EFFICACY OF PRE-HARVEST *ASPERGILLUS FLAVUS* BIOCONTROL TREATMENT ON REDUCING AFLATOXIN ACCUMULATION DURING DRYING

by

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To my parents, family, and friends

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CHAPTER 1. INTRODUCTION

1.1 The Global Threat of Food Insecurity

Food insecurity is a major global challenge to about 700 million worldwide, with about 90% of this population residing in Asia and Africa (World Health Organization, 2018). It is estimated that the global human population will reach 8.5 billion in 2030 and 9.7 billion by 2050 (Thornton et al., 2011). Therefore, world's demand for food is expected to increase rapidly, and cereal production will need to increase to 60-100% by 2050 to meet the growing demand for food (Rayfus & Weisfelt, 2012; Thornton et al., 2011). Agriculture is the economic backbone in many Sub-Saharan Africa countries serving two-thirds of livelihoods in this region The African economy is inherently dependent on agriculture given that 60% of its 1.66 billion people live in rural areas. However, the agricultural growth rate of Sub-Saharan Africa has declined in the 2000s, and food security remains a major concern (Kimatu et al., 2012).

Maize is a staple food to about 1.2 billion people around the world, making it an important component for food security (Outreach, 2017). In sub-Saharan Africa, 300 million people every year depend on maize farming for income (Shiferaw et al., 2013). Therefore, it is important to identify appropriate steps that will boost maize yields, prevent pre and post-harvest losses and prevent human health hazards (Kimatu et al., 2012). Maize farmers can achieve high crop yields by adopting better cultivation practices such as timely planting and harvesting of maize and by planting high quality variety of maize. (Tito et al., 2018). Unfortunately, over the years, pre- and post-harvest losses have led to declined supply of maize by farmers worldwide (Kimatu et al., 2012). Poor post-harvest management has led to more than 40% loss in grains; an estimated monitory value of more than US $4 billion annually. Post-harvest grain loss occurs as

a result of bacterial and fungal infection, insect pest and rodents infestation, and lack of suitable storage facilities (Sauer & Burroughs, 1980; Tefera et al., 2011). To date, one of the major health concerns related to maize is contamination of mycotoxins during pre and post-harvest periods (Kimatu et al., 2012).

1.2 Mycotoxins

Mycotoxins are secondary metabolites produced by both field and storage fungi which grow on seed, grain and feed (Pozzi et al., 1995). These toxic compounds have the potential of causing a variety of ill effects to humans and animals, from allergic responses to immunosuppression and death (Pitt, 2000; Wagacha & Muthomi, 2008). Toxin production on crops is mostly caused by pre-harvest infection, delayed harvest, wet conditions during harvest periods, inadequate grain drying and high moisture during storage and transportation. Most common mycotoxins of public health concern are aflatoxin, deoxynivalenol (DON), nivalenol, zearalenone and fumonisms (Pitt, 2000; Reddy et al., 2008). The research presented in this thesis focuses on aflatoxin contamination of maize, which is a major issue in Sub Sahara Africa.

1.2.1 Aflatoxin

Aspergillus flavus and *Aspergillus parasiticus* are Ascomycota fungi and the most studied producers of aflatoxins (Cotty, 1994; Dorner et al., 1999; Klich, 2007). These fungi are ubiquitous and can be found in many kinds of soil; therefore, can easily infect corn in the field and can be brought to storage places, leading to aflatoxins contamination (Horn, 2003). Aflatoxins are classified into four major types, designated as B1, B2, G1 and G2. *Aspergillus flavus*, the most encountered species, produces aflatoxin B1 and B2, although strains that produce small sclerotia can produce G1 and G2 aflatoxins (Ojiambo et al., 2018). *Aspergillus*

parasiticus produces aflatoxin B1, B2, G1 and G2 (Chang et al., 2005). The B-group aflatoxins (B1 and B2) exhibit blue fluorescence under ultraviolet (UV) light, while the G-group (G1 and G2) exhibit yellow-green fluorescence (Wacoo et al., 2014). This difference in fluorescence emitted is important in identifying and differentiating between the two groups. Aflatoxin M1 and M2 are metabolites of aflatoxin B1 and B2 which are found in milk and urine of animals after consuming contaminated feeds.

Two sclerotial groups are recognized in *A. flavus*, designated as L and S. L-strains produce relatively large sclerotia (> 400 µm in diameter) compared to those of S-strains (< 400 µm) (Cotty, 1994; Horn, 2003). The L-strains also produce more conidia. Toxigenic L-strains produces B aflatoxins; whereas toxigenic S- strains have the potential to both B and G aflatoxins (Probst et al., 2014).

1.3 Health effects of aflatoxin

Aflatoxin was first discovered and characterized in 1960 after more than 100,000 turkey died in England after consuming groundnuts contaminated with *Aspergillus flavus* (Blount, 1961). Thereafter, it became apparent that aflatoxins were present in many agricultural products, such as maize, peanuts, groundnuts, cotton seeds and tree nuts (Lizárraga-Paulín et al., 2011). When these contaminated products are consumed, health disorders develop due to the carcinogenic and mutagenic nature of aflatoxin (Scheidegger and Payne, 1986). Ingesting high levels of aflatoxin can result in acute aflatoxicosis (Bandyopadhyay et al., 2016). AFB1 interacts with a cytochrome P450 enzyme in the liver leading to epoxidation of the toxin, resulting in a reactive agent that can bind to proteins and DNA (Williams et al., 2004; Wu & Khlangwiset, 2010). DNA-bound can induce hepatocellular carcinoma (HCC), by mutation of tumor

suppressing gene, P53 (Williams et al., 2004). Other symptoms of acute aflatoxicosis include hemorrhage, acute liver damage, edema and even death (Probst et al., 2007). Consumption of aflatoxin-contaminated maize increases the risk (up to 30 folds) of liver cancer among people with Hepatitis B and Hepatitis C (Ngindu et al., 1982; Wu et al., 2009). Scientist have also found an association between impaired child growth and aflatoxin exposure through consumption of contaminated milk from their mothers or dairy. Children that consume AFB1 in any form are also more predisposed to liver cirrhosis (Dhanasekaran and Shanmugapriya, 2011: Zheng & Zhang, 2012).

