflatoxin after a 6-day incubation of the kernels in the dark at 30°C. Kernels were kept in 15 mL falcon tube at -20°C prior to conidia count. Conidia were washed from the surface of kernels by adding 2 mL of 0.2% Tween 20 solution into the 15 mL tube and vortexing at the maximum speed for 1 min prior to pouring the suspension into a well in a 12-well multi-dish (Waltham, MA). Conidia were counted using a hemocytometer. Kernels were kept at -20°C prior to aflatoxin quantification.

Mature kernel assay for the IBM RILs. Kernels of the IBM population that had been grown at Clayton, NC were subjected to the established assay. Prior to inoculation with the toxigenic *A. flavus* strain, kernels of each of the 190 lines/field replicate were visually assessed and sorted into large and small size categories. Sample inoculation and incubation was based on a completely randomized design. Data was collected on percent kernel colonization and aflatoxin level after 6 days of kernel incubation in the dark at 30°C.

Aflatoxin quantification. Aflatoxin was quantified from a 0.5-gram (NAM founder lines) and 1-gram (IBM population) samples using Vicam AflaTest® method (Waters, Watertown, MA). Briefly, kernels were removed from -20°C and thawed at room temperature for 15 minutes prior to pounding using a mortar and pestle. A sub-sample of 0.5 g (NAM founder lines) or 1-gram (IBM population) was weighed into a sterile falcon tube prior to addition of NaCl and 80% methanol in the ratios of 1:10:10, respectively. The slurry was vortexed at a maximum speed for 3 minutes, and allowed to settle for 10 minutes. The supernatant (extract) was decanted in a sterile 14 mL tube, diluted at 1 mL of extract: 4 mL of DD H_2O and mixed well prior to passing through a microfiber filter (Waters, Watertown, MA). Two milliliters of the filtered diluted extract was passed through an AflaTest® column (Waters, Watertown, MA) at a flow rate of 1-2 drops/second. The column was rinsed twice using 5 mL of DD H_2O water, and eluated with 1 mL of HPLC grade methanol into disposable cuvette. One milliliter of AflaTest developer was added to the 1 mL eluate prior to reading in a Vicam flourometer calibrated at 110/-2.0 (ppb) per AflaTest protocol. The flourometric readings were

multiplied by the appropriate dilution factors. Samples with aflatoxin above the flourometric calibration limit of 300 ppb were diluted and re-tested.

Statistical Analysis. Statistical analysis were done using JMP software version 11 (SAS Institute Inc., 2012, Cary, NC.). Data were transformed using methods that enabled it to fit the assumptions of statistical models used. For the pilot experiment, data on the percent of the kernel surface colonized by *A. flavus* collected at assay development was arcsine square root transformed prior to computing area under disease progress curve (AUPDC) (46) for each replicate of a treatment. For the larger NAM founders experiment, percent kernel colonization was cube root transformed prior to statistical analysis. Similarly, conidia count and aflatoxin data were fourth root transformed. For the IBM population, aflatoxin and percent kernel colonization were cube root transformed prior to input in statistical models. Back-transformed means and least square means were reported in summary tables. Statistical assessment of the response of inbred lines across the E_es was based on 21 genotypes and three environments (3E_es; Missouri, 2007 and 2009; and Puerto Rico, 2008) for which we had enough seed stock (Table 1).

Results

The pilot mature kernel assay experiment showed that the 10 inbred lines differed significantly (P<0.0001) in colonization when inoculated with *A. flavus* conidial suspension at either 10^6 or 10^7 conidia/mL (Fig. 1). Most kernels were colonized and germinated at the 7th day. Significant (P<0.05) differences were observed among the lines at the 5th and 6th days post-inoculation, but not later. The 6th day after inoculation was chosen as the sampling date for subsequent assessment of percent kernel colonization. The latent period among the inbred lines ranged from 38 hours (in OH7B, NC358 and CML322) to 96 hours (in CML52), and had a mean of 55 hours at inoculum concentration of 10^7 conidia/mL and 60 hours at inoculum concentration of 10^6 conidia/mL. The latent period and the AUDPC did not differ between the inoculum concentrations of 10^6 and 10^7 conidia/mL (P>0.05). The latent period was negatively correlated with AUDPC (r=-0.53, P=0.0021) (Fig. 1 and 2).

A pair-wise comparison of kernel colonization, sporulation, and aflatoxin for kernels from the seven E_es showed a strong correlation between aflatoxin and sporulation, but a marginal correlation between aflatoxin and percent kernel colonization (Table 2). Aflatoxin was significantly correlated with colonization in kernels from four (Aurora, Blacksburg, Missouri in 2009, and Puerto Rico in 2008) of the seven E_es, but the grand correlation was insignificant (r=0.37, P>0.05). Aflatoxin was positively correlated with sporulation in kernels from all E_es, and the two traits had a grand significant positive correlation (r=0.52, P=0.0055) (Table 2). Kernel colonization and sporulation were highly correlated (r=0.85, P<0.0001), and the correlations ranged from 0.42 (Florida, 2007) to 0.91 (Blacksburg, 2009) (Table 2).

Ear environment and the response of maize to *Aspergillus flavus*. A summary of the response of the 26 inbred lines to the toxigenic *A. flavus* in the kernel screening assay is presented in Tables 3-5. Kernels of lines that were grown in Blacksburg in 2009 (VA_09) and in Florida in 2007 (Fl_07) were less susceptible to colonization and aflatoxin accumulation compared to other environments (Table 3-5). The most susceptible were kernels that were grown in Aurora, NY in 2009 (Au_09) and in Puerto Rico in 2008 (PR_08) (Table 3-5). Except for kernels of Mo17 and B73, kernels of the rest of the inbred lines that were grown from across different E_es showed inconsistency in response to colonization and aflatoxin accumulation (Table 3-5). Kernels of B73 were the most consistent line, and were generally ranked in the top resistant category across most of E_es (Tables 3-5). Mo17 had lower kernel colonization compared to B73 in Au_09 kernels (36%, B73; 12%, Mo17), and in Missouri 2007 (MO_07) kernels (15%, B73; 5%, Mo17), but Mo17 had a higher aflatoxin accumulation than half of the rest of the lines across the E_es.

By using kernels of a subset of 21 inbred lines from the 3 E_es (Missouri in 2007 and 2009; and Puerto Rico in 2008) for which we had enough seed stock, the variances of the genotypes, the environment and their interaction were compared (Table 1). Maize genotype accounted for the largest aflatoxin variance, but for the lowest variances in percent kernel colonization and sporulation compared to ear environment (Table 1). E_e did not influence aflatoxin accumulation, but significantly influenced percent kernel colonization and sporulation (Table 1-3). The interaction of the maize genotype and the E_e significantly influenced the three traits (Table 1).