Aflatoxin is commonly found in most animal feed ingredients such as corn, peanut meal, cotton seed meal, soybean produce, sorghum and fish meal (Table 1.1[a]). Experimental studies on pigs and sheep have also shown reduced weight gain and growth rate with increase in aflatoxin concentration in their food (Atherstone et al., 2016). Fish and poultry, especially turkey, are also known to be extremely sensitive to AFB1. Although chickens are relatively resistant to the toxin, broilers tend to be more susceptible than layers (Atherstone et al., 2016; Rawal et al., 2010).

The health effects caused by aflatoxins precipitated regulatory agencies worldwide to limit the levels of aflatoxins in food and feed. United State Food and Drug Administration (FDA) has set its limits at 20 ppb (Table 1.1[b]), while European Union (EU) has set limits much lower at 4ppb. In Sub Saharan Africa, most countries have regulatory threshold of 20ppb for total aflatoxin in food; with countries such as Kenya having lower limits of 10ppb for total aflatoxin and 5ppb for AFB1 (Mutegi et al., 2018).

Table 1.1 FDA Action Levels for Aflatoxin in Human Food, Animal Feed and Animal Feed Ingredients.

Intended Use	**Grain, Grain By-Product, Feed or other Products[a]**	**Aflatoxin Level[b] [parts per billion (p.p.b.)]**
Human consumption	Milk	0.5 p.p.b. (aflatoxin M1)
Human consumption	Foods, peanuts and peanut products, brazil and pistachio nuts	20 p.p.b.
Immature animals	Corn, peanut products, and other animal feeds and ingredients, excluding cottonseed meal	20 p.p.b.
Dairy animals, animals not listed above, or unknown use	Corn, peanut products, cottonseed, and other animal feeds and ingredients	20 p.p.b.
Breeding cattle, breeding swine and mature poultry	Corn and peanut products	100 p.p.b.
Finishing swine 100 pounds or greater in weight	Corn and peanut products	200 p.p.b.
Finishing (i.e., feedlot) beef cattle	Corn and peanut products	300 p.p.b.
Beef, cattle, swine or poultry, regardless of age or breeding status	Cottonseed meal	300 p.p.b.

Table obtained from (FDA, 2011).

1.4 Environmental factors favoring growth of *A. flavus*

Studies on soil moisture and temperature have shown significant relationship between drought stress on crops and preharvest invasion of crops by *A. flavus* (Hill et al., 2017; Sanders et al., 1984). Also, insects may contribute to kernel infection in various ways: transporting inoculum to the ears; transferring inoculum from silks to ears or by injuring the kernels, facilitating colonization and infection (Magan et al., 2003). In a four-year experiment, Payne et al. (1986) studied effects of drought stress on *A. flavus* invasion and aflatoxin accumulation in maize. They observed that high levels of aflatoxin occurred during years with drought stress and that irrigation reduced stress and lead to reduced contamination with aflatoxin. A two-year study conducted in Benin by Sétamou et al., (1997), also shows a relationship between insect damage and aflatoxin levels following *A. flavus* infection. In their experiment, they observed lower aflatoxin levels in less damaged maize ears compared to those that were more damaged (Sétamouet et al., 1997).

Delayed harvest on maize has shown to significantly reduce moisture content in the grains, but it does not reach the required safe storage moisture content of 13% (Hell et al., 2008; Akowuah et al., 2015; Kaaya et al., 2005). Aflatoxin levels also increases by 4 and 7 times after three and four weeks of delayed harvest (Kaaya et al., 2005). Development of storage fungi and accumulation of aflatoxin is also affected by moisture content in the produce, amount of inoculum prior to storage, high relative humidity, lack of aeration in the store, storage time and insect and rodents' activity. Maize stored for a longer period might have higher aflatoxin levels compared to those stored for a short period, as found by Kaaya (2006) where they observed higher aflatoxin levels in maize stored for more than six months compared to those stored for less than two months. They showed that maize harvested from agroecological zones with higher

temperatures and relative humidity had higher aflatoxin levels compared to those grown drier agroecological zones (Kaaya & Kyamuhangire, 2006).

1.5 Aflatoxin management strategies

1.5.1 Pre-harvest management of maize

Aflatoxin management of maize prior to harvest must take into consideration all the environmental factors such as high temperature and cultural practices that lead to crop infection with *Aspergillus flavus* and aflatoxin accumulation (Champ and Highley, 1994; Torres et al., 2014). Although elimination of aflatoxin is currently not realistically achievable in the field, there are several integrated practices that can be used to reduce its impact. These management strategies include modification of cultural practices, development of resistant crops, biological control, and development of field treatments that would block aflatoxin production, pest management, and breeding (Klich, 2007).

1.5.1.1 Biological control

One successful strategy used to manage aflatoxin contamination is application of biological controls to the crops (Bhatnagar-Mathur et al., 2015; Brown et al., 1991). Numerous microorganisms, including bacteria, yeasts and non-toxigenic *A. flavus,* have been tested for ability to reduce aflatoxin contamination. Bacteria such as *Bacillus subtilis*, *Lactobacillus sp. Pseudomonas sp.* can inhibit the growth of *Aspergillus sp.* and aflatoxin production under laboratory conditions (Dorner, 2004; Yin et al., 2008). However, the efficacy of these organisms is poor under field conditions (Dorner, 2004). Like bacteria, saprophytic yeasts, *Candida krusei* and *Pichia anomala* also have shown reduction of aflatoxin by 96% and 99% respectively *in*

vitro (Hua et al., 1999). Therefore, more experimental studies should be done to test the efficacy of these microorganisms as biological control agents (Yin et al., 2008).

There is some evidence that non-aflatoxigenic strains of *A. flavus* can reduce aflatoxin contamination under both, laboratory and field conditions, and they have the potential to be used as biological control agents (Dorner, 2004). These strains are referred to as atoxigenic. Studies on atoxigenic *A. flavus* strains have shown reduction of aflatoxin levels in maize (Abbas et al., 2006; Atehnkeng et al., 2008; Cotty & Bhatnagar, 1994; Dorner et al., 2009, 2010; Dorner & Cole, 2002). The atoxigenic biocontrol strains applied to the soil must occupy the same environment as the native toxigenic strains and out compete them on the host (Dorner & Cole, 2002).

Cotty (1990) tested the ability of atoxigenic strains in reducing aflatoxin levels in cottonseeds (Cotty, 1990). In a greenhouse experiment, Cotty et al., (1994) tested the ability of five atoxigenic strains on preventing a toxigenic strain from contaminating cottonseed with aflatoxin. All strains reduced contamination but one strain AF36, was more effective (average reduction of 95.33%) when inoculated simultaneously with the toxigenic strain (Cotty & Bhatnagar, 1994). Additionally, this strain was further tested by Brown et al., (1991) for control of aflatoxin in maize. This atoxigenic strain reduced preharvest aflatoxin contamination 80-95% in maize when applied simultaneously with or one day prior to a toxigenic strain (Brown et al., 1991). This led to its registration with the United States Environmental Protection Agency as biological control products for aflatoxin control (AF36™); which is produced and distributed by Arizona Cotton Research and Protection Council for the use on cotton, maize and pistachio (Abbas et al., 2011). AF36 strain does not produce aflatoxin because of a mutation gene *aflC*

(*pksA*), which encodes the polyketide synthase involved in aflatoxin biosynthesis (Ehrlich & Cotty, 2004).

Aspergillus flavus NRRL21882, has also been tested for its efficacy in reducing aflatoxin contamination in fields in both pre- and post-harvest of peanuts (Dorner & Cole, 2002). At harvest, aflatoxin levels in untreated peanuts were almost 10 times more than those in treated peanuts. Similar observations were made after storage. Further experiments on maize showed a maximum reduction of 76% aflatoxin after fields were treated with *A. favus* NRRL 21882. This strain was later registered as AflaGuard which is produced and distributed by Syngenta for use on maize and peanuts (Dorner, 2004). In a similar way to AF36, NRRL 21882 misses the entire cluster of genes responsible for the biosynthesis of aflatoxin (Chang et al., 2005).

Studies on efficacy of *A. flavus* biocontrol strains in reducing aflatoxin have been conducted in other countries, including in Sub-Saharan Africa (Bandyopadhyay et al., 2016). In Nigeria, a mixture of four non-toxigenic strains, named AflaSafe®, has gained provisional registration. Several AflaSafe products, each of a different set of four atoxigenic strains are being tested on farms across African countries (Bandyopadhyay et al., 2016). AflaSafe KE01 has been developed for use in maize in Kenya and has shown efficacy in reducing aflatoxin levels in all treated fields (Bandyopadhyay et al., 2016; Migwi, 2016). In Senegal, similar field trials have been done one on AflaSafe SN01 biocontrol showing reduction of aflatoxin levels in maize by 75 to 93% at harvest (Bandyopadhyay et al., 2016; Grace et al., 2015). In Europe, experimental studies on *A. flavus* MUCL54911 have shown more than 90% reduction in aflatoxin levels in treated maize (Mauro et al., 2018; Ojiambo et al., 2018). Further experiments on commercial product AF-X1 which is made of this strain are still in progress in Italy.

Formulation and application protocols for these biocontrol products have been extensively researched. Biocontrol strains are delivered to the field on small seeds, such as sorghum, wheat, barley and rice (Ojiambo et al., 2018; Tafesse et al., 2017). The seeds act as nutritive substrates to the atoxigenic fungi and provide stability and easy handling. The grains are first sterilized (Dorner, 2004; Yin et al., 2008) and the atoxigenic strains are allowed to colonize. After drying, the product is cast to the soil at 5 kg to 10 kg per acre, depending on the crop (Cotty & Mellon, 2006). At these rates, a dominant population of competitive atoxigenic strains is established in the field at the time the maize is susceptible to infection by toxigenic *A. flavus*. The best times for application to the soil surface of non-toxigenic strains are V7 and V12 stages of growth (Dorner, 2004). After application, growth and sporulation of the biocontrol strains provide enough inoculum to achieve a competitive advantage (Dorner, 2004).

1.5.1.2 Chemical control

Insect pests and fungal contamination increases susceptibility of mycotoxins to crop. These two menaces can be eliminated by application of insecticides and fungicides in the field to reduce insect damage and fungal infection (Kabak, Dobson, & Var, 2006; Magan & Lacey, 2009). Some of the fungicides available to eliminate *Aspergillus* species include itraconazole and amphotericin B (Wagacha & Muthomi, 2008).

1.5.1.3 Breeding and biotechnology

A lot of laboratory and field studies have identified several maize crops resistant to *A. flavus* infection, aflatoxin production and insect infestation (Kabak et al., 2006). Using corn cultivars resistant to ear feeding insects is a very successful strategy to control insects and fungal infection. In the US, Bt maize that produce insecticidal proteins from *Bacillus thuringiensis* (Bt) are used widely for insect management. (Mendelsohn et al., 2003). This Bt toxin attacks the gut

cells of the insect causing lysis and eventual death (Wu, 2006). Agrisure Viptera is also a genetically modified maize seed that contains Vip and Cry toxins originally found in *B. thuringiensis*, designed to protect maize plants from a range of common pests including earworms, black cutworm and stalk bores. Viptera reduces insect damage that enable infection of *A. flavus* and accumulation of aflatoxin.

1.5.1.4 Cultural Control strategies

Cultural control strategies such as crop rotation, irrigation and proper tillage practices can be used to modify conditions that encourage *A. flavus* infection in the field (Suleiman & Rosentrater, 2015). Water stress is one of the major factors contributing to high levels of aflatoxin in the field. In a three-year study conducted by Payne et al., they observed that high levels of aflatoxin in maize occurred during years that experienced drought (Payne, 1986). Drought stress was proposed to affect plant by reducing leaf area, hence making silk more accessible to conidia of the fungus. They concluded that irrigation and subsoiling tillage reduces stress among the crops, increases maize yield and reduced aflatoxin contamination. Therefore, maintaining high kernel water activity until time of harvest can be used as a defense mechanism against growth of aflatoxigenic fungi (Pettit et al., 1971). Late season irrigation also combats heat and drought stress. However, this practice is very difficult to do in some areas especially if it is semi-arid and arid with limited water supply.

Crop rotation can be used to break the build-up of *A. flavus* in the soil and reduce infection in the following years (Mejía-Teniente et al., 2011; Kabak et al., 2006). However, to ensure maximus efficacy of this strategy, other factors such as tillage practices, weed control and irrigation should still be considered.

1.5.2 Post-harvest management of maize

To meet the demands in the dry seasons, most maize produced in Africa is stored and hence susceptible to deterioration if pre-harvest and harvest practices were no adequate. The success of the storage depends on the moisture content, air temperature, relative humidity and percentage of kernels (Domenico et al., 2016). Most aflatoxin contamination of food occurs during post-harvest storage as opposed to pre-harvest conditions (Dorner and Cole, 2002). Therefore, controlling aflatoxin contamination during post-harvest periods is paramount (Wu & Khlangwiset, 2010).