Data for the MO_07, MO_08 and PR_08 kernels showed that B73 was the most resistant line (Table 3-5). Puerto Rico kernels were more susceptible to colonization than kernels from the two-year trials at Missouri (Table 3). Similarly, the highest sporulation was observed for the PR_08 kernels (Table 5). Although the interaction between the GxE_e influenced colonization, sporulation and aflatoxin, the trend of the lines was consistent between the two years in kernels that were produced at Missouri. A significant rank correlation was observed for mean aflatoxin in MO_07 and MO_09 kernels (ρ=0.46, P= 0.037) (Table 6).

The strong effect of the GxE_e effect was seen between MO_07 and PR_08 kernels, where negative correlations were observed in the levels of colonization (ρ= -0.46, P=0.04) and sporulation (ρ=-0.53, P=0.013) (Table 6 & Fig. 3). The lines with the least colonization and sporulation in kernels produced at Missouri in 2007 were CML322, NC358 and Ky21, but these genotypes were ranked among the top 5 with most colonization and sporulation in PR_08 kernels (Fig. 3). Kernels of B73 were marginally influenced by the interaction, and the line was ranked as moderately resistant to colonization across the two contrasting E_e (Fig. 2).

We compared the MO_07, MO_08 and PR_08 kernel responses of the inbred lines with available data from three years of inoculated field trials that had been conducted in Mississippi (36,37). The pair-wise ranks of mean aflatoxin levels for the lines were dissimilar between the two

studies and the aflatoxin levels were not correlated (r=-0.12, P=0.65; Table 7). Similarly, sporulation in mature kernel assays was not correlated with the kernel infection coefficient in field-inoculated trials (r=-0.35, P=0.21).

The 188 IBM RILs that had been grown in Clayton, NC in 2005 differed in the extent of kernel colonization by *A. flavus* but not in aflatoxin accumulation (Table 8). Aflatoxin level of the RILs ranged from 0 ppb to 3,150 ppb. The aflatoxin ranges of the parental lines in the same E_e were 5.5 to 575 ppb for B73, and 2 to 1,000 ppb for Mo17. The aflatoxin mean of the IBM population (260±306 ppb) was between those of the parental lines (185±170 ppb, B73; 306±297 ppb, Mo17), but the standard deviations were very large. The percent kernel colonization of the RILs ranged from 4 to 42%. The ranges of colonization of the IBM parental lines in the same E_e were 6 to 25% for B73, and 6 to 38% for Mo17. The mean percent kernel colonization (17±7%) of the IBM RILs was similar to that of the parental lines but closer to that of Mo17 (17±9%) than to that of B73 (13±6%).

Aflatoxin accumulation differed significantly (P<0.0001) between kernel size categories within the field replicates of the IBM population (Table 8, Fig. 4). Larger kernels had significantly higher toxin levels than the small kernels (73 ppb v. 10 ppb; t-ratio=-27.4, P<0.0001, df=750; Fig. 4). No significant differences were observed in percent kernel colonization between the kernel size categories (Table 8). Interestingly, the kernels from different field replicates differed in kernel colonization (replicate 2, 18%; replicate 1, 13%), but did not differ in aflatoxin level (Table 8).

A pair-wise comparison showed that the concentration of sulfur and magnesium in maize grain was correlated with percent kernel colonization and/or aflatoxin. A low but statistically significant correlation was observed between sulfur and aflatoxin (r=-0.1, P=0.024), and between sulfur and percent kernel colonization (r=-0.13, P=0.035). Similarly, grain magnesium content was found to be negatively correlated (r=-0.12, P=0.006) with kernel colonization (Table 9).

Discussion

We used a mature kernel screening assay that relied on a few kernels of each maize genotype to give insights into different aspects of the *A. flavus* – maize pathosystem. The assay was effective in differentiating the response of the diverse inbred lines to colonization by the toxigenic *A. flavus*. The study showed how ear environment (E_e) could influence the colonization of maize by *A. flavus* and subsequent aflatoxin accumulation. In addition, certain grain mineral elemental profiles (sulfur and magnesium) were associated with reduced colonization and aflatoxin accumulation. Brown et al. (17,18) used a kernel screening assay to identify aflatoxin resistance, and to develop molecular markers for breeding for resistance in West and Central African maize. Chen et al. (55) combined a mature kernel screening assay with proteomics and expression analysis to identify aflatoxin

resistance-associated proteins that were proposed as potential molecular markers for aflatoxin resistance breeding. The genotypic trends in the mature kernel assay were not similar to those of the field inoculated trials, implying that distinct mechanisms exist for resistance to pre- and post-harvest colonization of maize by *A. flavus* and aflatoxin accumulation.

A higher correlation was observed between sporulation and aflatoxin than between percent kernel colonization and aflatoxin. The percent kernel colonization was a visual assessment of the severity of the colonization on the surface of the kernel. Post-harvest internal kernel colonization could occur when the fungus enters the kernel via the peduncle or through a ruptured surface. The observed differences in the correlations between the three traits could be attributed to the role of environmental factors on kernel integrity and other physical and chemical characteristics of the kernel, and their effect on fungal growth and toxigenicity. Previous *in-vitro* studies have shown that fungal growth and formation of secondary metabolites is influenced by the nutrient status of the substrate (40). Because non-motile fungi use spore formation as a strategy to escape from harsh conditions, nutrient starvation may lead to reduced mycelial formation, and increased sporulation and aflatoxin production in *A. flavus* (40,1,35,49). Additionally, aflatoxin production by *A. flavus* has been correlated with increased oxidative stress, a condition that is influenced by the nutrient status of the media (30,12). While maize endophytes could be another cause of the observed differences in correlations across environments, we only observed insignificant random (<1% of the samples) colonization of kernels by *A. niger*. The consistent positive correlation between sporulation and aflatoxin across environments suggests that that sporulation could be used as an aflatoxin proxy in mature kernels that are inoculated with a single *A. flavus* strain.

The inbred lines differed in susceptibility to colonization and aflatoxin accumulation, but the magnitude of the response varied across the E_es. Significant GxE has been reported in most of the previous studies, but GxE_e has not been investigated in mature kernel screening assays and in such a diverse set of inbred lines (54). NAM founders consist of inbred lines of different climatic adaptation, but no obvious trends in response *A. flavus* could be associated with the weather conditions during seed production or adaptation to either tropical or temperate conditions (22). The tropical lines, developed by the International Maize and Wheat Improvement Center (CIMMYT), would have been expected to be consistent in kernels that were grown at Peuerto Rico and Florida, while temperate lines would be consistent at Missouri and Aurora (NY). Colonization was lower (2%) in kernels of CML322 that were produced in Florida in 2007 compared to those that had been produced in Puerto Rico in 2007 (93%) and at Puerto Rico in 2008 (88%). Similarly, a lower colonization (9%) was observed in kernels of CML52 that were produced in Florida in 2007 compared to those that were grown in Puerto Rico in 2008 (39%) and in Missouri in 2009 (38%). These findings imply a role of other factors beyond the climatic adaption of maize, and the interaction between the maize genotype and environmental edaphic factors is a good candidate cause of the observed variability.