Maize is often harvested at a moisture content of 17-20% (Bandyopadhyay et al., 2016). Therefore, it must be dried to the ideal moisture content of 13.5% before storage to reduce infection with *A. flavus*. Dissemination and production of mycotoxins during the post-harvest may be the result of floods and unseasonal rains that interfere crop harvesting (Bhatnagar-Mathur, Sunkara, Bhatnagar-Panwar, Waliyar, & Sharma, 2015). In both 1981 and 2004, two aflatoxin outbreaks took place in Kenya; they were favored by unseasonal rains during maize harvest (Lewis et al., 2005; Ngindu et al., 1982). In Sub Sahara Africa, the post-harvest period is often delayed because of rain and reduced solar conditions, which increases the risk of having high moisture maize that favors the growth of *A. flavus* and aflatoxin accumulation. Losses can be extensive when harvest and drying periods are delayed. A report in India found aflatoxin contamination higher during seasons with extensive rain and when atmospheric conditions during storage was warm and humid (Ahmad, 1993). Hell and Mutegi suggested that the faster the process of drying the grain is, the lesser rates of fungal growth (Hell & Mutegi, 2011).

Many studies have evaluated drying of maize to ideal moisture content before storage and even recommended fast drying processes. However, most of these studies evaluated drying

alternatives used at large scale and did not consider the elementary system still used by small and middle holder farmers in Sub Saharan Africa. Most farmers in Africa do not use rapid drying equipment because large capital is needed to acquire them. They rely on solar drying to dry their produce on platforms and drying mats outside the field. In an experiment carried out in Nigeria on rapid drying of peanut kernels, it was observed that artificial drying after 4 to 6 days in the field resulted on uncontaminated kernels, whereas samples left for 8-12 days or sundried for 10-26 days resulted on high aflatoxin levels of 25-50 ppb. The longer the crop was in the field before artificial drying, the higher the toxin level concentration in the kernels.

A survey carried out in Ghana reported that 69% of the farmers interviewed did not harvest their maize based on physiological maturity (Akowuah et al., 2015). Instead, most used inaccurate traditional practices like observing the dried tassels of cobs and biting into kernels as sign of maturity and dryness of the maize. Therefore, maize may still have high moisture content during storage, increasing its susceptibility to fungal infection and aflatoxin contamination. Effective solar drying and drying equipment should therefore be effectively used to dry maize to this targeted moisture content (Diao et al., 2014).

During storage, maize is prone to excessive heat, high humidity and lack of aeration in the storage facilities. Insects and rodents can also attack maize kernels leading to spread of *A. flavus* spores and subsequent increase in aflatoxin contamination (Atehnkeng et al., 2015). The heat generated in this microenvironment, coupled with high moisture content, increases fungal invasion (Danso et al., 2017; Magan et al., 2003). One of the mechanisms adopted to reduce fugal growth and aflatoxin contamination during storage in developing countries, is the use of silo bags among middle and large holder farmers (Hell at al., 2010). These bags are quite inexpensive and can hold approximately 150 to 200 tons of the stored grains. The use of

hermetic bags has also gained popularity among many farmers to store dry gains (Maina et al., 2016). Unlike the polypropylene woven bags commonly used in most developing countries, the advanced triple layered hermetic bags are airtight to water and gases. Therefore, they work by depriving oxygen to the insects and fungi and dehydrating the environment (Baributsa, et al., 2014; Lane & Woloshuk, 2017; Maina et al., 2016).

1.6 Research Objective

One of the pivotal ways to inhibit aflatoxin contamination after harvest, is to dry maize to safe moisture levels of 13%, which inhibits the growth of *A. flavus*. However, in Sub Saharan Africa, the post-harvest period is delayed often because of rain and reduced solar conditions. The objective of this research was to address the hypothesis that preharvest treatments of maize fields with biocontrol strains will reduce aflatoxin accumulation during the postharvest drying period. Here we tested maize collected from Texas and North Carolina fields treated with Aflaguard and AF36. We adjusted the grain moisture to 20% to simulate a midpoint in the postharvest drying period. By incubating the grain for six days, we were able to evaluation the changes in kernel infection, A. flavus populations, and the accumulation of aflatoxin.

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CHAPTER 2. EFFICACY OF PRE-HARVEST ASPERGILLUS FLAVUS BIOCONTROL TREATMENT ON REDUCING AFLATOXIN ACCUMULATION DURING DRYING

2.1 Abstract

Maize is a major calorie source for people living in Sub-Sahara Africa. In this region, *Aspergillus flavus* causes ear rot diseases in maize, contributing to food insecurity due to aflatoxin contamination. The biological control principle of competitive exclusion has been applied in both the United States and Africa to effectively reduce aflatoxin levels in maize at harvest by introducing atoxigenic strains that out-compete toxigenic strains. The goal of this study was to determine if the efficacy of preharvest biocontrol treatments carry over into the drying period, which is often delayed in Sub-Sahara Africa by the complexities of postharvest drying practices and lack of modern drying machinery. Maize was collected from fields in Texas and North Carolina that were treated with commercial biocontrol, and control fields that were untreated. To simulate moisture conditions similar to those experienced by farmers during drying in Sub-Sahara Africa, we adjusted the grain to 20% moisture content and incubated it at 28 ℃ for 6 days. Although the initial number of infected kernels in most samples were high, less than 24% of kernels were infected with *Aspergillus flavus* and aflatoxin levels were low (<4ppb). Both toxigenic and atoxigenic strains increased and spread through the grain over the incubation period, and aflatoxin levels increased, even in samples from biocontrol-treated fields. Our molecular analysis suggests that applied biocontrol strains from treated fields migrate to untreated fields. The results also indicate that the population of toxigenic *A. flavus* in the harvested grain will grow and produce aflatoxin during the drying period when moisture is high. Therefore, any potential postharvest reduction in aflatoxin accumulation will depend on how

effective the biocontrol strain was at displacing the toxigenic populations prior to harvest.

2.2 Introduction

Maize in Sub-Sahara Africa is often infected by the aflatoxin-producer *Aspergillus flavus* prior to harvest and during post-harvest handling. In most areas, the moisture content of maize at harvest is above 23% and should be dried quickly to 13% to prevent spoilage by *A. flavus* and other fungi (Hell et al., 2010). However, achieving safe moisture by the sun-drying methods used in some Sub-Sahara regions is difficult, hence conditions are conducive for the growth of *A. flavus*, which can grow at moisture contents above 17.5% (Harris, 2016; Mestres et al., 2004; Oyebanji and Efiuvwevwere, 1999; Trenk and Hartman, 1970). Hell et al., (2000) showed that aflatoxin contamination increased when maize harvest was delayed for more than five days. After harvest, maize is often stooked in the field, hung on racks, or heaped on the ground or in storage facilities for an extended period. As high humidity and temperature in the heaped maize provides favorable conditions for fungal growth, contamination is accelerated by delayed drying (Hell et al., 2000). These practices and unfavorable weather conditions occur in many areas of Africa, leading to a widespread problem of aflatoxin contamination across the continent.

As a result of *A. flavus* growth in maize, many individuals in Sub-Sahara countries are chronically exposed to aflatoxin in their diet. Ingesting high levels of aflatoxin can result in acute aflatoxicosis, which manifests as hepatotoxicity, cancer, immunosuppression, stunted growth in children and impaired food conversion (Bandyopadhyay et al., 2016; Hell and Mutegi, 2011; Wu et al., 2009). Outbreaks of aflatoxin contamination in Kenya have caused human deaths repeatedly since 1981 (Lewis et al., 2005; Probst et al., 2007). Deaths were also reported in Tanzania in 2016 (Kamala et al., 2018). Due to these tragic incidences, efforts have focused on methods to reduce aflatoxin accumulation in maize through improved cultural practices, post-

harvest handling procedures, grain-drying technologies, and the application of biocontrol products (Bandyopadhyay et al., 2016; Hell et al., 2003; Hell et al., 2000).

Among the strategies that have been investigated to manage aflatoxin contamination, application of atoxigenic strains of *A. flavus* to the crop appears to be the most promising. This strategy seeks to competitively exclude aflatoxin producers from crops with atoxigenic strains of *A. flavus*. A study conducted in the United States by Brown et al., (1991) showed efficacy of the application of atoxigenic *A. flavus,* which reduced pre-harvest aflatoxin contamination by 80-95%. Abbas et al., (2006) also found that atoxigenic *A. flavus* strains CT3 and K49 reduced aflatoxin contamination in maize by 68% and 37%, respectively. A screen of 4200 *A. flavus* isolates in Nigeria identified 20 as potential biocontrol strains (Donner et al., 2010). Additional studies on performance found four strains reduced aflatoxin levels in maize by up to 99% and hence were used to the formulate biocontrol product AflaSafe® in Africa (Atehnkeng et al., 2008; Bandyopadhyay et al., 2016).

In the US, two commercial biocontrol products are available, Afla-Guard™ and AF36™. The Afla-Guard strain lacks the entire cluster of genes responsible for aflatoxin biosynthesis; whereas, the AF36 strain has a mutation in the *aflC* (*pksA*) gene, which encodes the polyketide synthase involved in aflatoxin biosynthesis (Chang et al., 2005; Ehrlich and Cotty, 2004). Brown et al., (1991) showed that maize treated with AF36 and a toxigenic strain of *A. flavus* aflatoxin concentrations in the harvested grain was lower than treatments with the toxigenic strain alone (Brown et al., 1991). A study in Texas by Dorner (2009) on AflaGuard treatment of maize showed a maximum reduction of 76% in aflatoxin accumulation. AflaSafe®, which has gained provisional registration, is being tested on farms in several African countries (Bandyopadhyay et al., 2016; Donner et al., 2010). AflaSafe KE01, applied to maize in Kenya, reduced aflatoxin

levels (<4 ppb) in all treated fields. Similar results were found in in Senegal, with a 75 to 93% reduction of aflatoxin in fields treated with AflaSafe SN01 (Bandyopadhyay et al., 2016).

The study described here attempts to address the question of whether the benefits of preharvest application of biocontrol strains carry over into the postharvest period. Atehnkeng et al., (2014) attempted to answer this question with maize from AflaSafe-treated and non-treated fields. Bags of the maize where placed in water for 4 h to increase the moisture level and then allowed to slowly dry for a 10-day period. In all treatments, the starting level of aflatoxin was significantly higher in maize from non-treated fields and the level of aflatoxin contamination increased dramatically. The authors concluded that the AflaSafe treatment reduced postharvest aflatoxin contamination by 57% - 99%, although aflatoxin concentrations in all treatments were well above safe levels. Our study also tested this hypothesis that treatment of maize fields with biocontrol strains during pre-harvest period will reduce aflatoxin accumulation during the postharvest drying period. Here we examined changes in maize collected from North Carolina and Texas fields treated and untreated with biocontrol when incubated at a high postharvest grain moisture.

2.3 Materials and Methods

2.3.1 Maize samples

In 2017, maize samples were collected from AF36-treated (TX) and untreated fields (Control TX) in Hill and Burleson Counties in Texas. AflaGuard-treated (NC) and untreated samples (Control NC) were also collected from fields in North Carolina (NC). In 2018, maize samples were collected from two Texas field sites. The Greenville (GV) site, located at 33.1696N, 96.1683W, had a history of previous treatment with AF36. Samples came from

experimental plots at the field-site treated with AF36 and AflaGuard, which were applied at the V7 and V9 stages of maize development. Maize from a non-treated plot was also collected. The second collection site in Texas was Field 219 located at 30.5476N, 96.4289W. This field was free of biocontrol treatment for seven years. Maize was collected from plots treated with AflaGuard applied when the plants were at V9. These plots were also treated with the aflatoxin-producing strain NRRL3357 five days after the start of silking. Samples also came from non-treated plots.

2.3.2 Storage experiment

All maize samples from 2017 and 2018 were dried and shipped to Purdue University, where they were stored in hermetic bags until use. Grain moisture was adjusted to 20% (wet basis) by the method described by Williams et al., (2014) with some modifications. About 1500 g of the maize with the appropriate amount of water were placed in a rotary tumbler (C&M Topline Goleta. CA) for 2 hours. Thereafter, the grain was incubated at 4°C for 72 hr with periodical shaking to achieve uniform moisture distribution. The moisture content of the grain after conditioning was confirmed by the air-oven method adopted by ASAE (ASABE, 2012; Grabe, 1989). The conditioned grain was then divided into 3 equal subsamples of 500 g and placed into 1 L glass jars (L× W ×H – 8cm × 8cm × 16cm) with perforated lids (lid diameter - 8.5cm). These jars were incubated for 6 days at 28 ^{o}C. For the 2017 maize, 120 kernels were collected from the jars after 0, 2, 4, and 6 days of incubation. For the 2018 maize, 110 kernels were collected after 0 and 6 days. At the same timepoints, a 50 g sample was collected and stored at -20 C until analyzed for aflatoxin.