The response of the lines based on the current mature kernel assay was not correlated with the findings of the artificially-inoculated field trials. Mideros et al. (37) carried out 3-year field inoculation trials of the same inbred lines in Mississippi, reporting significant differences among the inbred lines. Although we report significant differences in the response of the inbred lines based on the mature kernel screening assay, the trend of the current data is not similar to that observed by Mideros et al. (37). For example, Mo17 was moderately resistant to colonization in the current study, but was ranked among the most susceptible based on the field inoculated trials (37). Similarly, while B73 was found to be the resistant based on the current study, but it was listed among the most susceptible by Mideros et al. (37). CML322 and CML247 were ranked among the resistant category in the field inoculated trials, but tended to be among the most susceptible in the current study. These findings could imply that the pre- and post-harvest resistance mechanisms are distinct. Given that the inbred lines were not grown in the same environments, it is possible that the observed distinct trends in response of the genotypes between the two experiments were due to GxE. In the current study, evidence of the strong GxE_e is reflected in the negative correlation between the resistance components of the maize grown at Missouri 2007 and in Puerto Rico 2008.

The IBM RILs included in the kernel screening assay differed in kernel colonization but not in aflatoxin accumulation. The recombinant inbred line population of 250 was developed from a B73 × Mo17 biparental cross, and its high mapping resolution has enabled mapping of genes of multiple quantitative traits (32). The kernels used in the current study were kindly provided from a maize ionome trial that was conducted at Clayton, NC. While the field design was not intended for aflatoxin experiments, we used the available materials to test for an association between susceptibility and the grain elemental profiles and to investigate segregation that might have allowed mapping of aflatoxin resistance in the panel. Although the IBM population has been useful in other quantitative traits, the lack of genotypic differences in aflatoxin levels, a low correlation between colonization and aflatoxin, and the experimental design in the current study did not permit QTL mapping. In the current study, the IBM parental lines were found to be resistant (B73) and moderately resistant (Mo17) to colonization by *A. flavus* and aflatoxin accumulation and the RILs did not show consistent reactions. Either the parental lines did not differ in major QTL for resistance to aflatoxin-related traits, or the extent of error variation was too great to allow us to detect differences.

We observed significant differences in aflatoxin level between kernels of different sizes of the same genotype. The kernels had been sorted as large v. small, and were assumed to have originated from different positions in the maize ear as follows: small kernels from the terminal, and the large kernels from the middle parts of the ear. Previous studies had shown a huge aflatoxin range (0–80,000 ppb) in kernels from different positions of the same ear (31). The differences in colonization and aflatoxin accumulation among the kernels could be caused by variation in kernel integrity (e.g., insect feeding) or could be due to intra-ear variation in chemical or structural characteristics. Recent studies

showed uniform protein and starch content along decile positions of the maize ear (45). Baxter et al. (6) showed significantly higher grain sulfur and iron content at the tip compared to the middle or the base ear positions. Given that we found a negative correlation between sulfur and kernel colonization or aflatoxin, it is possible that the differences in aflatoxin levels between the large and the small kernels were due to differences in sulfur content. Although the observed correlation coefficients are small, there is need for confirmation of these findings and a further investigation of the mechanism through which the elements could reduce colonization and aflatoxin accumulation.

The observed differences in responses of kernels between the two field replicates of the IBM population implies the expected great variability in response of maize to *A. flavus* across environments. The strong GxE requires multiple replications within and among environments to allow credible inferences about aflatoxin resistance in maize. The findings of this study imply that soil fertility management could influence the aflatoxin contamination in maize during storage in the African context, where conditions often favor the accumulation of mycotoxins. To facilitate further understanding of the *A. flavus* – maize pathosystem, and hopefully guide future aflatoxin breeding efforts, there is need to carry out parallel studies involving pre-harvest artificial inoculation, and developing and mature kernel assays.

Tables and Figures

Table 1. Analysis of variance for effect of genotype, seed source and their interactions on percent kernel colonization, aflatoxin and sporulation in mature, dry kernels of 21 nested association mapping (NAM) founder inbred lines that were artificially-inoculated with Aspergillus flavus in the laboratory. Kernels included in this analysis were of the 21 inbred lines from three ear environments, 3 E_es (Missouri_07, Missouri_09 and Puerto Rico_08) for which we had enough seed stock. Replicates were considered to have a random effect in the model. Aflatoxin and conidia (sporulation) were fourth root transformed, while percent kernel colonization was cube root transformed prior to input in respective models.

	Aflatoxin (ppb)		
Source	DF	Sum of squares	Mean squares
Genotype (G)	20	926.88	46.34***
E_e	2	9.82	4.91
G*E_e	40	665.85	16.65**
Lab. replicate	2	52.19	26.10
Error	119	1,108.05	9.31
C. Total	183	2,731.46	
	Percent Kernel colonization		
G	20	44.94	2.25***
E_e	2	83.80	41.90***
G*E_e	40	72.29	1.81***
Lab. replicate	2	5.58	2.79**
Error	122	64.63	0.53
C. Total	186	272.50	
	Sporulation (conidia count)		
G	20	13,052.45	652.62***
Ee	2	18,496.64	9,248.32***
G*Ee	40	20,903.68	522.59***
Lab. replicate	2	230.44	115.22
Error	119	17,827.00	149.81
C. Total	183	71,992.90	

*, **, ***; significant at 0.05, 0.001 and <0.0001 respectively.
[1]location and year where the inbred line was grown.

Table 2: Correlation between kernel infection factors and aflatoxin (ppb) in an *in-vitro* assay. Inbred lines of the kernels were grown at the following environments, years: Aurora, 2009 (Au_09), Blacksburg, 2009 (VA_09), Florida, 2009 (Fl_09), Missouri, 2007, 2009 (MO_07, 09), and Puerto Rico, 2007, 2008 (PR_07, 08). Kernels were inoculated with toxigenic *A. flavus* and incubated at 30°C for six days in the dark prior to score for kernel colonization and aflatoxin quantification. Correlation analysis using data from the three experimental replications in which aflatoxin was analyzed.

		Seed source						
Correlation between	Grand	Au_09	Bl_09	Fl_07	Mo_07	Mo_09	PR_07	PR_08
Kernel colonization & Aflatoxin	0.37	0.30*	0.43**	0.24	0.01	0.25*	0.23	0.29*
Kernel colonization & Sporulation	0.85***	0.69***	0.90***	0.42**	0.57**	0.63***	0.57***	0.51***
Sporulation & Aflatoxin	0.52**	0.28	0.50**	0.58***	0.35**	0.28*	0.45***	0.68***
Latent period & Sporulation		ND	ND	ND	-0.36	-0.60***	-0.49**	-0.26
Latent period & Aflatoxin		ND	ND	ND	-0.16	-0.23	-0.21	-0.25
Kernel colonization & latent period		ND	ND	ND	-0.45**	-0.58***	-0.36	-0.17

*, **, ***; significant at 0.05, 0.001 and <0.0001 respectively.

Table 3. Percent kernel colonization by *Aspergillus flavus* based on kernel screening assay of mature, dry kernels of the NAM founder inbred lines. Inbred lines of the kernels were grown at the following environments, years: Aurora, 2009 (Au_09), Blacksburg, 2009 (VA_09), Florida, 2009 (Fl_09), Missouri, 2007, 2009 (MO_07, 09), and Puerto Rico, 2007, 2008 (PR_07, 08). Kernels were inoculated with *A. flavus* and incubated at 30°C for six days in the dark prior to score for percent kernel colonization.

	Percent kernel colonization (±Standard deviation)								
Inbred line	**Au_09**	**VA_09**	**FL_07**	**PR_07**	**PR_08**	**MO_09**	**MO_07**	LSM of 3 E_e	Mean of 3 E_e
CML228				23±18		36±23			
Il14H	16±14		2±1	14±9		3±2	17±10		
M162W	84±9	1±0	11±9	54±42	44±34	24±4			
Mo17	12±3		5±0	13±12		8±5	5±4		
MO18W			29±12	10±2		49±31	19±14		
B73	36±31	21±14	3±0		9±5	1±0	15±12	3 D	3.8
CML103	92±8	25±15	15±4	26±17	10±9	2±1	9±0	6 CD	5.6
P39			2±1	32±4	20±4	1±0	14±12	6 CD	5.8
M37W	84±3	0	1±0	12±5	49±23	1±0	8±7	7CD	7.5
B97	39±6		4±0	4±3	16±15	24±15	4±0	9 BCD	8.7
CML333			56±12	53±20	70±28	5±3	3±2	13 ABCD	12.8
MS71	40±38		10±8	9±4	45±31	21±17	3	13 ABCD	13.2
NC350				23±17	61±43	8±3	5±4	15 ABCD	14.9
Oh7B	61±23	1±0	5±2	7±0	43±35	13±6	8±3	17 ABCD	16.8
NC358	96±3	26±20	14±9		88±11	13±11	2±1	17 ABCD	17.1
Oh43	34±24	3±0		4±2	32±23	18±10	13±12	17 ABCD	17.1
Ki11		10±5		33±25	55±8	21±19	3±1	17 ABCD	17.3
CML52			9±2	18±8	39±25	38±29	19±14	19 ABCD	18.9
CML69		15±3		17±10	46±21	13±10	14±17	20 ABCD	19.7
Ky21	49±34	1±0	22±18	39±32	78±16	20±4	2	21 ABCD	20.6
CML247					24±21	59±15	44±29	21 ABCD	21.5
CML322	100±0		2±0	93±6	88±6	34±27	2	22 ABC	22.5
Tx303		60±10		50±24	60±10	16±8	15±11	25 ABC	24.7
CML277		78±11		22±15	89±9	47±7	12±8	34 AB	33.9
HP301	78±7	20±24		39±27	53±24	32±22	28±16	34 AB	34.1
Ki3	79±20	2±1		27±6	100±0	67±7	16±12	44 A	44.2
E. mean					40^{c}	13^{b}	6^{a}		

Means were compared for 21 inbred lines that were grown at the 3 Ear Environments, E_e: MO_07, MO_09 and PR_08 using Tukey's HSD (α=0.05): means followed by similar capital letter imply that the inbred lines do not differ significantly. Means followed by superscript small letter imply that the environments do not differ significantly.

Table 4. Aflatoxin (ppb) based on based on kernel screening assay of mature, dry kernels of the NAM founder inbred lines. Inbred lines of the kernels were grown at the following environments, years: Aurora, 2009 (Au_09), Blacksburg, 2009 (VA_09), Florida, 2009 (Fl_09), Missouri, 2007, 2009 (MO_07, 09), and Puerto Rico, 2007, 2008 (PR_07, 08). Kernels were inoculated with *A. flavus* and incubated at 30°C for six days in the dark prior to aflatoxin quantification.

	Aflatoxin (ppb) (± standard deviation)									
Line	Au_09	VA_09	FL_07	PR_07	PR_08	MO_09	MO_07	LSM of 3 E_e		Mean of 3 E_e
CML228	-	-	-	277±156	1	2,123±653	-			-
M162W	16,544±21	3,307±142	905±622	2,988±27	10,722±18	1,909±4	-	0		-
Mo17	3,317±4	-	2,211±45	75	-	3,342±282	1,473±52	-		-
MO18W	-	-	548±11	123±26	-	4,072±1,203	447±39	-		-
B73	109±3	-	246±4	-	0	2	603±11	9	D	14
M37W	10,624±6	-	-	2,021±1,206	200±22	1±1	45±91	14	D	14
Il14H	34±23	-	81±69	30±17	-	935±596	-	-		32
P39	-	-	5	271±60	53±2	47±5	81±2	33	CD	33
CML103	5,769±53	444±315	1,982±120	3,140±166	7±1	470±59	335±278	109	BCD	108
NC358	6,020±8	787±109	1,000 ±111	225±900	2,463	5±1	15±0	119	BCD	119
CML333	-	-	393±0	2,700±17	4,643±1	138±27	297±242	714	ABCD	580
CML247	-	-	-	16	410±6	5,448±28	333±37	661	ABCD	772
Tx303	-	2,657±110	-	4,115±16	6,783	7±2	348±4	501	ABCD	794
CML69	-	2,876±75	-	533±317	1,858±30	1,281±212	414±368	1,012	ABCD	1,014
CML52	-	-	69±1	25	2,284±28	712±428	1,138±605	1,040	ABCD	1,040
CML277	-	4,248±559	-	1,567±789	833±97	7,244±1	63±99	1,139	ABCD	1,136
B97	600±67	-	277±868	47±188	2,739±31	2,362±1,262	1,061±118	1,932	ABCD	1,941
Ky21	695±17	173±8	940±355	3,459±113	652±372	6,477±30	1,644±475	2,138	ABCD	2,135
Ki3	2,484±8	105±100	-	215±847	6,649±404	2,734±91	406±250	2,293	ABCD	2,288
Oh7B	1,903±37	537±17	1,677±88	411±46	2,658	5,149±3	1,666±61	2,918	ABC	2,913
HP301	8,880±570	402±298	-	452±398	2,955±179	1,367±840	12,335±8	4,096	AB	3,479
Oh43	1,169±4	-	-	33±27	1,642±13	11,708±6	3,676±2	4,499	AB	4,488
MS71	3,364±8	-	489±1	2,200±24	3,020±264	3,281±726	11,700±203	5,122	AB	5,124
Ki11	-	2,437±35	-	3,487±65	2,204	1,494±867	27,029±54	5,547	A	5,548
NC350	-	-	-	14,645	8,289±17	13,822±21	1,038±37	5,703	A	5,695
CML322	2,515±36	-	5±3	2,917	5,640±9	17,190±32	1,775±508	6,190	A	6,188
E. mean					1,348[a]	1,300[a]	943[a]			