2.3.3 Isolation and enumeration of fungi

Kernels from each timepoint were placed into flasks containing 0.05% Triton X-100 solution. After stirring for 1 min, the kernel-wash was collected, and fungal counts were determined by dilution plating onto Rose Bengal agar medium amended with chloramphenicol (25 μg/ml). Subsequently, the washed maize kernels were surface-sterilized in a sodium hypochlorite solution (3-5%) for 2 min, rinsed three times with sterile distilled water, and 100 kernels were plated onto Rose Bengal agar medium. Both the dilution-plates and kernel-plates were incubated for 5 days at 28 °C. Fungal colonies from surface-washed and maize kernels were identified base on morphological characteristics. Colony counts were expressed as colony forming units (CFU). Percent of infected kernels also was enumerated. *A. flavus* from infected kernels was isolated into pure cultures and stored at -80 until further characterization.

2.3.4 Characterization of A. flavus isolates

Conidia from *A. flavus* isolates were inoculated into 5 ml culture tubes containing 1 ml of YEPD broth (Yeast Extract Peptone Dextrose; 0.3% yeast extract, 1.0% peptone, 2.0% glucose). After stationary incubation for 72 hours at 28 °C, mycelial mats were transferred into 1.5 ml micro centrifuge tubes and stored at -80 °C for subsequent DNA isolation. The culture broth was transferred to a 1.5 ml micro centrifuge tubes and stored at -20 °C until aflatoxin analysis.

2.3.5 Aflatoxin analysis

For aflatoxin analysis, about 50 g of maize were ground in a coffee grinder and a subsample (0.5 g) was extracted overnight in 2 ml of chloroform: methanol (2:1, v/v). The resulting extract was analyzed by thin-layer chromatography (TLC). Aflatoxin was extracted from culture broth by adding an equal volume of chloroform and mixing with a vortex for 1 min.

The chloroform phase was analyzed by TLC. TLC plates (silica gel 60 F_{254}) were developed in chloroform: acetone: acetic acid (88:12:0.1, v/v/v), digitally photographed under UV and the image analyzed with ImageJ (http://rsb.info.nih.gov/ij). Quantification was based on comparisons with aflatoxin standards that were included on each TLC plate.

2.3.6 Genotyping of A. flavus isolates

DNA was purified from isolates of *A. flavus* that failed to produce aflatoxin in YEPD medium by a previously described CTAB method (Cubero et al., 1999; Rogers, 1985). Purified DNA was used as the template in a PCR amplification of *aflC* with primers *aflC*-forward (5'-TTAGATCGGTCCCTTTACTTT-3') and AFLC-reverse Donner et al.; Donner et al.; (5'-GGTGGTCAGTCCTTGTCTCTGTA-3'). As a DNA quality control, a 600 bp ITS region was amplified also with the primer pair ITS1 (5'-TCCGTATGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 54°C (AflC) or 56°C (ITS) for 30 s, 72°C for 1 min, and one cycle at 72°C for 7 min. PCR products were separated by electrophoresis in 1% agarose gels. Isolates that did not yield *aflC* PCR product were designated as Aflaguard-like. A subset of these isolates were also analyzed for gene *aflQ*, which is near the end of the aflatoxin gene cluster and encodes the enzyme involved in the last step in aflatoxin biosynthesis (Yu, 2012). Primers *(aflQ*-forward 5'-TTAAGGCAGCGGAATACAAG-3' and *aflQ*- reverse 5'-GACGCCCCAAAGCCGAACACAAA-3') were used with the reaction conditions 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and one cycle at 72°C for 7 min. For non-aflatoxin-producing isolates resulting in a PCR amplified *aflC*, the product was gel-purified and sequenced by the Purdue Genome Core Facility. Sequence results

were compared to the *aflC* from wildtype and AF36 strains. Isolates with a mutation (G→A) at nt 591 were designated as AF36-like (Elrich and Cotty, 2004).

2.4 Results

2.4.1 Effects of storage conditions on fungi

In 2017, we received maize harvested from fields in Texas and North Carolina that were treated with and without biocontrol strains of *A. flavus*. The moisture content of the grain was conditioned to 20% from an initial moisture of about 12%. At the start of the experiment, the number of infected kernels was high (>80 %) in the North Carolina samples and the control sample from Texas (Table 2.1). The kernels from the AF36-treated field were only 40% infected. The number of surface fungi paralleled kernel infection levels with over 10^6 CFU in the samples for North Carolina and the Texas control (Table 2.1). Over the six-day incubation period, the kernel infection levels and number of surface fungi increased (Table 2.1). Visible signs of fungi on the kernels were observed at days 4 and 6.

In 2018, we received samples from Texas experimental plots that were being tested with several biocontrol strains. Samples from the Greenville site had a kernel infection level between 41% and 63%, with surface fungi exceeding 2×10^6 CFU (Table 2.1). The kernel samples from the Field 219 site were nearly all infected and the surface fungi were greater than 3×10^7 CFUs. As observed with the 2017 samples, fungi infection and surface fungal counts increased during the six days of incubation.

Fungal observed on dilution plates and infected kernels included *Fusarium* sp., *Penicillium* sp., *Alternaria* sp., *Aspergillus niger* and *A. flavus*. *Fusarium* sp. were predominant in both AF36-treated fields, while *A. niger* infection dominated maize from the control field (TX). *Aspergillus flavus* dominated both the kernel wash and the plated kernels from the

Greenville location. While most *A. flavus* isolates collected during this study produced either large sclerotia or none in culture, 5% and 19% of the *A. flavus* isolates from Greenville field and Field 219 sites produced small sclerotia respectively.

Table 2.1 Changes in surface fungi and infected kernel during storage[a].

Location	Treatment	Surface Fungi [b]				Infected kernels [c]			
		0 [d]	2	4	6	0	2	4	6
2017									
North Carolina (NC)	AflaGuard	1.1	3	188	120	88	100	100	100
	Control	57.5	1.4	22.1	85.8	99	94	100	100
Texas (TX)	AF36	0	0	0.4	2.2	40	53	66	64
	Control	1.1	2.1	6.4	39.6	90	94	100	99
2018									
Greenville (GV)	AflaGuard	11.25	-	-	388	62	-	-	100
	AF36	2.25	-	-	49.38	41	-	-	100
	Control	16	-	-	40	63	-	-	100
Field 219 (219)	AflaGuard	40.88	-	-	368	96	-	-	99
	Control	30.2	-	-	265	100	-	-	100

[a] Maize moisture content was adjusted to 20% and stored in jars at 28˚C.