Means were compared for 21 inbred lines that were grown at the 3 Ear Environments, E_e: MO_07, MO_09 and PR_08 using Tukey's HSD (α=0.05): means followed by similar capital letter imply that the inbred lines do not differ significantly. Means followed by superscript small letter imply that the environments do not differ significantly.

Table 5. Sporulation (conidia count) based on kernel screening assay of mature, dry kernels of the NAM founder inbred lines. Parental lines of the kernels were grown at the following environments, years: Aurora, 2009 (Au_09), Blacksburg, 2009 (VA_09), Florida, 2009 (Fl_09), Missouri, 2007, 2009 (MO_07, 09), and Puerto Rico, 2007, 2008 (PR_07, 08). Kernels were inoculated with *A. flavus* and incubated at 30°C for six days in the dark prior to conidia count.

	Sporulation (or conidia count ± standard deviation)x1^4								
Line	Au_09	BK,VA_09	FL_07	PR_07	PR_08	MO_09	MO_07	LSM of 3 E_e	Mean of 3 E_e
CML228				51.5±10.1		60.6±11.4			-
M162W	70.8±4.2	37.7±8.7	65.6±30.8	68.5±17.6	73.2±4.7	64.4±4.6			-
Mo17	55.0±7.1		58.3±6.0	20.2±22.3		50.6±2.3	42.7±15.0		-
MO18W			51.9±8.2	48.5±2.7		69.1±5.4	41.0±15.0		-
Il14H	50.8±4.8		40.2±11.6	43.0±7.5		39.6±7.4	16.8±15.3		28.2
B73	56.5±16.1	37.7±12.7	39.7±13.2		47.4±4.6	25.2±12.4	43.4±8.6	38.7C	38.7
P39			36.6±9.0	55.2±12.0	53.0±9.5	35.2±4.4	40.5±9.3	42.9BC	42.9
M37W	78.5±5.9	32.5±2.6	30.9±1.5	50.2±11.3	77.4±6.3	31.0±5.8	29.3±25.7	45.9BC	45.9
B97	63.4±7.4		45.6±9.0	46.0±4.4	26.9±38.0	67.1±8.8	38.1±11.9	43.8BC	46.2
CML69		59.6±4.5		64.1±15.9	63.0±4.6	41.0±3.6	53.5±4.5	52.7BC	51.2
CML333			62.3±3.4	66.9±7.4	79.2±6.6	42.5±2.9	38.3±10.2	53.3BC	53.3
Ky21	56.6±2.9	26.2±5.0	62.5±12.4	56.5±11.0	75.8±5.1	64.8±14.3	28.5±10.4	56.4BC	53.9
Oh7B	66.8±3.1	38.3±4.4	46.3±18.3	31.9±27.7	65.3±15.9	55.9±11.4	42.4±7.8	54.5BC	54.5
CML52			48.7±15.4	52.9±19.6	62.6±4.3	53.4±4.9	49.1±13.3	55.1BC	55.1
NC358	84.8±1.3	60.6±8.4	50.0±4.2		86.8±3.5	46.9±4.7	34.8±7.3	56.2BC	56.2
NC350				69.7±6.5	80.7±16.2	52.7±8.8	38.2±6.2	57.2BC	57.2
CML103	78.5±4.5	58.3±2.8	59.1±13.1	69.0±15.1	59.3±3.2	57.0±25.6	55.2±0.9	57.2BC	57.4
HP301	73.1±4.5	56.4±11.5		47.9±1.0	57.1±14.1	51.9±10.7	66.0±19.3	58.1BC	57.4
CML322	77.9±10.0		31.1±10.2	85.8±4.6	85.7±10.0	61.6±15.1	25.7±22.9	57.6BC	57.6
Oh43	59.8±1.5	37.5±9.2		40.0±2.6	69.8±8.8	56.7±6.2	47.1±15.9	57.8BC	57.8
CML247					47.4±13.0	71.4±5.0	58.4±12.2	59.1ABC	59.1
CML277		77.7±4.0		65.9±29.3	75.5±12.5	72.2±7.2	33.3±8.2	60.3AB	60.3
Ki11		52.4±2.7		68.5±3.2	75.5±4.6	61.6±7.3	46.7±11.9	61.3AB	61.3
MS71	68.3±11.5		48.9±1.3	58.9±8.8	76.3±12.9	58.3±9.6	51.1±25.8	61.9AB	61.9
Tx303		75.3±7.5		78.0±3.2	80.7±9.8	54.6±4.2	52.6±3.9	62.6AB	62.6
Ki3	71.6±6.7	36.6±1.6		64.3±3.2	103.0±28.0	82.1±8.9	55.3±10.6	80.1A	80.1
E. mean					69^c	54^b	44^a		

Means were compared for 21 inbred lines that were grown at the 3 Ear Environments, E_e: MO_07, MO_09 and PR_08 using Tukey's HSD (α=0.05): means followed by similar capital letter imply that the inbred lines do not differ significantly. Means followed by superscript small letter imply that the environments do not differ significantly.

Table 6: Rank correlations of genotypic means of aflatoxin, kernel colonization and sporulation based on laboratory kernel screening assay of 21 maize inbred lines (a subset of NAM founders) among the environments, Missouri in 2007 and 2009, and at Puerto Rico in 2008. Kernels were inoculated with *A. flavus* and incubated at 30°C for six days prior to score for percent kernel colonization, counting of conidia and aflatoxin quantification.