[b]. Values are mean CFU x 10^6/sample from 3 replicates jars.

[c] Value are mean percent of infection from 3 replicate jars.

[d] Days of incubation.

2.4.2 Aspergillus flavus

Despite the high incidence of kernel infection in the three of the 2017 samples, the percentage infected with *A. flavus* was 1-2% (Table 1.2). Kernels from the AF36-treated field had no detectable *A. flavus* at the start of the storage experiment. The fungus clearly spread to other kernels during the 6 days of incubation. Although the 2018 samples from Texas followed a similar trend, the percent of kernels initially infect with *A. flavus* was higher, and the fungus spread more rapidly during the incubation period, especially in the Greenville samples.

Table 2.2 Percent of kernels infected with *A. flavus* before and after storage of maize grain from two locations in North Carolina and nine locations in Texas.

Location	Treatment	Kernels infected by Aspergillus flavus (%)			
		0 [a]	2	4	6
2017					
North Carolina (NC)	AflaGuard	2	16	26	35
	Control	2	9	38	39
Texas (TX)	AF36	0	1	6	10
	Control	1	1	7	7
2018[b]					
Greenville (GV)	AflaGuard	17	-	-	86
	AF36	24	-	-	92
	Control	6	-	-	96
Field 219 (219)	AflaGuard	19	-	-	62
	Control	6	-	-	49

[a] Days of incubation.

[b] Samples were collected before incubation and after 6 days of incubation.

2.4.3 Aflatoxin accumulation in grain

At the start of the storage experiment, aflatoxin was not detected in any of the 2017 samples (Table 1.3). However, by the end of the six-day incubation period, high aflatoxin levels were found in the Texas samples and the AflaGuard-treated sample from North Carolina. Although 39% of the kernels from the North Carolina control sample were infected with *A. flavus*, no aflatoxin was detected. In the 2018 maize samples, only the control sample from Greenville had detectable aflatoxin at the start of the experiment. After six days, measurable aflatoxin was found in all except the AF36-treated sample from the Greenville site. Only the control samples at the Field 219 had measurable aflatoxin. Because the values were low, we extended the storage to 10 days. Aflatoxin continued to accumulate in the samples and became detectable in all Field 219 samples. However, Greenville AF36 sample remained free of aflatoxin.

Table 2.3 Production of AFB1 in maize samples from North Carolina and Texas at day 0, 6 days and 10 days of incubation.

Location	Treatment	Aflatoxin (ppb/g (range)		
		Day 0	Day 6	Day 10
2017				
North Carolina (NC)	AflaGuard	0	11(0-33)	-
	Control	0	0	-
Texas (TX)	AF36	0	211(33-333)	-
	Control	0	77(33-200)	-
2018				
Greenville (GV)	AflaGuard	0	8	14
	AF36	0	0	0
	Control	4	2	6
Field 219 (219)	AflaGuard	0	0	1
	Control	0	1	1

2.4.4 Characterization of A. flavus isolates

From the 2017 samples, we characterized 187 *A. flavus* isolates from North Carolina and 96 from Texas (Table 1.4). Only 17 % and 9% of the isolates from the AflaGuard-treated and control, respectively, produced aflatoxin in culture. Of the 84 isolates from the AflaGuard-treated samples that did not produce aflatoxin, 17 isolates were missing the *aflC* gene. Similarly, 78 isolates from the control field did not produce aflatoxin and 6 of these did not have the *aflC* gene. Sequence analysis of those containing the *aflC* gene indicated none with the hallmark AF36 mutation. About 62% of the isolates from the AF36-treated field did not produce aflatoxin in culture. About half of these isolate did not have the *aflC* gene and the other half contain the AF36 mutation in the gene. In the corresponding control sample, over half of the non-aflatoxin producers contained the *aflC* gene and none had the AF36 gene mutation.

From the 2018 samples, a total of 458 *A. flavus* isolates from the Greenville field site and 298 isolates from the Field 219 site were characterized (Table 1.5). Less than 13% of the isolates were aflatoxin producers. Furthermore, only aflatoxin B_1 was produced by the small sclerotial isolates from these fields. Of the atoxigenic isolates from AF36 treated field, 50 isolates did not have both *aflC* and *aflQ* genes. Likewise, 79 of the atoxigenic isolates from the control field had both *aflC* and *aflQ* genes missing. Sequencing analysis on isolates containing *aflC* gene showed high incidence (76%) of atoxigenic isolates containing the AF36 gene mutation from control field. In the AflaGuard-treated and the control field 68% were atoxigenic isolates missing the *aflC* gene, with 31% and 43% of the isolates missing both the *aflQ* and *aflC* genes, respectively. Only 6% of the 213 atoxigenic isolates from Field 219 site had the AF36 gene mutation.

Table 2.4 Genotype determination of *A. flavus* isolates from infected kernels of treated and untreated fields from Texas and North Carolina[a].

Location	Treatment	Days	Total A. flavus isolates	Toxigenic[b]	aflC +	aflC -	AF36-like[c]
2017							
North Carolina (NC)	Aflaguard	0	6	2	1	3	0
		2	34	0	30	4	0
		4	30	11	15	4	0
		6	31	4	21	6	0
		Total	101	17	67	17	0
	Control	0	8	0	7	1	0
		2	18	1	16	1	0
		4	30	3	24	3	0
		6	30	4	25	1	0
		Total	86	8	72	6	0
Texas (TX)	AF36	0	0	0	0	0	0
		2	3	1	1	1	0
		4	17	2	10	5	7
		6	29	6	14	9	7
		Total	49	9	25	15	14
	Control	0	3	1	2	0	0
		2	4	1	2	1	0
		4	19	3	7	9	0
		6	21	1	15	5	0
		Total	47	6	26	15	0
2018							
Greenville (GV)	Aflaguard	0	50	0	33	17	21
		6	120	1	67	52	59
	AF36	0	71	0	64	7	64
		6	100	0	28	72	27
	Control	0	17	1	3	13	1
		6	100	7	86	7	82
Field 219 (219)	Aflaguard	0	18	8	10	0	8
		6	104	23	19	62	3
	Control	0	56	22	23	10	2
		6	120	32	59	29	1

[a] DNA purified from the isolates was used in PCR with primers that amplified the *AflC* gene. $AflC^{+}$ = correct PCR product and $AflC^{-}$ = no PCR product produced. All DNA samples yielded a PCR product with ITS primers.