		Missouri_07			Missouri_09			Puerto Rico_08	
		Aflatoxin	Colonization	Sporulation	Aflatoxin	Colonization	Sporulation	Aflatoxin	Sporulation
Missouri_07	Colonization	-0.12							
	Sporulation	0.09	0.74***						
Missouri_09	Aflatoxin	0.46*	-0.18	-0.14					
	Colonization	0.29	0.00	0.18	0.63**				
	Sporulation	0.21	-0.21	0.05	0.68**	0.83***			
Puerto Rico_08	Aflatoxin	0.36	-0.23	0.04	0.30	0.32	0.21		
	Colonization	0.00	-0.46*	-0.31	0.18	0.32	0.21	0.48*	
	Sporulation	-0.02	-0.53*	-0.32	0.13	0.02	0.08	0.58**	0.81***

*, **, ***; significant at 0.05, 0.001 and <0.0001 respectively.

Table 7. Means and ranking of sporulation, infection coefficient and aflatoxin in field inoculated [data from Mideros et al. (36)] and *in-vitro* inoculation of mature kernels (this paper) of diverse inbreed lines. Infection coefficient (proportion of *A. flavus* DNA in total DNA of colonized maize) was estimated using quantitative polymerase chain reaction (36). Sporulation was analyzed through counting of conidia that were washed from colonized mature kernels after 6 days of incubation in the dark at 30°C. Means sporulation represents fourth root transformed conidia count.

	In-vitro inoculation of mature kernels[2]			Field inoculation at 50% silking[1]		
Line	Sporulation	Aflatoxin (ppb)	Aflatoxin (ppb) rank	Infection coefficient	Aflatoxin	Rank for aflatoxin
B73	38.7C	9 D	1	0.85 BCDE	4,596 AB	14
M37W	45.9BC	14 D	2	0.2 E	541 BCDEFG	5
P39	42.9BC	33 CD	3			
CML103	57.2BC	109 BCD	4	1.08 ABC	5,173 AB	15
NC358	56.2BC	119 BCD	5	0.26 E	661 BCDEF	6
Tx303	62.6AB	501 ABCD	6	0.38 CDE	874 ABCDEF	8
CML247	59.1ABC	661 ABCD	7	0.37 CDE	233 EFG	2
CML333	53.3BC	714 ABCD	8			
CML69	52.7BC	1,012 ABCD	9			
CML52	55.1BC	1,040 ABCD	10	0.31 CDE	136 FG	1
CML277	60.3AB	1,139 ABCD	11			
Mo17	46 BC	1,514 ABCD	12	1.63 AB	6,596 A	16
B97	43.8BC	1,932 ABCD	13	1.75 A	3,932 ABC	13
Ky21	56.4BC	2,138 ABCD	14	0.91 ABCD	1,046 ABCDEF	10
Ki3	80.1A	2,293 ABCD	15	0.23 E	268 DEFG	3
Oh7B	54.5BC	2,918 ABC	16		3,423 ABCD	12
HP301	58.1BC	4,096 AB	17			
Oh43	57.8BC	4,499 AB	18	0.71 BCDE	1,440 ABCDE	11
MS71	61.9AB	5,122 AB	19	0.73 BCDE	705 BCDEF	7
Ki11	61.3AB	5,547 A	20			
NC350	57.2BC	5,703 A	21		889 ABCDEF	9
CML322	57.6BC	6,190 A	22	0.28 CDE	389 CDEFG	4

1 Pre-harvest inoculation of the inbred lines with aflatoxigenic NRRL 3357 *A. flavus* strain, in 2008, 2009, and 2010 at the R. R. Foil Plant Science Research Center at Mississippi State University.

[2]Inbred lines were grown in Missouri in 2007 and 2009 and in Puerto Rico in 2008. Kernels of Mo17 were not available from Puerto Rico in 2008.

Table 8: Analysis of variance for effect of genotype and kernel size on percent kernel colonization and aflatoxin based on kernel screening assay of mature, dry kernels of 188 Intermated B73xMo17 (IBM) recombinant inbred lines which were grown at Clayton, NC in 2005. Genotype and field replicate were considered to have a random effect in the model. Kernel size was based on sorting into large or small grain of each RIL within a field replicate, and hence had a random effect.

	Kernel colonization (%)		
Source	DF	sum of squares	Mean squares
Genotype (G)	187	237.43	1.27***
Field Rep	1	50.24	50.24**
Kernel size[Field replicate]	2	0.42	0.21
Error	561	246.80	0.44
C. Total	751	534.89	
	Aflatoxin (ppb)		
G	187	173.66	0.93
Field Rep	1	0.02	0.02
Kernel size[Field replicate]	2	776.97	388.48***
Error	561	554.73	0.99
C. Total	751	1505.38	

*, **, ***; significant at 0.05, 0.001 and <0.0001 respectively.

Table 9: Correlation between maize kernel ionomic content, colonization and aflatoxin. Mature, dry Kernels of the IBM RILs grown at Clayton, NC in 2005 were analyzed for mineral elemental content, and a subset of samples were inoculated with toxigenic *A. flavus* and incubated at 30°C for six days in the dark prior to score for percent kernel colonization and aflatoxin quantification.

Element	Kernel colonization	Aflatoxin
Magnesium	-0.12 (**)	-0.10
Sulfur	-0.13 (**)	-0.10 (*)
Calcium	-0.06	0.06
Copper	-0.02	0.05
Manganese	-0.12	0.04
Zinc	-0.04	0.01
Iron	-0.03	0.00
Potassium	-0.07	-0.01
Phosphorous	-0.08	-0.03

*, **, ***; significant at 0.05, 0.001 and <0.0001 respectively. NS; not significant.