[b] Strains failed to produce aflatoxin when grown on YEPD medium.

[c] Strains containing the AF36 mutation at NT-591 in *aflC* (Ehrlich & Cotty, 2004).

2.5 Discussion

At harvest, maize, as well as other grains, have many fungal genera on the surface and inside the grain. These fungi can be identified by traditional plating techniques and by microbiome techniques, which utilize high throughput DNA sequencing. The latter methodology has revealed that the fungal populations are diverse in structure, and likely influenced by geographic location, weather, and prior management practices (Klich, 2007). By plating we identify several fungi on the grain surface and inside the kernels, including *Aspergillus*, *Fusarium* and *Penicillium*. These three genera are often the core fungi in the microbiomes of grains at harvest and in storage. Lane et al., (2018) found that in maize collected from 30 farms in Makueni County, Kenya, all contained *Fusarium* and *Penicillium* in their microbiomes and 23 farm samples contain *Aspergillus* species. Similarly, studies conducted in Brazil and Nigeria identified species of *Fusarium, Penicillium,* and *Aspergillus* in harvested maize (Atehnkeng et al., 2008; Oris et al., 2000).

Maize harvested in the US and other developed countries is quickly shelled and dried to moisture levels that inhibit fungal growth. In Sub-Saharan Africa countries, where majority of farmers lack modern drying machinery, the postharvest period begins with maize plants cut and stacked or stooked in fields and may remain stooked for as long as 3 weeks, depending on the weather conditions and availability of labor. Similarly, the next drying-steps, which include removing the cobs from the stalks, husking the ears, shelling, and spreading on tarpaulins for solar drying, takes several days to weeks before a safe moisture content of 13% is attained. Throughout this period, maize is at moistures and temperatures that encourage fungal growth and mycotoxin accumulation. In our study, maize harvested by traditional US methods was adjusted to a moisture content of 20%, which is a mid-point between the high at harvest and the recommended storage level of 13%. The samples were then incubated at an optimum

temperature of 28°C for fungal growth, conditions similar to those a farmer in most Sub-Saharan Africa countries would experience during their drying process.

In our 2017 study, the level of kernel infection in samples from the AF36-treated Texas field was low compared to the two North Carolina samples and the control sample from Texas. As such, the spread of infection was slower in the AF36-treated samples during the incubation period. In 2018, samples from the Field 219 site had a higher initial fungal infection than samples from the Greenville site. The reason for the difference in the initial fungal infection levels is likely due to environmental conditions during the growing season, such as drought during grain filling as observed by Jones et al. (1980). High temperatures and insect damage also may have contributed (Hell et al., 2008; Hesseltine et al., 1981). Our results indicate that the number of fungi increased and spread in maize during the 6-day incubation period. A study in Nigeria by Oyebanji and Efluvwevwere (1999) showed similar results when maize at various moisture contents was stored at ambient temperatures for 180 days. Higher fungal loads were observed with increasing moisture content and maize at the highest MC (17% and 20% MC) was more prone to deterioration during the storage period.

Because of the application strategy for biocontrol products, one would expect that biocontrol-treated fields to have a high incidence of *A. flavus*-infected kernels. In 2011, Isakeit (2013) found that Texas maize treated with Aflaguard and AF36 consistently had higher *A. flavus* infection rates than the control fields. In our 2018 analysis, we observed at the Greenville location that infection by *A. flavus* was nearly 50 % and 30% in kernels from Afla-Guard and AF36-treated fields, respectively. At the Field 219 location, 19 % of the kernels in the Afla-Guard sample were infected with *A. flavus*, which was more than the 6 % infection in the control field. Only the control sample from the Greenville field was contaminated with aflatoxin, which

also had a 6 % infection with *A. flavus*. The samples collected in 2017 were unusual in that very few kernels were infected by *A. flavus* regardless of treatment, and these samples also were all free of aflatoxin. This variability was also observed in a 2015 study in three Texas counties, where kernel infection and aflatoxin contamination were low in maize fields treated with Afla-Guard (Isakeit et al., 2015).

Our genotyping analysis was based on the absence of *aflC* in Afla-Guard-like isolates and a specific nucleotide change in the gene in AF36-like isolates. Many of the atoxigenic isolates contained the *aflC* gene but lacked the AF36 mutations. Furthermore, many isolates lacking the *aflC* possessed *aflQ* suggesting that other mutations can render the fungus atoxigenic. Chang et al., (2005) characterized isolates from natural populations and found a variety of mutations within the aflatoxin gene cluster, including isolates with deletions in the middle of the cluster between *aflC* and *aflQ*. Our results also suggest movement of the biocontrol strains, especially at the 2018 Greenville, Texas site, which had experimental plots near each other. Similar spread of AF36 was observed by Cotty (1994) in cotton, where as high as 25 % of the isolates in untreated cotton plots were the biocontrol strain. Dorner et al., (1999) also observed spread of the biocontrol strain to ears in an untreated field, which was 1 km from the biocontrol-treated field.

In conclusion, results from our study do not support the hypothesis that treatment of maize fields with biocontrol strains during pre-harvest period will reduce aflatoxin accumulation during the postharvest drying period. Both atoxigenic and toxigenic *A. flavus* associate with the grain at the start of the experiment increased and spread during the 6 days of incubation, and growth of biocontrol strains did not reduce aflatoxin levels. Therefore, the potential and magnitude of aflatoxin increase during the drying period will depend on the proportion of toxigenic strains in the infected grain at harvest. The study by Atehnkeng et al. (2014) supports

this conclusion. These authors observed that aflatoxin increase tremendously in both AflaSafe and control grain during a 10-day period after rewetting. The control grain had significantly higher amounts of aflatoxin, likely because it had a higher population of toxigenic *A. flavus* at the start of the experiment. Our results indicate that preharvest biocontrol applications will not replace the need for better postharvest practices that reduce the drying time between harvest and storage. Therefore, to ensure efficacy of these biological controls during drying periods, fast drying methods and equipment should be used by farmers. Other factors such as frequency of biocontrol application in fields and time of application should also be considered. In addition, field management strategies such as irrigation, crop rotation and tillage practices should also be integrated in these fields to reduce *A. flavus* infection and aflatoxin levels in maize during post-harvest periods (Lavkor & Var, 2017; Okoth et al., 2012).

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