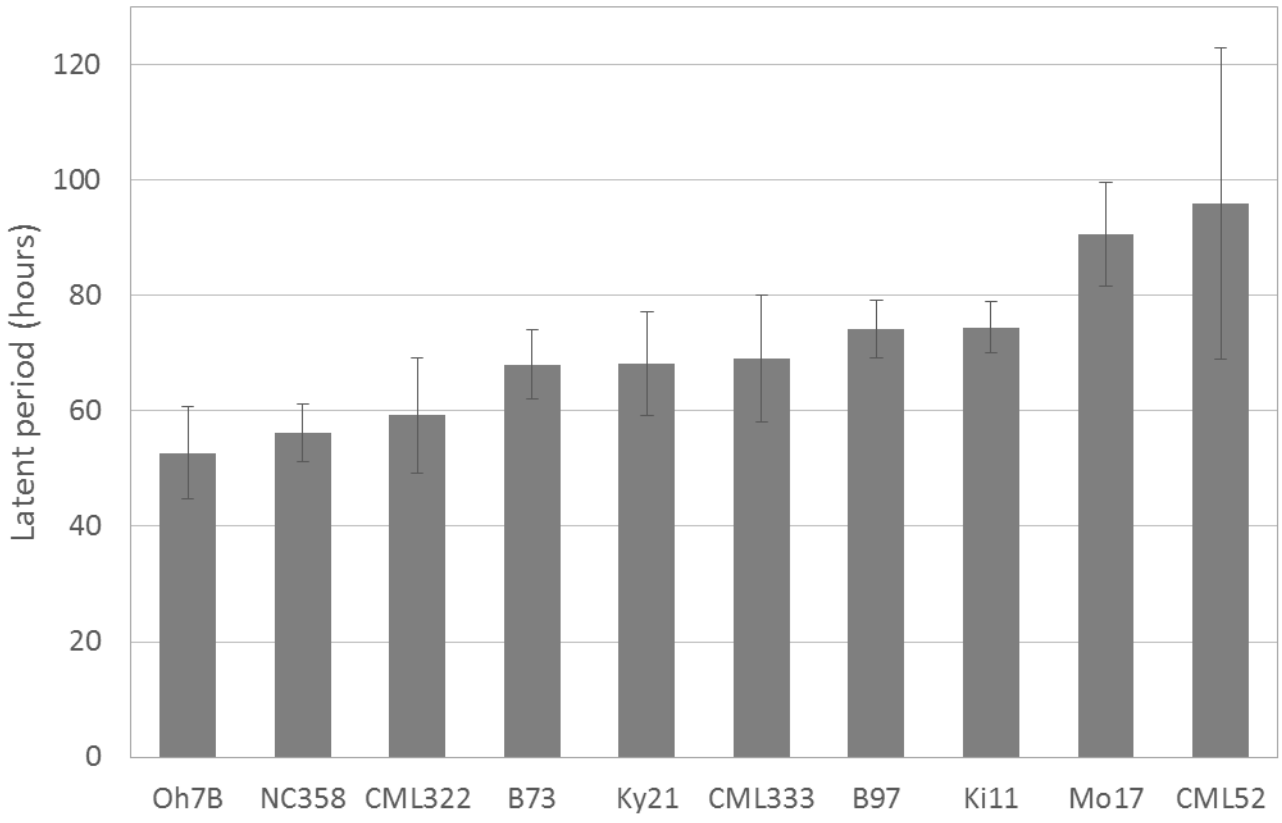

Fig. 1. Latent period for colonization of 10 maize inbred lines by *Aspergillus flavus*. Data were collected during the pilot experiment for the establishment of a mature kernel assay using maize inbred lines that were grown at Cornell's Robert Musgrave Research Farm, Aurora, NY in 2008.

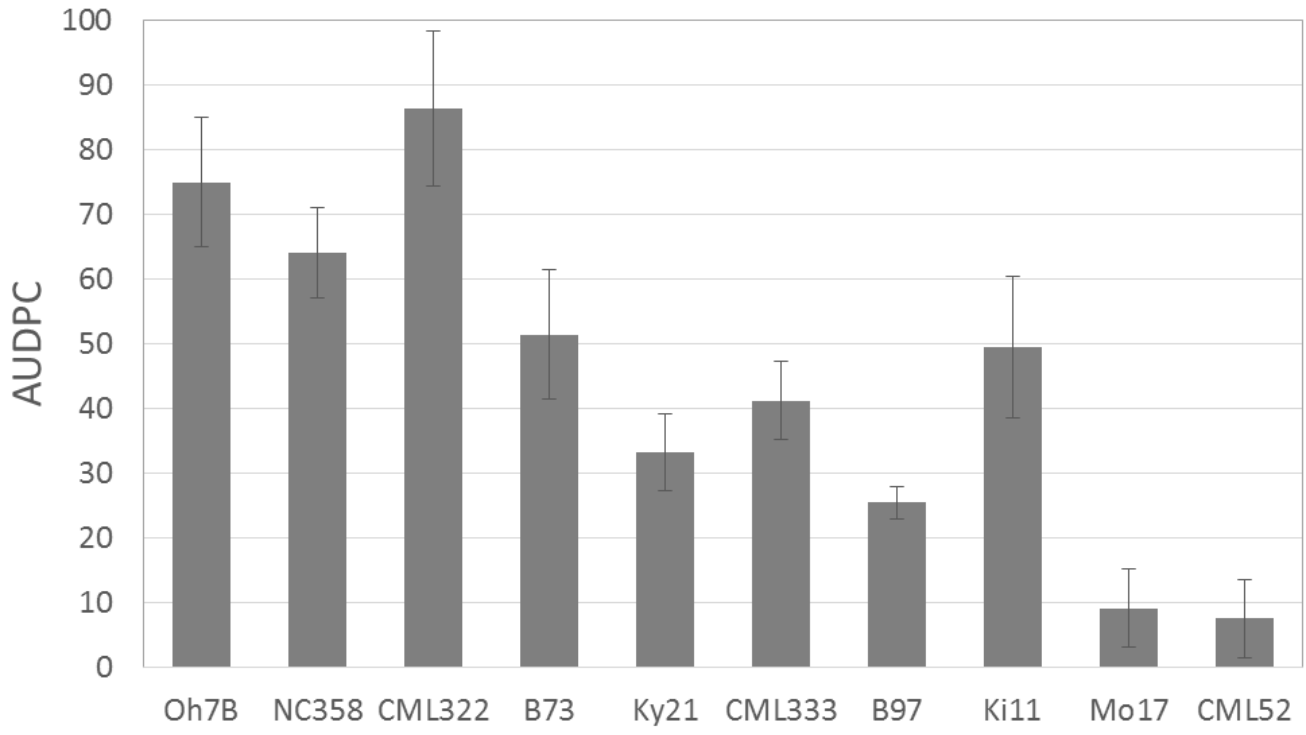

Fig 2: Area under disease progress curve (AUDPC) derived from percent kernel colonization (PKC) of 10 maize inbred lines by *Aspergillus flavus*. Data were collected during the pilot experiment for the establishment of a mature kernel assay using maize inbred lines that were grown at Cornell's Robert Musgrave Research Farm, Aurora, NY in 2008.

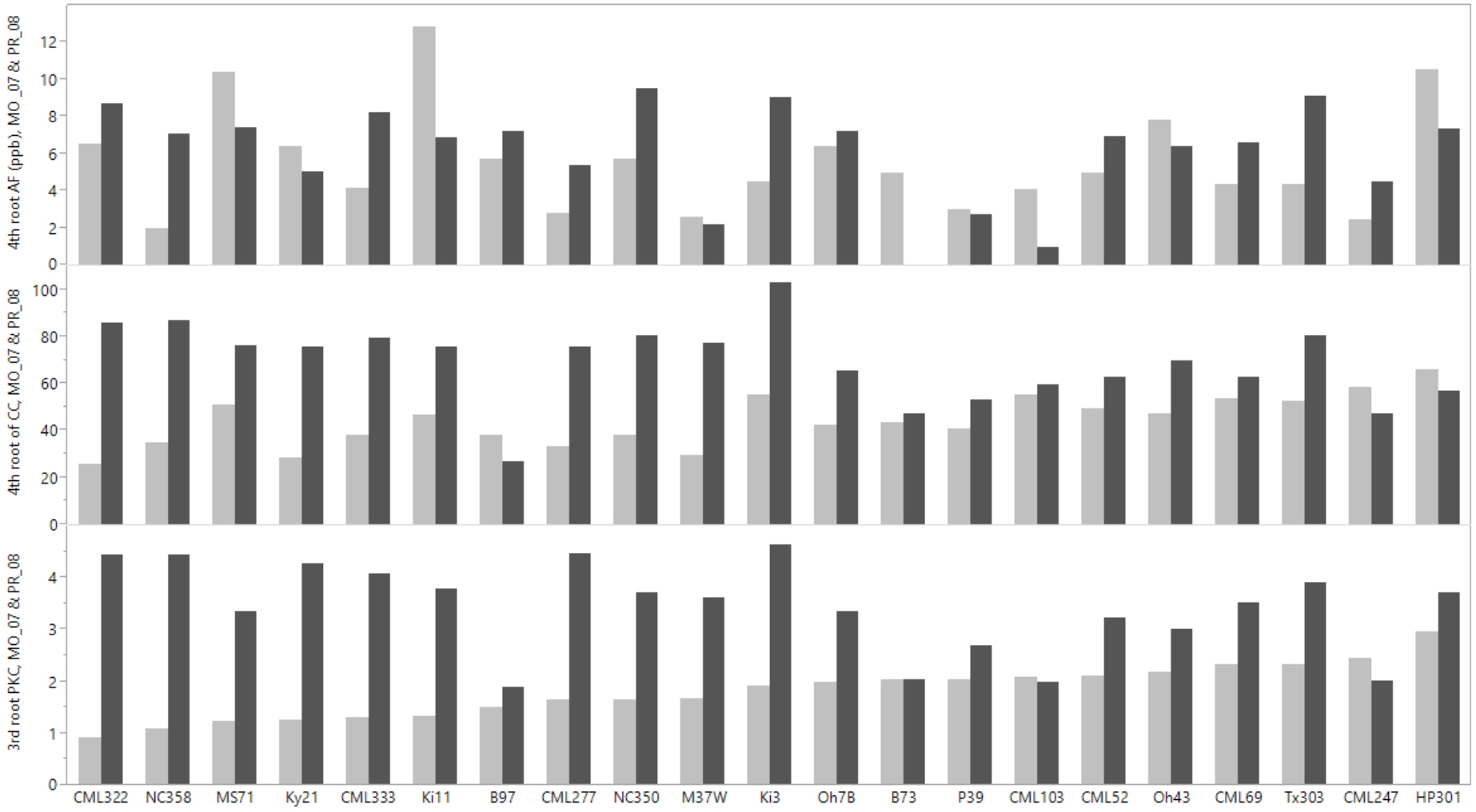

Fig. 3. Colonization (PKC), sporulation (CC) and aflatoxin accumulation (AF) in seed of maize inbred lines that had been grown in Missouri in 2007(light grey bars) and in Puerto Rico in 2008 (dark grey bars). Graph is ordered by percent kernel colonization in inbred seed that had been grown in Missouri to enhance visualization of the trend of related traits across the environments. The of PKC of maize grown in Puerto Rico in 2008 and that grown in Missouri in 2007 show an inverse relationship across the inbred lines (bottom graph).

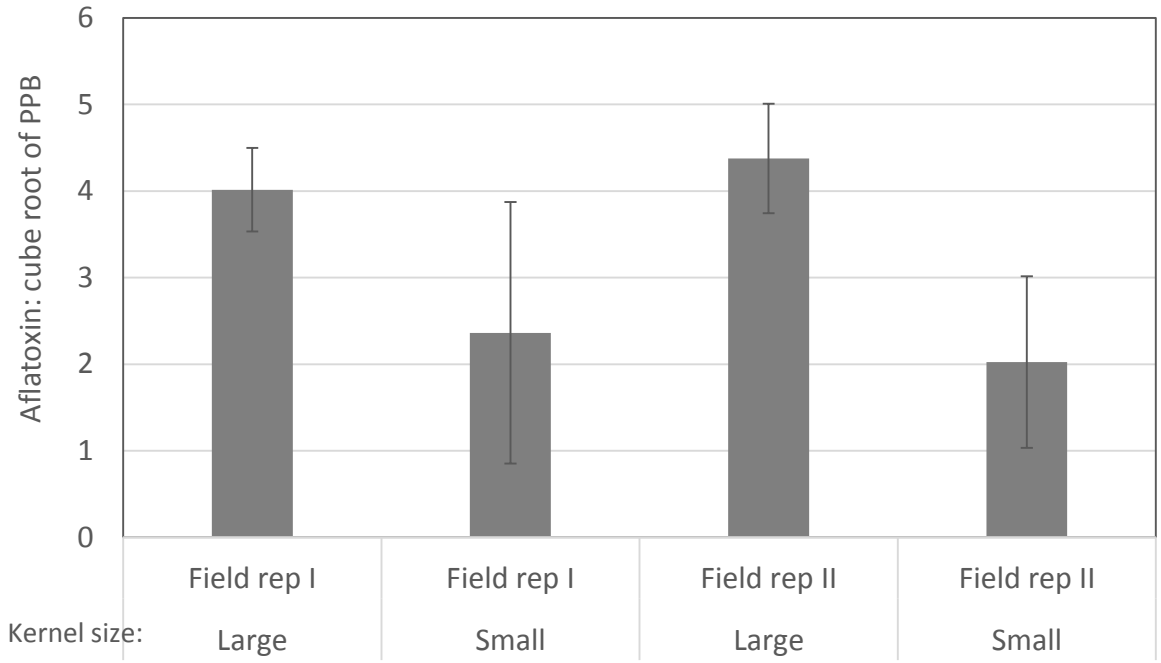

Fig. 4. Aflatoxin level in mature, dry kernels of the Intermated B73xMo17 (IBM) maize population by *Aspergillus flavus* based on a laboratory-based assay. Kernels from two field replicates grown at Clayton, NC, 2005 were divided into two groups of large and small kernel sizes within each genotype. Aflatoxin was extracted from 1 gram of each sample. Aflatoxin was cube root transformed prior to mean comparison. Error bars denote standard deviation.

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